



HAL
open science

Décryptage du Polymorphisme de Compatibilité dans l'interaction entre *Biomphalaria glabrata* et *Schistosoma mansoni* : une approche intégrative

Anaïs Portet

► **To cite this version:**

Anaïs Portet. Décryptage du Polymorphisme de Compatibilité dans l'interaction entre *Biomphalaria glabrata* et *Schistosoma mansoni* : une approche intégrative. Sciences agricoles. Université de Perpignan, 2017. Français. NNT : 2017PERP0035 . tel-01968022

HAL Id: tel-01968022

<https://theses.hal.science/tel-01968022>

Submitted on 2 Jan 2019

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Délivré par
UNIVERSITE DE PERPIGNAN VIA DOMITIA

Préparée au sein de l'école doctorale
Énergie Environnement (ED205)

Et de l'unité de recherche
**Interactions Hôtes-Pathogènes Environnements
(IHPE)**
UMR5244 (UPVD-CNRS-IFREMER-UM)

Spécialité : **Biologie**

Présentée par
Anaïs PORTET

**Décryptage du Polymorphisme de Compatibilité
dans l'interaction entre *Biomphalaria glabrata*
et *Schistosoma mansoni* : une approche
intégrative**

Soutenue le **17 Octobre 2017** devant le jury composé de :

Mme. **Christine BRAQUART-VARNIER**, Pr, Université de Poitiers

Rapporteur

M. **Franck PRUGNOLLE**, DR, Université de Montpellier

Rapporteur

Mme. **Elisabeth HUGUET**, Pr, Université de Tours

Examineur

M. **Guillaume MITTA**, Pr, Université de Perpignan

Examineur

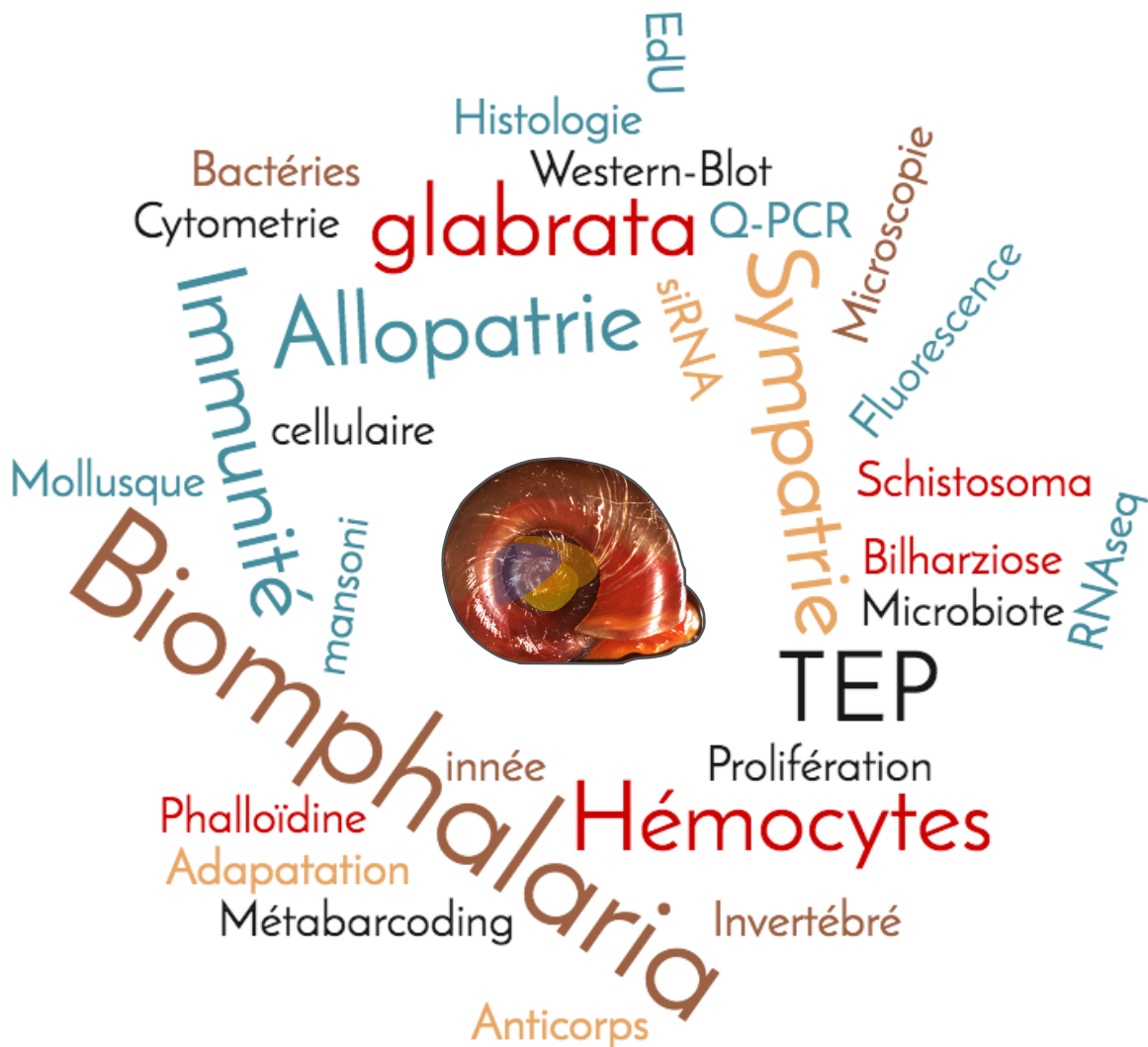
M. **Benjamin GOURBAL**, MCF, Université de Perpignan

Directeur de thèse

M. **Richard GALINIER**, IR, Université de Perpignan

Co-directeur de thèse

Décryptage du **Polymorphisme de Compatibilité** dans l'interaction entre *Biomphalaria glabrata* et *Schistosoma mansoni* : une approche intégrative



Anaïs PORTET

Sous la supervision de Benjamin GOURBAL et Richard GALINIER

À Papé

Sommaire

INTRODUCTION	1
DE LA THÉORIE DE L'ÉVOLUTION À LA THÉORIE SYNTHÉTIQUE DE L'ÉVOLUTION	1
<i>L'ÉVOLUTION EXPÉRIMENTALE</i>	2
<i>GRANDS TYPES D'INTERACTIONS BIOTIQUES</i>	4
<i>LES INTERACTIONS HÔTES / PARASITES</i>	6
BIOMPHALARIA GLABRATA / SCHISTOSOMA MANSONI	7
<i>L'INTERACTION BIOMPHALARIA GLABRATA / SCHISTOSOMA MANSONI</i>	7
<i>L'HISTOIRE ÉVOLUTIVE ENTRE BIOMPHALARIA GLABRATA ET LE SCHISTOSOME</i>	9
<i>LE POLYMORPHISME DE COMPATIBILITÉ</i>	15
L'IMMUNITÉ	17
<i>L'IMMUNITÉ DES INVERTÉBRÉS</i>	17
<i>LA RECONNAISSANCE</i>	18
<i>LES VOIES DE SIGNALISATIONS</i>	26
<i>LA RÉPONSE CELLULAIRE</i>	27
<i>LA RÉPONSE HUMORALE</i>	31
<i>L'INTERACTION IMMUNITAIRE ENTRE BIOMPHALARIA GLABRATA ET SCHISTOSOMA MANSONI</i>	35
INFLUENCE DE FACTEURS ENVIRONNEMENTAUX SUR LA COMPATIBILITÉ HÔTE / PARASITE	39
<i>FACTEURS ABIOTIQUES</i>	39
<i>FACTEURS BIOTIQUES</i>	41
CHAPITRE I : DU POLYMORPHISME DE COMPATIBILITÉ À L'ADAPTATION LOCALE ENTRE BIOMPHALARIA ET SCHISTOSOMA.	45
<i>INTRODUCTION</i>	45
1. L'INTÉRÊTS DES OMICS DANS LA MISE EN ÉVIDENCE DU DIALOGUE MOLÉCULAIRE ENTRE BIOMPHALARIA GLABRATA ET SCHISTOSOMA MANSONI	48
<i>Publication : Integrated multi-omic analyses in Biomphalaria-Schistosoma dialogue reveal the immunobiological significance of FREP-SmPoMuc interaction.</i>	48
2. L'ÉTUDE DU COMPLEXE IMMUNITAIRE FREP/SMPOMUC	61
<i>Publication : A multistrain approach to studying the mechanisms underlying compatibility in the interaction between Biomphalaria glabrata and Schistosoma mansoni.</i>	61
3. L'IMPACT DE L'ADAPTATION LOCALE SUR L'INTERACTION IMMUNOLOGIQUE ENTRE BIOMPHALARIA GLABRATA ET SCHISTOSOMA	88
<i>Publication : Towards an integrative view of local adaptation theory in host/parasite systems.</i>	88
<i>DISCUSSION</i>	119
CHAPITRE 2 : DU POLYMORPHISME DE COMPATIBILITÉ VERS LA COMPRÉHENSION D'UN SYSTÈME IMMUNITAIRE COMPLEXE.	128
<i>INTRODUCTION</i>	128
1. ÉTUDE D'UN FACTEUR CLÉ DE LA RÉPONSE IMMUNITAIRE CELLULAIRE : LA BGTEP	131

<i>Publication : The Thioester-containing protein, a key factor of the Biomphalaria glabrata immune cellular response?</i>	131
2. MISE EN ÉVIDENCE D'EFFECTEURS IMMUNITAIRES CHEZ BIOMPHALARIA GLABRATA EN RÉPONSE À DIVERS PATHOGÈNES	162
<i>Publication : Specific pathogen recognition by multiple innate immune sensors provides support to Medzithov's cooperative concept in a non-model invertebrate.</i>	162
3. MISE AU POINT D'UNE TECHNIQUE D'INVALIDATION FONCTIONNELLE SUR LES CELLULES IMMUNITAIRES DE BIOMPHALARIA GLABRATA	175
<i>Publication : An improved method for siRNA gene silencing in Biomphalaria glabrata hemocytes</i>	175
DISCUSSION	188
<u>CHAPITRE 3 : L'INFLUENCE DU MICROBIOTE SUR L'INTERACTION BIOMPHALARIA / SCHISTOSOMA.</u>	<u>197</u>
INTRODUCTION	197
1. CARACTÉRISATION DE VIRUS RÉSIDENTS CHEZ BIOMPHALARIA	200
<i>Publication : First characterization of viruses from freshwater snails of the genus Biomphalaria, the intermediate host of the parasite Schistosoma mansoni.</i>	200
2. INFLUENCE DE LA RÉPONSE IMMUNITAIRE SUR LE MICROBIOTE BACTÉRIEN CARACTÉRISÉ DE NOVO	209
<i>Publication : Bacterial communities homeostasis a reciprocal interplay between microbiota and immune system in the lophotrochozoan snail Biomphalaria glabrata</i>	209
DISCUSSION	233
<u>DISCUSSION</u>	<u>242</u>
<u>ANNEXES</u>	<u>258</u>
ANNEXE 1	258
<i>Publication : The aerolysin-like proteins family acquired from bacteria are key diversified immune weapons in an invertebrate</i>	258
ANNEXE 2	299
<i>Publication : Polymorphic sets of immune receptors support specificity in the innate immune memory of the Schistosomiasis vector snail, Biomphalaria glabrata</i>	299
ANNEXE 3	339
<i>Publication : Clearance of schistosome parasites by resistant genotypes at a single gene region in Biomphalaria glabrata snails involves cellular components of the hemolymph.</i>	339
<u>REMERCIEMENTS</u>	<u>346</u>
<u>BIBLIOGRAPHIE</u>	<u>350</u>

Sommaire des illustrations

FIGURE 1 : DEUX SCÉNARIOS DE CO-ÉVOLUTION HÔTE / PATHOGÈNE	3
TABLE 1 : PRINCIPAUX TYPES D'INTERACTIONS BIOTIQUES RETROUVÉES DANS LE MILIEU NATUREL	4
FIGURE 2 : HYPOTHÈSE D'ÉVOLUTION DES TYPES D'INTERACTIONS	6
FIGURE 3 : CYCLE DE VIE DU PARASITE SCHISTOSOMA MANSONI	8
FIGURE 4 : REPRÉSENTATION DES ARBRES PHYLOGÉNÉTIQUES DE BIOMPHALARIA SP. ET SCHISTOSOMA SP.	10
FIGURE 5 : HISTOIRE ÉVOLUTIVE DE BIOMPHALARIA SP. ET SCHISTOSOMA SP.	12
FIGURE 6 : REPRÉSENTATION SCHÉMATIQUE DU POLYMORPHISME DE COMPATIBILITÉ	16
FIGURE 7 : INTERACTION IMMUNITAIRE ENTRE BIOMPHALARIA GLABRATA ET SCHISTOSOMA MANSONI	36
FIGURE 8 : DU POLYMORPHISME DE COMPATIBILITÉ À L'ADAPTATION LOCALE ENTRE B. GLABRATA ET S. MANSONI	127
FIGURE 9 : DU POLYMORPHISME DE COMPATIBILITÉ VERS LA COMPLEXITÉ DE LA RÉPONSE IMMUNITAIRE	196
FIGURE 10 : CARACTÉRISATION DU MICROBIOTE ET ÉTUDE DE SON INFLUENCE SUR L'INTERACTION BIOMPHALARIA ET SCHISTOSOMA	239
FIGURE 11 : RELATION TRIPARTITE ENTRE L'IMMUNITÉ DE L'HÔTE SON MICROBIOTE ET LES PATHOGÈNES	249
FIGURE 12 : SCHÉMA DE CONCLUSION DU DÉCRYPTAGE DU POLYMORPHISME DE COMPATIBILITÉ	256

Introduction

Introduction

Ce travail de thèse s'intègre dans un projet plus large sur l'étude et la compréhension des interactions l'hôte / parasite. Nous portons un intérêt tout particulier à la plasticité phénotypique intervenant dans la dynamique co-évolutive entre hôte et parasite. En populations naturelles, cette co-évolution longue entre l'hôte et le parasite implique un équilibre entre les mécanismes de virulence du parasite et de défense de l'hôte. Ainsi, au sein d'une même population d'hôte et de parasite du fait de cette plasticité, c'est parfois le parasite qui gagne et infeste l'hôte et parfois l'hôte qui gagne et élimine le parasite.

Le travail de cette thèse s'inscrit dans ce contexte et vise donc à une meilleure compréhension des interactions immunologiques dans le modèle impliquant le mollusque *Biomphalaria glabrata* et le trématode *Schistosoma mansoni*.

De la théorie de l'évolution à la théorie synthétique de l'évolution

L'évolution est un ensemble des changements subis au cours des temps géologiques par les organismes vivants, ayant pour résultat l'apparition de nouvelles formes de vie (Zimmer 2012).

La théorie de l'évolution voit le jour à partir du XIX^{ème} siècle. C'est Jean-Baptiste de Lamarck (1744-1829), naturaliste français, qui pour la première fois propose une théorie de l'évolution des espèces. Il base ses recherches sur des phénomènes matérialistes et mécanistiques, on parle de théorie transformiste, la diversification ou la spécialisation des espèces se fait à la suite d'une adaptation aux contraintes ou spécificités du milieu, à partir de traits biologiques évoluant au cours de la vie de l'individu.

La biologie de l'évolution se voit ensuite révolutionnée par l'intermédiaire du naturaliste anglais, Charles Darwin (1809-1882), qui propose alors la théorie de la « sélection naturelle », selon laquelle toutes les espèces vivantes ont évolué au cours du temps à partir d'un seul ancêtre commun. Son ouvrage « De l'origine des espèces » paru en 1859, changea la vision de la biologie et de l'évolution, sa théorie constitue, encore aujourd'hui, les bases de la théorie moderne de l'évolution.

Plus tard, grâce aux découvertes génétiques réalisées par Mendel, les modèles de la théorie de l'évolution ont pu évoluer et s'affiner. En effet, depuis les années 30, nous

Introduction

parlons du néodarwinisme (ou théorie synthétique de l'évolution), cette théorie reprend les concepts de Darwin, la sélection naturelle, et ajoute la notion d'hérédité, qui était encore inconnue à l'époque de Darwin.

Enfin, le paléontologue américain, Stephen Jay Gould (1941-2002), fait encore évoluer la théorie de l'évolution énoncée par Darwin, rajoutant la notion d'évolution des espèces lors de périodes ponctuelles séparées de périodes stagnantes, et parle alors de la théorie des équilibres ponctués.

L'évolution expérimentale

L'évolution expérimentale est une branche de la biologie, qui consiste à mimer en laboratoire, le processus évolutif afin d'en étudier les règles de fonctionnement sous-jacentes. Elle constitue un pont entre les études conduites dans des conditions naturelles et les simulations réalisées par modélisation mathématique (Bataillon, et al. 2010).

Aujourd'hui, les expériences d'évolution expérimentale s'étendent à beaucoup d'organismes, des animaux aux plantes. Par extension, le terme d'évolution expérimentale désigne aussi les expériences en laboratoire et en milieu naturel, dès lors qu'elles envisagent des pas de temps de plusieurs générations et qu'elles visent à tester soit le processus de sélection, soit son issue. Il faut noter, que l'évolution expérimentale, est aujourd'hui utilisée pour analyser les évolutions entre les caractères au sein d'un organisme, les règles d'interactions entre les organismes et plus généralement les théories touchant l'évolution et l'écologie des organismes et des communautés (Bataillon, et al. 2010).

L'extension de l'approche d'évolution expérimentale à l'étude de la co-évolution a permis de tester, en environnement contrôlé, les concepts de base des interactions entre les hôtes et les parasites. Le terme co-évolution est généralement attribué à Ehrlich et Raven, grâce à leurs travaux fondateurs sur les plantes et les papillons, en 1964 (Ehrlich and Raven 1964). Mais le principe de la co-évolution n'est véritablement énoncé qu'en 1973 par Leigh Van Valen (Van Valen 1973; Tirard, et al. 2016)

Les phénomènes d'adaptation et de contre-adaptation peuvent se faire dans le temps ou dans l'espace. Des études d'évolutions expérimentales montrent qu'un parasite est capable de s'adapter spécifiquement à nouveau génotype d'hôte ou à une nouvelle espèce ; cette adaptation peut s'accompagner d'une baisse de performance sur d'autres hôtes voir sur l'hôte initial (Ebert 1998; Little, et al. 2006; Nidelet and Kaltz 2007). En

Introduction

parallèle, des travaux révèlent la capacité de l'hôte à évoluer rapidement sous la présence du parasite (Ebert, et al. 2004). Cependant, l'étude de l'évolution conjointe entre l'hôte et le parasite dans le temps, c'est-à-dire la co-évolution, est moins courante.

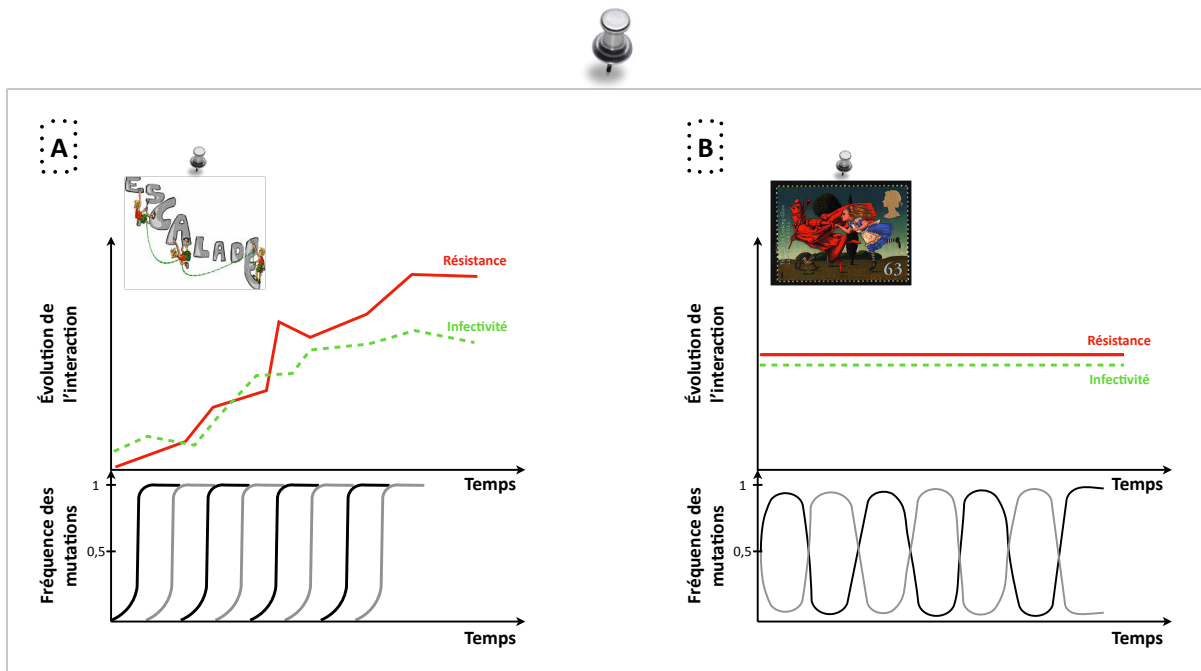


Figure 1 : Deux scénarios de co-évolution hôte / pathogène

Figure adaptée d'après (Bataillon, et al. 2010). A. Théorie de « la course aux armements », escalade des processus d'inféctivité du parasite et de résistance de l'hôte, caractérisé par un balayage de sélection successif et d'une augmentation régulière des capacités des deux acteurs. B. Théorie de « la Reine Rouge », inspirée par le livre de Lewis Carroll (Van Valen 1974), l'idée étant de courir pour rester à la même place, ce qui se traduit par une oscillation des fréquences de génotypes et les processus d'inféctivité du parasite et de résistance du mollusque sont stables au cours du temps.

Deux types de scénarios peuvent être à la base des processus co-évolutifs entre l'hôte et son parasite ; le premier conduit à un balayage sélectif successif, à l'apparition de parasites de plus en plus offensifs et d'hôtes de plus en plus résistants, il s'agit d'une évolution réciproque de l'hôte vis-à-vis du parasite et réciproquement, on parle de la « course aux armements » (fig. 1A). Dans ce scénario, la diversité génétique est donc transitoire et il n'y a pas de recyclage des génotypes au cours du temps ; il fut mis en évidence en 1974 (Van Valen 1974). Le second scénario se caractérise par des oscillations des fréquences des génotypes liées à de la sélection fréquence dépendante, appliquée à un système simple de type « gène pour gène » (fig. 1B). Dans ce scénario, l'hôte acquiert de nouvelles capacités, souvent conséquence de pressions de sélection du milieu, devenant moins permissif sa fréquence augmente dans la population. La

Introduction

parasite, lui, est soumis à cet environnement changeant et doit s'adapter aux nouvelles capacités de son hôte pour pouvoir toujours être capable d'infecter et survivre : il court pour rester à la même place, il s'agit de la théorie de la « Reine Rouge » (Van Valen 1974). Ici, nous avons un recyclage permanent des génotypes et un polymorphisme génétique stable. En effet la résistance de l'hôte et l'infectivité du parasite restent les mêmes au cours du temps (Bataillon, et al. 2010).

La structuration spatiale est un aspect très important dans l'évolution des interactions hôte-parasite. Dans la nature, toutes les populations sont plus ou moins structurées en un ensemble de sous-populations. Cette structuration spatiale peut augmenter le temps nécessaire pour que l'interaction entre l'hôte et le parasite se produise, en diminuant la probabilité de rencontre (Brockhurst, et al. 2007). La dynamique locale des sous-populations peut amener à une divergence entre populations ; cette structuration spatiale pouvant faire apparaître des phénomènes d'adaptation locale où le parasite est plus performant sur l'hôte avec lequel il interagit (Ebert, et al. 2004; Greischar and Koskella 2007; Bataillon, et al. 2010).

Finalement, le processus de co-évolution s'applique à tous les organismes ayant une interaction biologique quelque soit l'échelle. Nous proposons donc maintenant de mettre en évidence les principaux types d'interactions biotiques que nous pouvons rencontrer.

Grands types d'interactions biotiques

Dans le milieu naturel, nous retrouvons de nombreux types différents d'interactions biologiques. Une interaction biologique désigne un processus impliquant des échanges ou relations entre plusieurs éléments biologiques dans un écosystème. Ces interactions peuvent être abiotiques (facteurs climatiques, hydrologiques ou tout facteurs physico-chimique du milieu, etc.) ou biotiques (relations trophiques, intra/inter-spécifiques) et engendrer un phénomène de co-évolution ou d'adaptation, induit directement (relation proie-prédateur) ou indirectement (utilisation d'une même ressource).

Plusieurs types d'interactions biotiques sont retrouvés dans le milieu naturel, chacune ayant des impacts différents sur les espèces ou les individus. Les principales sont le neutralisme, le commensalisme, la symbiose, le mutualisme, la prédation, le parasitisme, la compétition et enfin l'amensalisme (**table 1**).

[Table 1 : Principaux types d'interactions biotiques retrouvées dans le milieu naturel](#)

Introduction

L'influence de chacun des types d'interactions entre les espèces. Les interactions peuvent être directes (D) ou indirectes (I). Le symbole "-" représente une nuisance pour l'espèce, le "+" un bénéfice et le "0", n'a pas d'effet. Grands principes de chacune des interactions d'après (Tirard, et al. 2016).

Types d'interactions	Effet sur l'espèce A	Effet sur l'espèce B	Grands principes
Neutralisme	0	0	Aucun effet bénéfique ou néfaste sur les différents organismes
Commensalisme D/I	0	+	Interaction bénéfique pour un seul organisme
Symbiose D	+	+	Interaction bilatérale obligatoire mutuellement profitable
Mutualisme D/I	+	+	Interaction bilatérale facultative, mutuellement profitable
Prédation D	-	+	Interaction unilatérale nutritionnelle bénéfique pour un seul organisme
Parasitisme D	-	+	Interaction unilatérale obligatoire pour le parasite, nuisible pour l'hôte
Compétition D/I	-	-	Interaction antagoniste pour les mêmes ressources
Amensalisme D/I	0	-	Interaction unilatérale nuisible pour un seul organisme

Dans l'ensemble de ces interactions complexes qui régissent le fonctionnement du vivant, je propose de porter mon attention sur les interactions se référant seulement au parasitisme que je vais décrire plus particulièrement ci-après.

Le parasitisme est un terme établi par Van Beneden en 1876, il correspond à une interaction biologique directe dans laquelle un des protagonistes (le parasite) tire profit (ressource, habitat, transport, etc.) et inflige des coûts (survie, reproduction, croissance, etc.) à l'autre protagoniste (l'hôte) (Beneden 1878). Les organismes parasites sont retrouvés dans tout les grands phyla du vivant allant des virus, bactéries aux métazoaires pluricellulaires. Pour les organismes hôtes, aussi, nous retrouvons une grande diversité allant des animaux vertébrés aux invertébrés sans oublier les plantes ainsi que les bactéries. Nous distinguons habituellement les ectoparasites, qui vivent à la surface du corps de leur hôte, les endoparasites, lesquels vivent à l'intérieur de leur hôte et enfin les mésoparasites, inféodés aux cavités naturelles de l'hôte ouvertes sur le milieu extérieur (Ramade 2003). Le coût infligé par le parasite sur l'hôte peut aboutir à une mort quasi-systématique de ce dernier, nous parlons alors de parasitoïdes. Dans ce cas-là, le parasite fini la plupart du temps par tuer son hôte, pour réaliser son cycle de vie.

Introduction

Au niveau évolutif, ces grands types d'interactions biologiques peuvent évoluer au cours de temps géologiques (fig. 2). En effet, nous pouvons imaginer que la symbiose peut être la première étape avant le mutualisme, qui peut se transformer en commensalisme, se dernier pouvant dériver jusqu'au parasitisme. Inversement, les parasites peuvent tendre à pérenniser leur interaction dans le temps avec leur hôte (interaction durable) ceci afin de maximiser leur transmission en réduisant les coûts associés à l'infection, et ce faisant, ils pourraient évoluer vers du commensalisme, voir même aller jusqu'au mutualisme.

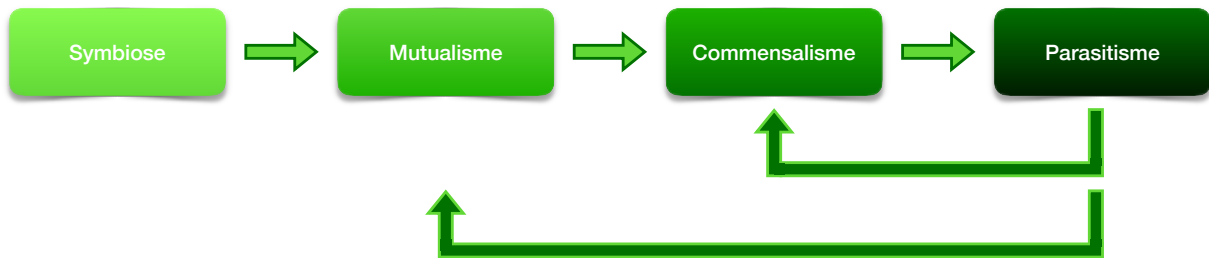


Figure 2 : Hypothèse d'évolution des types d'interactions

Évolution possible des types d'interactions biologiques entre les organismes, au cours des temps géologiques, depuis la symbiose vers le parasitisme ainsi que du parasitisme vers le mutualisme inspiré d'après (Ramade 2003; Bataillon, et al. 2010; Tirard, et al. 2016).

Au cours de mes travaux de thèse, j'ai porté un intérêt tout particulier au parasitisme de *B. glabrata* par *S. mansoni*, nous allons donc nous focaliser sur cette interaction dans la suite de mon mémoire de thèse.

Les interactions Hôtes / Parasites

Tout être vivant est soit un parasite, soit l'hôte potentiel d'un certain nombre de parasites (Tirard, et al. 2016). Les parasites exercent une pression de sélection importante sur leurs hôtes et réciproquement les parasites doivent évoluer pour contourner les adaptations de leurs hôtes visant à les éliminer. Il s'agit d'un système en co-évolution et/ou en adaptation permanente entre les hôtes et les parasites dans le but de contourner les armes développées par chacun des protagonistes dans un cas et conserver l'interaction dans l'autre cas. Dans ces interactions réciproques, de multiples génotypes hôtes et parasites coexistent. Seules certaines combinaisons peuvent conduire à la théorie de la "Reine Rouge" (Van Valen 1974) ou à de la co-évolution. Dans ce contexte, nous observons une variation cyclique dans le temps de l'abondance d'un génotype hôte et de son parasite (Tirard, et al. 2016).

Biomphalaria glabrata / *Schistosoma mansoni*

Biomphalaria glabrata est un mollusque Lophotrocozoaire d'eau douce, de la famille des Planorbidae, vivant en région intertropicale et plus précisément en Amérique latine. Ce planorbe est principalement connue pour être l'hôte intermédiaire de *Schistosoma mansoni*, vers plat parasite (plathelminthe, trematode). Ce dernier est l'agent responsable de la bilharziose intestinale, seconde endémie parasitaire humaine mondiale après le paludisme, décrite au 17^{ème} siècle par Theodor Maximilian Bilharz, médecin parasitologue allemand. La bilharziose touche aujourd'hui, 240 millions de personnes à travers le monde. Elle est reconnue comme un problème de santé humaine mondiale par l'Organisation Mondiale de la Santé (OMS), qui souhaite son éradication d'ici 2025. Cependant, aucun vaccin efficace contre cette maladie n'a pu être développé jusqu'à présent et la chimiothérapie limitée fait appel à une seule molécule active, le praziquantel pour lequel des cas de résistance ont pu être observés (Bergquist, et al. 2017). Il est donc opportun et urgent de pouvoir identifier de nouveaux moyens de lutte et de contrôle de cette maladie.

Dans ce contexte, il apparaît clairement qu'une meilleure compréhension de l'interaction entre le parasite et le mollusque, hôte intermédiaire, représente une voie de recherche prometteuse. Étant donné qu'il contribue de manière importante à la transmission de la Bilharziose humaine, il conviendrait de mettre l'accent sur le contrôle des escargots vecteurs pour développer de nouvelles stratégies visant à réduire la transmission de cette maladie (Fallon and Doenhoff 1994; Tennessen, et al. 2015). La compréhension des interactions immunologiques entre l'escargot et le parasite ainsi que des mécanismes moléculaires par lesquels les deux partenaires interagissent apparaît comme un prérequis à la découverte de nouvelles cibles thérapeutiques ou visant à développer de nouvelles stratégies de lutte sur le terrain. Ceci pourrait en partie passer par de la médiation vectorielle afin de limiter l'infection des mollusques (Tennessen, et al. 2015), comme ceci a pu être proposé et réalisé dans le passé chez le moustique dans la transmission du paludisme (Dinparast Djadid, et al. 2011; Iturbe-Ormaetxe, et al. 2011).

L'interaction *Biomphalaria glabrata* / *Schistosoma mansoni*

Comme toutes les espèces de trématodes, *S. mansoni* a besoin d'un mollusque comme hôte intermédiaire, ainsi que d'un hôte définitif, pour accomplir son cycle de vie

Introduction

hétéroxène (fig. 3). Son cycle commence chez son hôte définitif, vertébré, l'Homme, dans lequel il réalise sa reproduction sexuée. Les parasites adultes s'accouplent dans la veine porte hépatique migrent ensuite jusqu'aux veinules mésentériques de l'intestin pour que la femelle y pondre ses œufs. Les œufs vont alors pouvoir, traverser les épithéliums vasculaire et intestinal, afin de rejoindre la lumière de l'intestin et être évacués avec les fèces. Une fois dans l'eau douce, les œufs éclosent afin de libérer les miracidia, larve libre nageuse, phase infectante pour le mollusque.

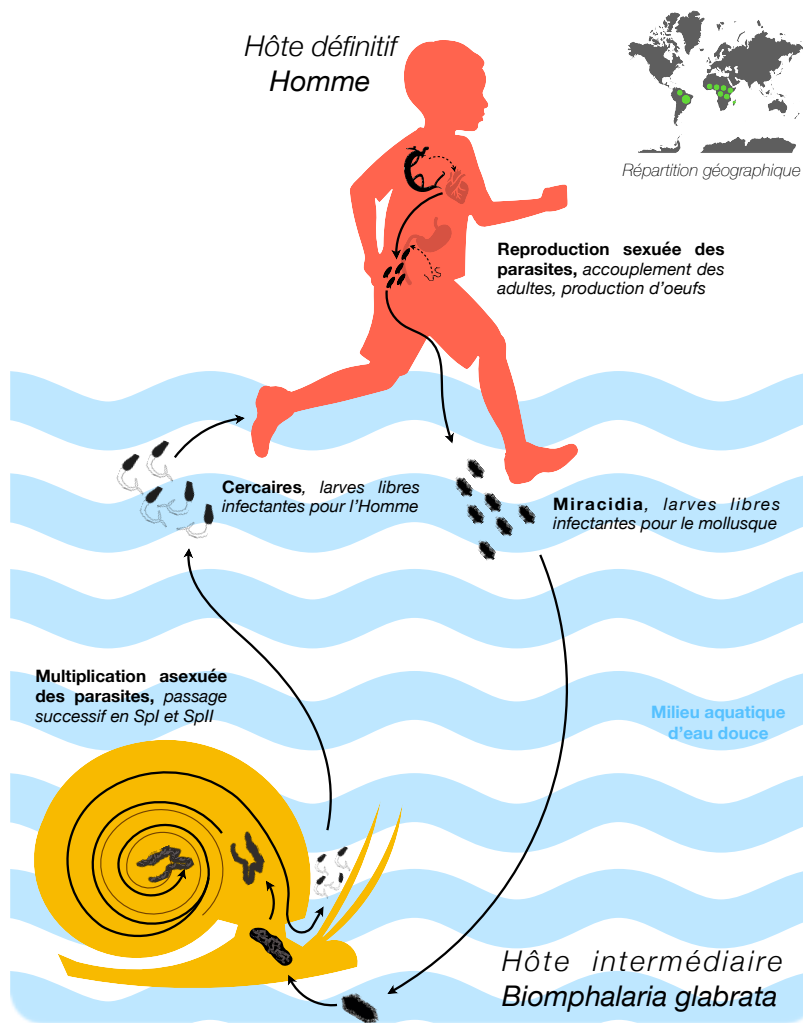


Figure 3 : Cycle de vie du parasite *Schistosoma mansoni*

Représentation schématique du cycle de vie complexe de *S. mansoni*, résumant les différents stades de la vie du parasite, à l'intérieur de son hôte définitif, l'Homme ainsi que de son hôte intermédiaire *Biomphalaria glabrata*. Répartition géographique de la Bilharziose causé par *S. mansoni* représenté par les points verts sur le planisphère.

Une fois dans le mollusque, les miracidia perdent immédiatement leurs plaques cillées, devenant alors des sporocystes primaires (Spl). Les Spl implantés dans les tissus du mollusque vont alors atteindre leur maturité en quelques jours et libérer des sporocystes secondaires (SpII) qui migreront jusqu'au centre de la coquille, à l'interface entre la glande digestive et la gonade. Une multiplication asexuée intense se produit à l'intérieur du Spl pour donner de nombreux SpII. Dans le SpII, une seconde multiplication asexuée intense aura lieu pour donner naissance à des milliers de cercaires, qui effectueront une migration

Introduction

inverse jusqu'au manteau du mollusque au niveau duquel elles sortiront par effraction vers le milieu extérieur aquatique. Le mollusque est alors le support d'une production cyclique, mais continue de cercaires, jusqu'à son épuisement et sa mort. Le parasite, au stade cercaire, larve libre nageuse libérée par le mollusque, recherche alors activement un hôte définitif, traverse activement la barrière cutanée de l'Homme en milieu aquatique, et migre jusqu'à la veine porte pour s'accoupler. Cette migration s'accompagne de la maturation des parasites qui mènera à la transformation de la cercaire en schistosomule (stade pré-adulte du parasite) jusqu'aux adultes sexuellement matures.

L'association mollusque/parasite est très spécifique et d'une importance centrale dans le cycle de vie du parasite.

L'histoire évolutive entre *Biomphalaria glabrata* et le Schistosome

Depuis plus de 20 ans, les scientifiques s'intéressent à l'évolution des espèces *Biomphalaria* et *Schistosoma* afin de reconstruire et comprendre leur histoire évolutive. Du côté du mollusque, les premières études ont été réalisées grâce à la paléontologie, avec l'étude des fossiles, elle ouvre des perspectives évolutives (Pierce 1993). Elles ont précédé les études moléculaires et le lien entre les deux a permis de confirmer le potentiel scénario évolutif des mollusques du genre *Biomphalaria*. Pour ce qui est des parasites, l'étude fossile étant impossible, seuls les études moléculaires ont permis d'avoir des informations sur leur histoire évolutive. C'est avec des études d'allozymes (utilisées par les phylogénéticiens comme marqueurs moléculaires) puis par la suite grâce à l'étude des ADN mitochondriaux ou bien ribosomiques, que le scénario évolutif de ces derniers a pu être retracé.

En 2000 et 2001, les équipes respectives de Campbell et DeJong étudient l'évolution biogéographique de *Biomphalaria*, grâce à l'analyse de séquences nucléotidiques telles que l'ADN mitochondrial et la sous-unité 1 de la cytochrome oxidase (CO1) (Campbell, et al. 2000) ou encore une séquence partielle de la sous-unité ribosomique 16S ainsi que les séquences complètes de l'ITS1 et l'ITS2 (nuclear ribosomal RNA internal transcribed spacer 1 or 2 regions) (DeJong, et al. 2001). Il apparaît alors que les distances génétiques entre les espèces africaines sont moins importantes que celles retrouvées chez les espèces néotropicales (sud-américaines), ce qui suggèrent une origine du genre *Biomphalaria* sur le continent sud-américain et plus récente sur le continent africain (Campbell, et al. 2000; DeJong, et al. 2001). Ce serait grâce à la capacité d'hermaphrodisme du mollusque, que l'expansion sur tout le continent africain

Introduction

aurait été possible. Ces nouvelles analyses phylogénétiques confirment les hypothèses avancées dans la fin des années 90, grâce à des études d'allozymes (Bandoni, et al. 1995; Woodruff and Mulvey 1997).

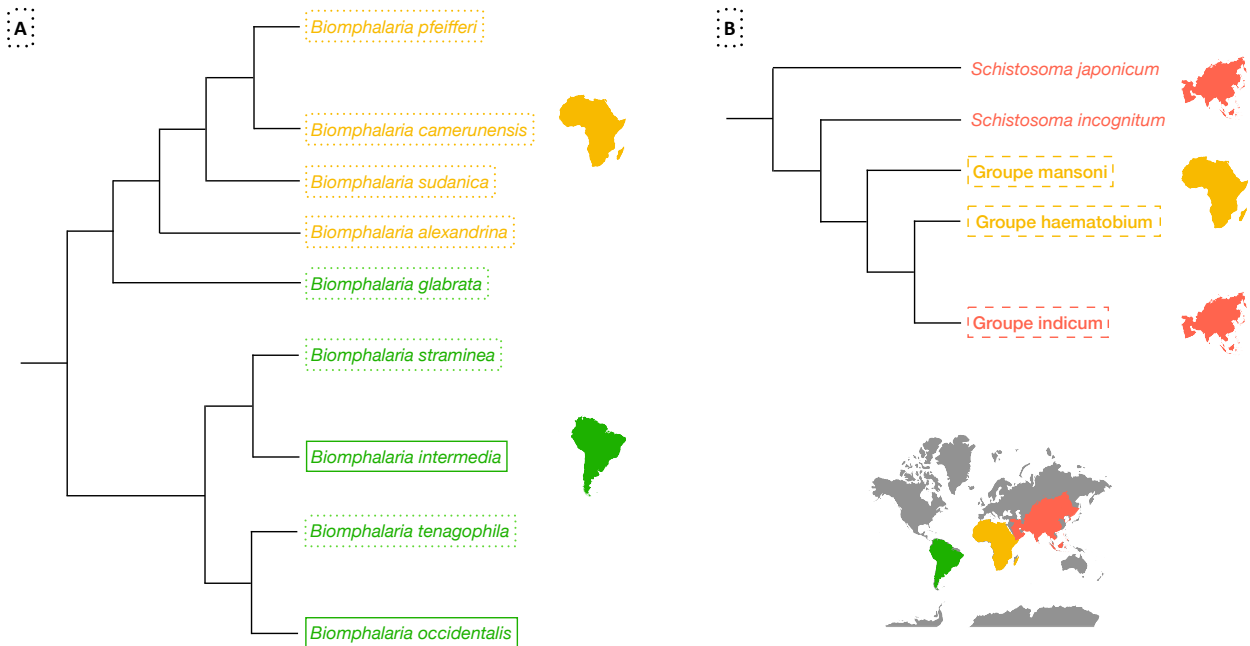


Figure 4 : Représentation des arbres phylogénétiques de *Biomphalaria* sp. et *Schistosoma* sp.

Basé sur (DeJong, et al. 2001; Lockyer, et al. 2003; Lockyer, et al. 2004). Les espèces (ou groupes) jaunes sont des espèces africaines, les rouges sont des espèces asiatiques et enfin les vertes sont des espèces sud-américaines. A. Arbre phylogénétique simplifié de *Biomphalaria* sp., les espèces encadrées par des pointillés sont naturellement susceptibles à *S. mansoni* alors que les espèces encadrées par des traits pleins sont naturellement résistantes. B. Arbre phylogénétique simplifié de *Schistosoma* sp., les groupes encadrés représentent un ensemble d'espèces de schistosomes.

De plus, l'étude phylogénétique révèle deux clades distincts du genre *Biomphalaria* (fig. 4A). En effet, les espèces africaines sont regroupées avec comme base, l'espèce sud-américaine, *B. glabrata* en un premier clade, qui apparaît comme un groupe monophylétique, quant au second, il contient toutes les autres espèces néotropicales, hormis *B. glabrata* qui s'en distingue (Campbell, et al. 2000; DeJong, et al. 2001). Les deux clades formeraient un groupe paraphylétique (Campbell, et al. 2000). Ceci confirme alors que le proto-*Biomphalaria glabrata* serait à l'origine de toutes les espèces africaines (DeJong, et al. 2001) et par conséquent *B. glabrata* serait plus proche génétiquement des taxa africains que des néotropicaux (Campbell, et al. 2000). Les études menées par l'équipe de Campbell confirment également que l'origine de l'expansion de *Biomphalaria* en Afrique daterait du Plio-Pleistocène (entre 1,8 et 3,6 millions d'années), cette

Introduction

colonisation récente est confirmée par la faible variance morphologique observable entre les différentes espèces africaines de *Biomphalaria* (Campbell, et al. 2000). Cette colonisation aurait été possible grâce aux masses d'œufs emprisonnées dans les pattes, le bec ou encore les plumes des échassiers migrants entre ces deux continents (Vuilleumier and Andors 1993). Les mollusques ont pu également être transportés par des amas de végétaux à la dérive à travers l'océan Atlantique, ce phénomène étant favorisé par la période interglaciaire du moment, qui favorise la reforestation de plaines.

En parallèle des études sur l'histoire évolutive du genre *Biomphalaria*, d'autres équipes travaillent sur celle du genre *Schistosoma*. Cette fois-ci, l'origine des *Schistosomatidae* serait d'origine asiatique (Lockyer, et al. 2003; Lockyer, et al. 2004; Morgan, et al. 2005; Crellen, et al. 2016). L'histoire évolutive des schistosomes est complexe et pleine de radiations (fig. 4B). En effet, *Schistosoma japonicum* serait à la base des schistosomes asiatiques et africains, avec *Schistosoma incognitum* (retrouvé exclusivement en Asie) (Lockyer, et al. 2003). Les schistosomes seraient arrivés en Afrique de l'Est (Morgan, et al. 2005; Crellen, et al. 2016) depuis l'Asie et auraient donné le groupe des mansoni, comprenant *Schistosoma mansoni* et *Schistosoma rodhaini* (Lockyer, et al. 2003; Lockyer, et al. 2004; Morgan, et al. 2005; Crellen, et al. 2016). Une seconde radiation serait survenue sur le continent africain, donnant le groupe africain haematobium, suivi d'une re-colonisation du continent indien et donner naissance au groupe indicum. Cette dernière origine ayant été confirmée par données moléculaires (Lockyer, et al. 2003).

En étudiant les histoires évolutives respectives de *Biomphalaria* et *Schistosoma*, tous les scientifiques se sont aujourd'hui accordés pour affirmer que les deux partenaires se sont rencontrés et ont commencé leur longue interaction sur le continent africain (fig. 5). Il semblerait que *Schistosoma mansoni* fut déjà présent sur le continent africain lors de l'arrivée de *Biomphalaria*. Nous assisterions donc ici à un changement d'hôte. Le premier hôte de *Schistosoma mansoni* reste un mystère, mais l'hypothèse qu'il provienne du genre *Lymnaea* ou *Bulinus* (Morgan, et al. 2005) a été avancée. En effet, ils sembleraient que ces deux genres de mollusques soient de bons candidats car ils nécessiteraient le moins d'adaptation à leur nouvel hôte, *Biomphalaria*.

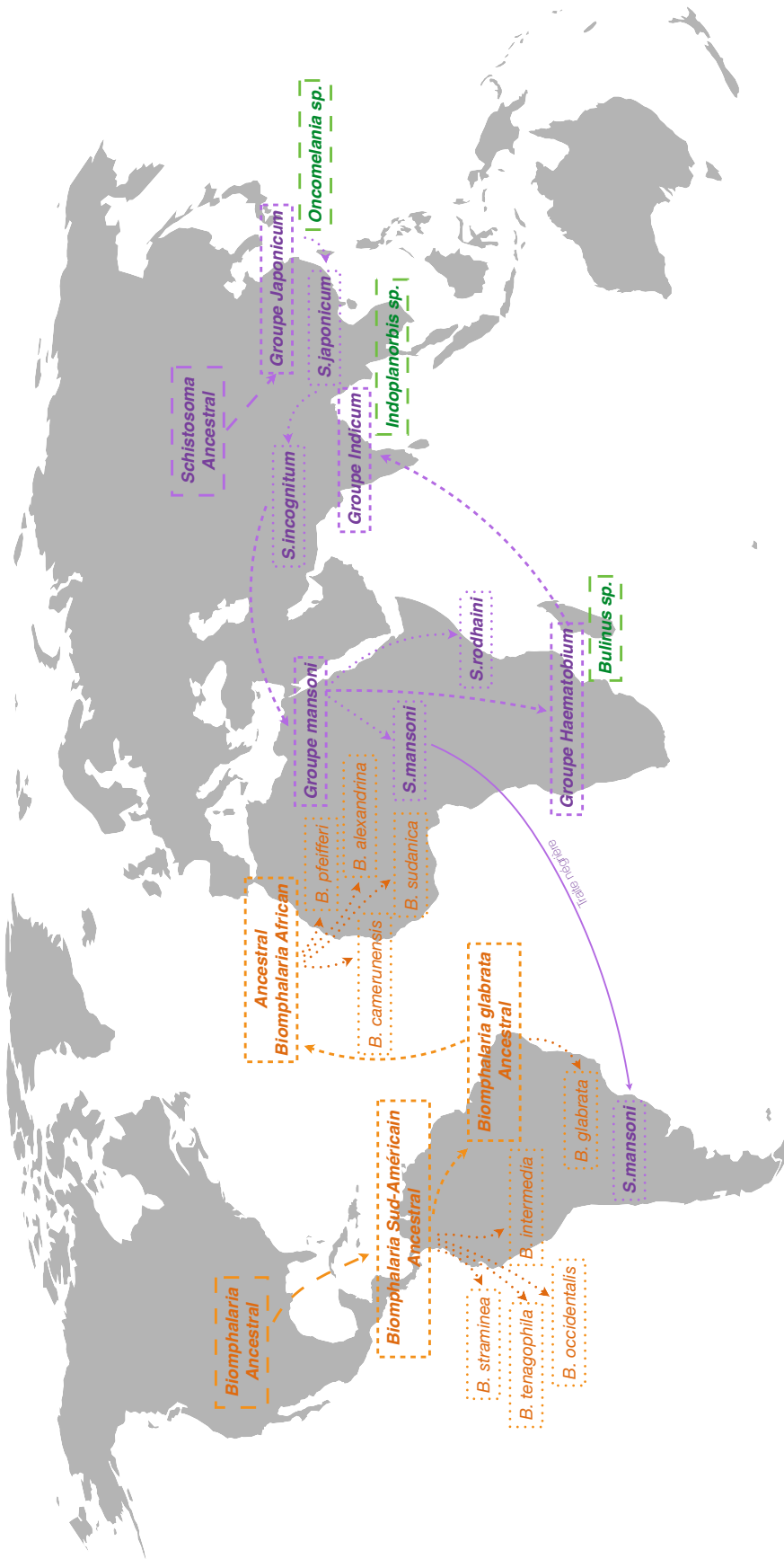


Figure 5 : Histoire évolutive de *Biomphalaria sp.* et *Schistosoma sp.*

Adapté d'après (Lockyer, et al. 2004). Représentation de l'histoire évolutive du mollusque et du parasite au cours des temps géologiques. Les deux encadrements par des grands tirets représentent les espèces ancestrales de mollusques et de parasites. Les encadrements pointillés représentent les espèces de mollusques et de parasites. Et enfin les encadrements par des petits tirés correspondent, pour le parasite, aux groupes d'espèces de parasites et pour le mollusque, aux espèces ancestrales donnant naissance à plusieurs espèces. Les flèches pointillées correspondent aux différentes évolutions. La flèche pleine correspond à l'arrivée récente de *S. mansoni* en Amérique latine au cours de la traite négrière.

Introduction

De plus, *Schistosoma incognitum*, qui est le schistosome à la base des parasites africains a comme hôte intermédiaire un mollusque du genre *Lymnaea* (Agatsuma, et al. 2002; Lockyer, et al. 2003), ce qui suggère que les premiers hôtes intermédiaires des schistosomes africains n'étaient pas des *Planorbidae*.

Beaucoup plus tard au XV^{ème} siècle, à la faveur de la traite négrière, les schistosomes ont été transportés jusqu'au nouveau monde, où ils ont retrouvé le descendant du *proto-Biomphalaria* présent en Amérique du Sud et appelé : *Biomphalaria glabrata* (fig. 5). En effet aujourd'hui, *Schistosoma mansoni* est un schistosome humain répandu à travers le monde entier, en Afrique, à Madagascar, ou encore dans la Péninsule Arabique, mais aussi dans le Nouveau Monde (Morgan, et al. 2005). Des études plus récentes se sont alors intéressées à essayer de définir l'origine africaine plus exacte du parasite sud-américain (Morgan, et al. 2005; Crellen, et al. 2016). Selon leurs répartitions sur le continent sud-américain, les parasites semblent plus ou moins proches phylogénétiquement. Les schistosomes du Nouveau Monde apparaissent phylogénétiquement plus proches de ceux retrouvés sur la côte ouest de l'Afrique (Morgan, et al. 2005).

En étudiant les différentes souches de parasites retrouvés en Amérique latine, il apparaît que plusieurs haplotypes soient aussi présents sur ce continent. Ces différents haplotypes ne semblent pas pouvoir provenir de phénomènes de mutations étant donné le faible temps géologique depuis lequel ils ont évolué sur le continent Américain. L'hypothèse serait plutôt une introduction multiple du parasite lors de la traite des esclaves (Morgan, et al. 2005). Malgré le fait de retrouver différents haplotypes sur le continent sud-américain, tous les haplotypes brésiliens sont identiques d'après leur ADN mitochondrial, ce qui suggère un effet fondateur de l'établissement de l'expansion du parasite dans ce pays (Morgan, et al. 2005). De plus, le faible nombre d'haplotypes différents (7), montre que la diversité génétique dans le Nouveau Monde a été réduite par rapport à l'ancêtre éloigné ouest-africain (Morgan, et al. 2005). À ce jour, plus de précision sur le pays d'origine des parasites sud-américains, est pour l'instant, très difficile à mettre en évidence, la seule hypothèse émise serait une histoire proche entre les parasites du Nouveau Monde et ceux provenant du Liberia. En effet en étudiant l'haplotype brésilien le plus communément retrouvé en Amérique latine, il apparaît identique à celui provenant du Liberia, mais la souche africaine étant maintenue en laboratoire depuis plus de 50 ans, ce n'est aujourd'hui qu'une hypothèse (Morgan, et al. 2005).

Introduction

Maintenant que l'histoire évolutive des deux partenaires de l'interaction est plus claire, quand est-il de l'histoire de leur interaction ?

Toutes les espèces du genre *Biomphalaria* ne jouent pas un rôle équivalent dans la transmission de la schistosomose, et les taxa de mollusques susceptibles aux différentes espèces de parasites ne sont pas homogènes suivant la phylogénie (fig. 4A).

Il apparaît chez les espèces Néotropicales de *Biomphalaria* que la susceptibilité à *Schistosoma mansoni* ne soit pas une synapomorphie. En effet, il semblerait que les hôtes les plus susceptibles appartiennent au clade de *B. glabrata* et des espèces africaines, mais pas exclusivement, des espèces, du second clade de *Biomphalaria sp.*, présentent aussi une susceptibilité à *S. mansoni* (DeJong, et al. 2001). En effet, sur le continent sud-américain, il semblerait que l'acquisition de la susceptibilité naturelle à *Schistosoma mansoni* soit apparue de manière indépendante chez les espèces comme *Biomphalaria straminea* et *Biomphalaria tenagophila* (DeJong, et al. 2001) (fig. 4A). Il apparaît que deux espèces de mollusques soeurs peuvent être, l'une naturellement hôte de *S. mansoni* et l'autre naturellement résistante (DeJong, et al. 2001). Ces profils suggèrent l'existence inhérente de mollusques susceptibles et résistants dans le milieu naturel au sein d'une même population. Il ne faut pas omettre les capacités intrinsèques d'adaptation du parasite à des mollusques dits résistants (DeJong, et al. 2001). En conclusion, il semblerait que la relation proche entre des espèces de mollusques susceptibles et résistantes démontre que le changement génétique affectant la susceptibilité n'est pas nécessairement accompagné d'une grande différence morphologique ou encore d'un isolement géographique (DeJong, et al. 2001).

Pour ce qui est du continent africain, *Biomphalaria pfeifferi* apparaît comme l'hôte naturel le plus important pour *Schistosoma mansoni* (DeJong, et al. 2001). Le genre *Biomphalaria* est l'hôte préférentiel pour le groupe des mansoni, alors que le groupe indicum réaliserait son cycle de vie dans des mollusques du genre *Indoplanorbis* et finalement le groupe des *haematobium* dans le genre *Bulinus* (Lockyer, et al. 2004).

La présence de plusieurs lignées de parasites distinctes en Afrique suggère des introductions de parasites indépendantes ainsi que des transferts latéraux de schistosomes entre les hôtes humains, rongeurs ou ongulés (Lockyer, et al. 2003). De plus la dispersion des rongeurs et petits mammifères sur le continent africain serait le facteur clé de la radiation des schistosomes en Afrique (Attwood, et al. 2003).

Finalement, il semblerait que la séparation géographique (et non l'hôte utilisé) apparaît être le facteur le plus important de la diversification des parasites (Morgan, et al. 2005).

Introduction

Si nous affinons l'échelle d'observation de l'interaction entre *Biomphalaria* et *Schistosoma*, et que nous nous focalisons sur l'interaction entre *B. glabrata* et *S. mansoni*, nous pouvons alors nous intéresser aux mollusques et aux parasites venant de plusieurs localités géographique d'Amérique latine. Nous disposons, au laboratoire de nombreuses souches de mollusques et de parasites venant du Brésil, du Vénézuéla et de Guadeloupe. Lorsque nous faisons des croisements, en laboratoire, de ces différentes souches, nous pouvons observer différents niveaux de susceptibilité du mollusque et d'infectivité du parasite, ceci nous amène alors au concept de polymorphisme de compatibilité en populations naturelles.

Le Polymorphisme de Compatibilité

Au sein d'une interaction hôte/parasite il est possible de suivre la virulence et la résistance de chacun des acteurs de l'interaction. Une sélection réciproque de la résistance de l'hôte et l'infectivité du pathogène amène à des processus de co-évolution ou d'adaptation de ces systèmes hôte-parasite. Le modèle *Biomphalaria glabrata/Schistosoma mansoni* présente une interaction complexe entre les mécanismes de défense du mollusque et les stratégies d'infection du parasite. Ainsi en fonction des phénotypes exprimés par chaque protagoniste en milieu naturel il est possible que certains couples hôte/parasite soient compatibles et d'autres incompatibles, voir qu'un même hôte puisse être infecté ou puisse éliminer différents individus d'une même espèce de parasite en milieu naturel. Ce phénomène appelé le « Polymorphisme de Compatibilité » fut décrit au laboratoire en 1998 (Langand and Morand 1998).

Introduction

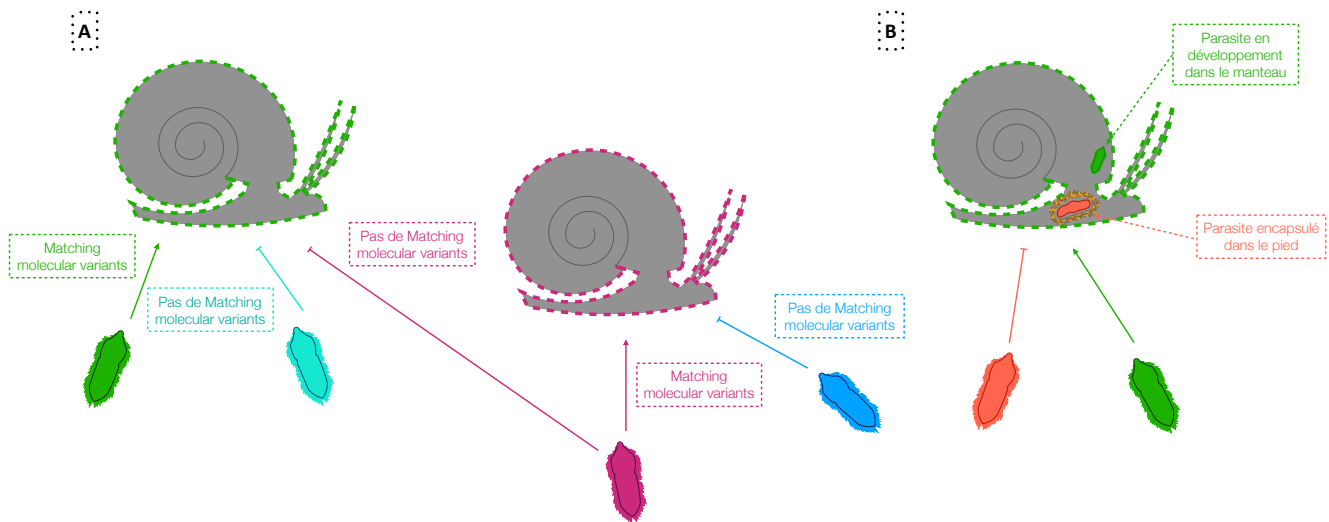


Figure 6 : Représentation schématique du Polymorphisme de compatibilité

D'après papier (Théron, et al. 1997; Langand and Morand 1998; Theron, et al. 2008). A. En population naturelle, dans une même population des couples hôte/parasite peuvent être compatibles, les variants moléculaires sont alors compatibles, et d'autres peuvent être incompatibles, cette fois ci les variants moléculaires des deux protagonistes ne sont pas compatibles. B. Interaction hôte/parasite à l'échelle individuelle, parasite en développement ou encapsulé dans les tissus d'un même individu hôte.

Le polymorphisme de compatibilité, met en évidence plusieurs concepts clés de l'interaction entre le mollusque et le parasite. En effet, des interactions seront dites incompatibles et d'autres compatibles. Une interaction est dite incompatible lorsque le parasite n'a pas les capacités d'infecter le mollusque et donc est incapable de compléter son cycle de vie. Il est alors reconnu par le système immunitaire de son hôte et éliminé. Par opposition, une interaction est dite compatible lorsque le parasite parvient à infecter le mollusque, et à compléter son cycle de vie et donc à pérenniser sa transmission. Ce polymorphisme de compatibilité met donc en évidence la résistance et la susceptibilité du mollusque face à l'infection parasitaire ainsi que les capacités d'infectivité du parasite (fig. 6).

En population naturelle, au sein d'une même population des couples hôte/parasite peuvent donc être compatibles alors que d'autres seront incompatibles (Basch 1975; Théron, et al. 1997; Webster and Davies 2001) (fig. 6A). De plus, lorsque la compatibilité est testée indépendamment pour chaque couple d'hôte/parasite, on observe à l'intérieur d'un même mollusque des parasites qui se développent et d'autres qui sont reconnus par le système immunitaire de l'hôte et encapsulés (Théron, et al. 2005) (fig. 6B).

Introduction

Lors d'une interaction hôte/parasite, il ne faut pas oublier que les deux acteurs jouent un rôle dans l'issue de l'interaction. Chacun exprime un profil moléculaire qui lui est propre et qui est à la base du phénotype de compatibilité dans la correspondance ou la non correspondance de ces molécules qui définissent alors l'hypothèse des « matching molecular variant » ou « matching phenotype » (Théron, et al. 2005; Mitta, et al. 2017). Ce concept met en évidence des mécanismes de résistance du côté du mollusque et de virulence du côté du parasite, nous sommes donc à une échelle individuelle. C'est l'ensemble de toutes ces interactions deux à deux entre les mollusques et les parasites qui établis alors la compatibilité ou l'incompatibilité d'une interaction visible à l'échelle individuelle et/ou populationnelle.

Par conséquence, l'infectivité du parasite et la susceptibilité du mollusque contribuent à une haute spécificité d'interaction.

Le dialogue moléculaire entre le mollusque et le parasite, responsable du polymorphisme de compatibilité que l'on observe dans l'interaction hôte/parasite est observé au niveau de la résistance ou susceptibilité du mollusque et de l'infectivité du parasite. Il semble donc que ce dialogue moléculaire s'exprime essentiellement au niveau des processus immunitaires entre les deux partenaires, il apparaît donc important de s'intéresser au concept d'immunité chez *Biomphalaria* et plus largement chez les invertébrés.

L'immunité

En biologie, l'immunité est la capacité intrinsèque d'un organisme à posséder des défenses biologiques et à se défendre contre une agression infectieuse (tels que des bactéries, virus, parasites), une maladie donnée ou encore contre des substances étrangères, ou la défaillance des cellules de l'organisme.

Le système immunitaire ou système immunologique, qui comprend un ensemble coordonné d'éléments de reconnaissances et de défenses qui permettront de lutter contre les différentes attaques.

L'immunité des invertébrés

Chez les invertébrés, lors de l'intrusion d'un pathogène, une succession d'étapes amène à la mise en place d'une réponse immunitaire coordonnée qui concourt dans le meilleur des cas à l'élimination du pathogène. Dans un premier temps, l'intrus est reconnu par des

Introduction

molécules de reconnaissances et des récepteurs immuns, par la suite des voies de signalisations et effectrices sont activées pour induire une réponse adaptée permettant l'élimination du pathogène. Deux grandes réponses sont mises en place, la réponse cellulaire dont font partie les hémocytes (= cellules immunitaires des invertébrés, retrouvées dans l'hémolymphe) et la réponse humorale, dont les molécules effectrices seront produites par les hémocytes mais également par d'autres tissus ou organes ayant des fonctions immunitaires (comme le corps gras, l'organe hématopoïétique, ou encore la glande albumen).

La reconnaissance

Chez les invertébrés, la reconnaissance est réalisée grâce à trois grands types de récepteurs, qui reconnaîtront spécifiquement des motifs moléculaires associés aux différents pathogènes appelés des (PAMP : Pathogen Associated Molecular Pattern). Certains récepteurs reconnaissent les composés des parois cellulaires des pathogènes (PGRP, GGBP, LGBP, PGBP, BGBP), d'autres récepteurs qui reconnaîtront des motifs moléculaires spécifiques (TLR, NLR) et enfin le dernier type de récepteur est constitué de molécules hypervariables (DSCAM, SRGR, Sp183/333, FREP, CREP et GREP) qui peuvent avoir des fonctions de lectines pour certains, pour d'autres la nature des ligands n'est pas connue. Les lectines sont d'ailleurs le dernier type de récepteur particulièrement bien décrit chez les invertébrés. Les capacités de reconnaissance des lectines sont particulièrement basées sur la liaison aux glycanes ou glycoprotéines portés par les pathogènes.

Les bactéries gram-négatifs, les bactéries gram-positifs, les levures ou les champignons présentent des composants de surfaces spécifiques qui pourront être reconnus différemment par différents récepteurs immuns. Les bactéries gram-négatifs, présentent une membrane externe composée essentiellement de lipopolysaccharides (LPS), les bactéries gram-positifs ne présentent pas de membrane externe et seront reconnues grâce aux peptidoglycanes (PGN) qui composent leur paroi cellulaire. Enfin les levures et les champignons possèdent une paroi cellulaire composée de β -glucanes qui seront reconnus par les récepteurs spécifiques. La reconnaissance de ces différents pathogènes sera possible grâce à des récepteurs immuns spécifiques.

Introduction

Reconnaissance des composés de la paroi cellulaire des pathogènes :

Les *Peptidoglycan Recognition Protein* (PGRP), sont classées selon leur formes courtes (S) et longues (L), dans la plupart des cas les formes longues sont retrouvées dans l'hémolymphe, ou encore à la surface des cellules du corps gras et des hémocytes alors que les formes courtes, elles, seront principalement exprimées par les hémocytes (Dziarski and Gupta 2006). Les PGRP (L/S) ont été mises en évidence dans la réponse immunitaire des insectes, des échinodermes, ainsi que chez *B. glabrata*. En effet, chez la drosophile, les PGRP-L, reconnaissent les LPS à la surface des bactéries gram-négatifs et sont impliquées dans leur élimination (Hoffmann 2003; Ferrandon, et al. 2007; Lemaitre and Hoffmann 2007) alors que les PGRP-S, eux, sont impliqués dans la réponse contre les bactéries gram-positifs, suite à la reconnaissance des PGN de surface des bactéries (Hoffmann 2003; Ferrandon, et al. 2007; Lemaitre and Hoffmann 2007). Chez les moustiques, principalement chez les *Anopheles sp.* et *Aedes sp.*, les PGRP-L sont impliqués dans la réponse anti-bactérienne (Christophides, et al. 2002; Dimopoulos, et al. 2002; Cirimotich, et al. 2011) ainsi que dans la réponse anti-plasmodique, mais uniquement contre *Plasmodium berghei* (Cirimotich, et al. 2011). Les PGRP-S, eux, sont retrouvés dans la réponse anti-plasmodique uniquement, mais cette fois-ci contre *Plasmodium falciparum* (Cirimotich, et al. 2011). Ici, non seulement, nous avons une reconnaissance des pathogènes bactériens, mais en plus, ces récepteurs sont capables de reconnaître spécifiquement deux souches de parasites *Plasmodium sp.*. Chez les échinodermes, 5 gènes de PGRP ont été identifiés (Hibino, et al. 2006) mais leur implication dans la réponse anti-bactérienne n'a pas encore été mise en évidence.

Chez *Biomphalaria glabrata*, 3 gènes de PGRP-L et 1 gène de PGRP-S ont été mise en évidence lors d'infection bactérienne (Deleury, et al. 2012; Coustau, et al. 2015; Adema, et al. 2017). De plus, lors d'une infection du mollusque par des trématodes parasites le niveau d'expression de PGRP-S est moins important que chez des mollusques naïfs (Coustau, et al. 2015; Adema, et al. 2017). Ce récepteur est potentiellement impliqué dans la reconnaissance des pathogènes. De plus 5 nouveaux gènes de PGRP ont récemment été identifié avec l'analyse du génome de ce mollusque (Adema, et al. 2017).

Les *Gram-Negative Binding Protein* (GNBP), sont des molécules principalement présentes dans l'hémolymphe. Comme les PGRP (L/S), elles ont été mises en évidence dans la réponse immunitaire des insectes et des échinodermes, ainsi que chez *B. glabrata*. En effet, chez la drosophile, les GNBP-1, reconnaissent les PGN de la paroi cellulaire des bactéries gram-positifs et sont impliquées dans leur élimination (Hoffmann

Introduction

2003; Ferrandon, et al. 2007; Lemaitre and Hoffmann 2007). Les β -glucanes des levures et des champignons, sont reconnus par les GGBP-3, et éliminés (Hoffmann 2003; Ferrandon, et al. 2007; Lemaitre and Hoffmann 2007). Chez les moustiques, les GGBP ont été mis en évidence dans la reconnaissance de pathogènes variés. En effet, ils reconnaissent à la fois les bactéries gram-positifs (Dimopoulos, et al. 1997), les plasmodiums (Christophides, et al. 2002; Cirimotich, et al. 2011) mais aussi le virus de la dengue (Xi, et al. 2008). Finalement, chez les échinodermes, comme pour les PGRP, 3 gènes de GGBP (Hibino, et al. 2006) ont été identifiés mais pas de mise en évidence dans la réponse contre de potentiels pathogènes.

Chez *Biomphalaria glabrata*, 1 gène de GGBP a été mise en évidence lors d'infection bactérienne et de trématode (Deleury, et al. 2012; Coustau, et al. 2015; Adema, et al. 2017). Comme pour les PGRP-S, il semblerait que le GGBP soit potentiellement impliqué dans la reconnaissance des pathogènes (Coustau, et al. 2015; Adema, et al. 2017). L'analyse du génome, permet de confirmer l'existence d'un seul gène (Adema, et al. 2017).

Chez les crustacés, 3 autres récepteurs de reconnaissances ont été mis en évidence dans la réponse aux bactéries et levures/champignons. Les *Lipopolysaccharide Binding Protein* (LGBP), impliquées dans la reconnaissance des bactéries gram-négatifs (Vazquez, et al. 2009), les *Peptidoglycan Binding Protein* (PGBP), impliquées dans la reconnaissance des bactéries gram-positifs (Vazquez, et al. 2009) et enfin les *β -1,3-glucan Binding Protein* (BGBP) impliquées dans la reconnaissance des levures et des champignons (Vazquez, et al. 2009).

Reconnaissance de motifs moléculaires spécifiques (TLR, NLR) :

Les Toll-Like Receptor (TLR), sont des protéines retrouvées dans tout le règne animal, ils appartiennent à la famille des récepteurs de reconnaissances de motifs moléculaires. Chez l'homme, ces récepteurs interviennent dans l'immunité innée et la reconnaissance de motifs très conservés (Hoffmann 2003). Ils sont souvent composés d'un domaine extracellulaire, qui sert de récepteur et d'un domaine intracellulaire permettant la transduction du signal d'activation ; ils contiennent de nombreux motifs LRR (Leucine-Rich Repeat). Identifiés chez les insectes, 9 TLR ont été mis en évidence chez *Drosophila melanogaster* (Du, et al. 2000; Tauszig, et al. 2000) et 10 chez *Anopheles gambiae* (Christophides, et al. 2002). À noter tout de même, les TLR ne semblent pas avoir tout à fait le même rôle chez les insectes que chez les mammifères. En effet, il

Introduction

apparaît que ces récepteurs joueraient non seulement un rôle de reconnaissance des pathogènes (Nakamoto, et al. 2012) mais également un rôle de cytokine dans la réponse immunitaire des insectes (Ferrandon, et al. 2004). Chez l'oursin, *Strongylocentrotus purpuratus*, la diversification de ce récepteur est impressionnante, l'étude du génome recense pas moins de 222 gènes codant pour des TLR (Hibino, et al. 2006), mais leur implication directe dans l'immunité n'a pas encore été démontrée.

Chez *Biomphalaria glabrata*, 56 gènes codants pour des TLR ont été mis en évidence dans le génome, dont 27 complet (Adema, et al. 2017). De plus une réelle implication de ces récepteurs dans la réponse immunitaire contre *S. mansoni* fut montrée récemment grâce à une expérience de knock-down (Pila, Tarrabain, et al. 2016).

Les Nucleotide-binding Oligomerization Domain (NOD)-Like Receptor (NLR), sont des protéines cytoplasmiques impliquées dans la reconnaissance de motifs moléculaires spécifiques ; impliquées notamment dans la reconnaissance des PGN de bactéries gram-négatifs lors de la réponse immunitaire innée chez l'Homme. Ces protéines cytoplasmiques, contiennent des domaines NOD et LRR, ce sont eux qui reconnaîtront la présence de pathogènes, cette reconnaissance entrainera alors un changement de conformation du récepteur. Chez les invertébrés, elles ont été mises en évidence uniquement chez l'oursin, *Strongylocentrotus purpuratus*, et comme pour les TLR, la diversification de ce récepteur est très importante ; toujours lors de l'étude du génome, 203 gènes codants pour ces récepteurs ont été mis en évidence, mais à ce jour leur implication dans la réponse immunitaire reste encore à démontrer (Hibino, et al. 2006).

Molécules hypervariables impliquées dans la reconnaissance des pathogènes :

Les Down Syndrome Cell Adhesion Molecules (DSCAM), sont des molécules qui appartiennent à la grande famille des immunoglobulines. Elles ont été initialement mise en évidence chez les vertébrés, comme étant impliquées dans l'adhésion cellulaire principalement dans la guidance axonique et la connectivité des neurones dans le système nerveux (Hattori, et al. 2007). Elles ont ensuite été identifiées dans le même contexte, de guide axonique, chez *Drosophila melanogaster* (Schmucker, et al. 2000). Puis les travaux de l'équipe de Watson, en 2005, ont montré l'implication des DSCAM dans la protection immunitaire des drosophiles (Watson 2005). Caractérisée, il y a 10 ans, chez *Drosophila melanogaster*, les études montrent que cette molécule est impliquée dans la défense immunitaire des insectes et le travail sur *Anopheles gambiae* indique qu'il s'agit d'un récepteur immunitaire hypervariable. Les études montrent aussi que la

Introduction

diversification somatique des DSCAM leur permet de produire une diversité inattendue de molécules immunitaires, posant l'hypothèse des bases mécanistiques de la spécificité de la réponse immunitaire spécifique chez les insectes (Armitage, et al. 2015). Cependant, il ne faut pas oublier, les hypothèses alternatives qui ont été envisagées dans les fonctions possibles des DSCAM, comme leur rôle dans les interactions cellule-cellule de l'hôte (Brites, et al. 2008). Nous sommes encore loin d'une compréhension complète du fonctionnement des DSCAM en relation avec les parasites et les agents pathogènes et leur véritable rôle dans le système immunitaire (Armitage, et al. 2017). De futures études devraient cependant mettre en évidence le rôle des DSCAM dans la reconnaissance et la réponse immunitaire des pancrustacés dans les prochaines années.

Nous savons qu'elles possèdent une vaste diversification somatique avec un potentiel d'expression de 18 000 isoformes (Watson 2005). Ce sont des protéines qui peuvent exister sous la forme transmembranaire, mais aussi soluble, qui sont sécrétées et détectées dans l'hémolymphe (Watson 2005). Ces DSCAM ont aussi été identifiées chez d'autres invertébrés comme chez *Anopheles gambiae* (Dong, et al. 2006), ainsi que chez les crustacés *Daphnia magna* et *Daphnia pulex* (Brites, et al. 2008) ou encore chez *Litopenaeus vannamei* (Chou, et al. 2009). Diverses expériences ont montré l'implication de ces molécules dans la réponse immunitaire des invertébrés. Des expériences d'ARN interférents montrent une diminution de l'activité de phagocytose des hémocytes d'environ 30 % chez les drosophiles et de 50 % chez les anophèles ainsi que la diminution de la survie du moustique suite à une infection bactérienne (Watson 2005; Dong, et al. 2006). De plus, l'infection du moustique, par un plasmodium, n'induit pas une augmentation de l'expression des DSCAM, mais un changement de répertoire des transcrits exprimés et ceux de manière différente selon la souche de plasmodium utilisée (*Plasmodium falciparum* et *Plasmodium berghei*) (Dong, et al. 2006). Chez la crevette, *Litopenaeus vannamei*, il semblerait que les DSCAM soient impliquées dans la réponse anti-virale (Chou, et al. 2009).

Les DSCAM sembleraient donc être des récepteurs immunitaires hautement diversifiés impliqués dans la reconnaissance immunitaire chez les pancrustacés (crustacés + hexapodes), jouant un rôle dans la réponse immunitaire face à différents stimuli (Armitage, et al. 2015; Hann, et al. 2015).

Les *Scavenger Receptor Cysteine-Rich* (SRCR), sont des protéines exprimées dans les hémocytes des invertébrés, découvertes dans un premier temps dans les coelomocytes (= hémocytes, cellules immunitaires) de l'oursin *Strongylocentrotus*

Introduction

purpuratus. Elles constituent une super-famille de protéines présentes dans différents groupes de métazoaires, allant jusqu'aux mammifères. La plupart des SRCR connues ont une fonction immunitaire et sont impliqués soit dans la reconnaissance soit dans la régulation de la réponse immunitaire (Pancer 2000). Chez les mammifères, les macrophages expriment divers récepteurs contenant des domaines SRCR qui sont impliqués dans la régulation de la cascade du complément, la liaison à des lectines ou encore à des bactéries (Martínez, et al. 2011). Chez les oursins, les protéines SRCR semblent avoir une fonction de reconnaissance et peuvent être liées aux membranes ou bien être sécrétées (Pancer, et al. 1999). Il existe 7 types de SRCR différents chez l'oursin, qui diffèrent par leur nombre de domaines SRCR ou bien par la présence d'autres domaines protéiques (domaine VonWillebrand, domaine de liaison à la matrice, motifs EGF (Epidermal-Growth Factor) etc.) tous étant potentiellement impliqués dans l'adhésion cellulaire, allant dans le sens d'une fonction de reconnaissance. Les SRCR sont codés par une famille multigénique qui code pour pas moins de 1 200 SRCR différents (Pancer 2000).

D'autres invertébrés possèdent des SRCR, c'est d'ailleurs le cas des cnidaires, pour lesquels l'équipe de Neubauer a mis en évidence un rôle dans la modulation de la réponse immunitaire et la tolérance de l'hôte face à son symbiote (Neubauer, et al. 2016).

Les Sp185/333, sont aussi des protéines retrouvées chez l'oursin *Strongylocentrotus purpuratus* exprimées par les coelomocytes. C'est une famille de molécules extrêmement diversifiées, composées d'environ 50 à 60 membres, dont les gènes sont organisés en cluster (Terwilliger, et al. 2006; Buckley, et al. 2008). La grande variabilité des Sp185/333 est basée sur la présence/absence de certains éléments permettant de distinguer 22 variants différents (Terwilliger, et al. 2006). De plus, à la suite d'une stimulation par des bactéries gram-négatifs ou des champignons, il a pu être observé non seulement une augmentation de l'expression des Sp185/333, mais aussi un changement des variants majoritaires exprimés (Terwilliger, et al. 2007; Brockton, et al. 2008).

Les études menées sur les oursins mettent en évidence 2 familles de molécules hypervariables, hautement diversifiées et/ou hautement polymorphes qui laisse penser qu'elles pourraient avoir des fonctions immunitaires, mais ceci reste encore à être démontré.

Les Fibrinogens-Related Proteins (FREPs), famille de protéines hypervariables identifiées, chez *Biomphalaria glabrata* ; composées d'un ou deux domaines de la super

Introduction

famille des immunoglobulines (Immunoglobulin SuperFamily (IgSF) domain) ainsi que d'un domaine fibrinogène carboxyl-terminal (Adema, et al. 1997; Zhang, et al. 2004). Ce type de lectine présente un haut niveau de polymorphisme par l'intermédiaire de diversifications et de mutations somatiques générant un répertoire individuel diversifié de FREP (Lee, et al. 2004; Zhang, et al. 2004; Gordy, et al. 2015). Les FREP se diversifient encore d'avantage grâce à différent mécanisme, y compris l'épissage alternatif et la diversification somatique à travers des recombinaisons ectopiques et des mutations ponctuelles (Zhang, et al. 2004; Hanington, et al. 2012). Plus tard, l'importance de FREP3 dans le processus de compatibilité a pu être mise en évidence grâce à un knock-down de cette lectine, qui entraîne une baisse partielle de la résistance du mollusque de 21 % (Hanington, et al. 2012). Les FREP constituent la famille de gène de reconnaissance immunitaire la plus diversifiée décrite chez *B. glabrata* et participent activement à la réponse immunitaire anti-schistosomes (Hanington, et al. 2010; Moné, et al. 2010; Mitta, et al. 2012; Coustau, et al. 2015). Grâce au séquençage massif des transcrits de *Biomphalaria glabrata*, la famille des FREP a pu être agrandie avec 14 sous-familles, qui diffèrent de par leur composition nucléotidiques et structurale (Dheilly, Duval, et al. 2015).

Les autres types de lectines :

Les lectines sont des protéines qui se lient spécifiquement et de façon réversible à certains types de carbohydrates. Elles interviennent, chez les vertébrés, dans divers processus biologiques, principalement au niveau de la reconnaissance entre les cellules, et également dans la réponse immunitaire (Turner 1996). *Drosophila melanogaster* présente une activité transcriptionnelle plus importante de la lectin-24A lors d'un challenge par une guêpe parasitoïde, qui suggère une implication dans les processus de reconnaissance de ces pathogènes (Keebaugh and Schlenke 2012).

Parmi ces lectines, les C-type lectines, qui sont des lectines calcium dépendantes ; interviennent chez les mammifères dans de nombreuses fonctions, notamment dans l'adhésion cellule-cellule, et jouent un rôle dans la réponse immunitaire contre les agents pathogènes ainsi que dans l'apoptose (Zelensky and Gready 2005). Chez les invertébrés, des protéines orthologues aux C-type lectines des mammifères sont retrouvées (Pees, et al. 2016). Chez *Anopheles gambiae*, 23 gènes codant pour des C-type lectines ont été mis en évidence lors de l'analyse du génome (Christophides, et al. 2002). De plus, en 2009, il a pu être démontré que 2 C-type lectines étaient capables d'activer les effecteurs de la réponse immunitaire contre des bactéries gram-négatifs (Schnitger, et al. 2009).

Introduction

Chez *Strongylocentrotus purpuratus*, 77 gènes codant pour des lectines ont été mis en évidence dans le génome de l'oursin (Pinsino and Matranga 2015). Parmi ces 77, une lectine a reçu une attention toute particulière, la SpEchinoidin, qui est exprimée uniquement par les phagocytes activés par des LPS (Terwilliger, et al. 2004).

Chez *Biomphalaria glabrata*, il a été mis en évidence récemment d'autres molécules présentant un ou deux domaines IgSF en partie N-terminale associé à différents domaines lectines en C-terminal ; parmi elles se trouvent, les C-type lectin-related proteins (CREPs) et les Galectin-related protein (GREP) très similaires aux FREP, elles sont désignées comme des molécules contenant des domaines variables de lectines ou immunoglobulines (VIgL - Variable Immunoglobulin and Lectin domain containing molecules) (Dheilly, Duval, et al. 2015). La récupération de 4 séquences de CREP complètes suggère qu'elles constituent une nouvelle catégorie de lectines chez *B. glabrata*. La GREP identifiée, possédant une structure unique, révèle l'existence d'une nouvelle catégorie de galectine. Les galectines appartiennent à une famille de lectine à liaison β -galactoside et font partie des lectines les plus conservées dans le règne animal (Kilpatrick 2002). Elles sont composées d'un à quatre domaines galectine (Vasta, et al. 2012) et impliquées dans les interactions hôte-pathogène, par la reconnaissance de ligands exogènes, comme les glycanes à la surface des virus, des bactéries, des champignons et des protozoaires (Vasta, et al. 2012). En outre, une première galectine de *B. glabrata* (BgGal) fut identifiées lors d'une précédente étude, elle se classe parmi les « tandem-repeat galectin » car elle possède deux domaines de liaison aux carbohydrates (domaine CRB) en tandem. BgGAL a une activité d'hémagglutination, elle n'est pas détectée sous forme circulante dans l'hémolymphe déplétée de cellules, et elle est retrouvée sur la membrane plasmique de certaine sous-population d'hémocyte. Cette liaison, pouvant être inhibée en présence de sucres contenant du galactose, suggère que BgGal peut servir de récepteur de reconnaissance et reconnaître certains pathogènes possédant des déterminants glycosylés appropriés, et en particulier la surface du tégument des schistosomes (Yoshino, et al. 2008). Toutes ces observations appuient davantage l'idée que les GREP pourraient également jouer un rôle dans la réponse immunitaire de *B. glabrata* (Dheilly, Duval, et al. 2015).

Chez *B. glabrata*, la mise en évidence de ces nouvelles molécules montre un répertoire plus large de lectines, comme les FREP, la Bgselectin (Guillou, et al. 2007; Coustau, et al. 2015), ou encore les CREP (Hathaway, et al. 2010) ; elles sont composées d'un ou deux

Introduction

domaines IgSF étroitement liés avec un domaine lectine, qui peut être soit un fibrinogène (FBG), une lectine de type C (CLECT), soit une galectine (GLECT).

Les voies de signalisations

Suite à la reconnaissance des agents pathogènes, la réponse immunitaire se met en place par l'activation de voie de signalisation. Chez les invertébrés, nous distinguons 3 voies de signalisations principales, la voie IMD, la voie Toll et enfin la voie JAK-STAT (Ferrandon, et al. 2004; Lemaitre and Hoffmann 2007; Buchon, et al. 2009b; Cirimotich, et al. 2010; Gendrin, et al. 2017).

La voie IMD « Immune Deficiency Pathways » :

La voie de signalisation IMD, fut, dans un premier temps, caractérisée chez la drosophile ; impliquée dans la réponse antibactérienne, elle se déroule dans les cellules du corps gras de l'animal (Ferrandon, et al. 2004; Lemaitre and Hoffmann 2007; Buchon, et al. 2014). Cette voie de signalisation est activée par des molécules de reconnaissance telles que les PGRP-L, suite à l'infection par des bactéries gram-négatifs. L'activation de la voie Imd conduit à la translocation nucléaire du facteur de transcription κ B (NF- κ B) : Relish pour activer l'expression des peptides antimicrobiens (Antimicrobial peptide - AMP) tels que la *Diptericin* ; elle induit aussi la biosynthèse des espèces réactives de l'oxygène (ROS), avec notamment l'induction de la dual oxidase (Duox) (Ferrandon, et al. 2004; Lemaitre and Hoffmann 2007; Buchon, et al. 2014). Chez *Anopheles gambiae*, nous retrouvons aussi la voie IMD ; elle est induite lors d'une infection par *Plasmodium falciparum* ou des infections bactériennes (Cirimotich, et al. 2010). De plus, toujours chez *Anopheles sp.*, une activité de régulation de la charge microbienne, composant le microbiote, implique aussi la voie de signalisation IMD (Clayton, et al. 2014; Gendrin, et al. 2017).

Toll Pathways (Toll) :

La voie de signalisation Toll, fut, elle aussi caractérisée, en premier chez la drosophile ; impliquée dans la réponse antibactérienne et antifongique, la reconnaissance des pathogènes et son activation se déroulent dans l'hémolymphe, grâce à des récepteurs solubles, et dans des cellules du corps gras, pour la fabrication des AMP (Ferrandon, et al. 2004; Lemaitre and Hoffmann 2007; Buchon, et al. 2014). Cette voie de signalisation est activée par des molécules de reconnaissance telles que les PGRP-S et

Introduction

les GGBP-1 en réponse aux bactéries gram-positifs et les GGBP-3 en réponse aux levures et champignons. L'activation de la voie Toll active une cascade protéolytique qui aboutit à la maturation de la cytokine Spätzle, qui conduit finalement à la translocation nucléaire du facteur de transcription NF- κ B Dif pour activer l'expression de AMP tels que la *Drosomycine* (Ferrandon, et al. 2004; Lemaitre and Hoffmann 2007; Buchon, et al. 2014). Chez le moustique, cette voie de signalisation est décrite de manière très complète dès 2002 (Christophides, et al. 2002) ; par ailleurs, elle est impliquée dans la réponse antivirale contre le virus de la dengue chez *Aedes sp.* (Xi, et al. 2008), ainsi que dans la réponse anti-plasmodium contre *Plasmodium berghei* chez *Anopheles sp.* (Cirimotich, et al. 2011; Garver, et al. 2012; Dennison, et al. 2016). De plus, toujours chez *Anopheles sp.*, une activité de régulation de la charge microbienne, composant le microbiote, implique aussi la voie de signalisation Toll (Clayton, et al. 2014; Dennison, et al. 2016).

Janus Kinase (JAK)-STAT Pathways (JAK-STAT):

La voie de signalisation JAK-STAT comporte trois composantes cellulaires principales : le récepteur Domeless, la Kinase Janus (JAK) et le facteur de transcription STAT (Agaisse and Perrimon 2004). Malgré une connaissance moins précise des fonctionnements de cette voie de signalisation, il semblerait que, chez *Drosophila melanogaster*, elle soit plutôt impliquée dans la réponse anti-virale ainsi que dans la réponse aux dommages causés par l'infection ou par des toxines (Ferrandon, et al. 2004; Lemaitre and Hoffmann 2007; Buchon, et al. 2014). Il semblerait que l'activation de la voie JAK-STAT ne soit pas induite pas des produits microbiens, mais par la sécrétion, par les hémocytes, de la cytokine Upd3 (Vanha-aho, et al. 2016), qui activerait ensuite la voie de signalisation (Ferrandon, et al. 2004; Lemaitre and Hoffmann 2007; Buchon, et al. 2014). Le rôle précis de cette voie et sa contribution globale à la défense de l'hôte reste à établir. Chez les moustiques, il semblerait que cette voie de signalisation se soit spécialisée dans la réponse antivirale uniquement, elle est d'ailleurs activée en réponse aux virus de la dengue, du chikungunya ou encore de Zika chez *Aedes sp.* (Jupatanakul, et al. 2017).

La réponse cellulaire

Finalement, lors de la réponse immunitaire contre des pathogènes, une réponse cellulaire se met en place en plus de la réponse humorale, dans le but d'être le plus efficace

Introduction

possible dans l'élimination des intrus. Cette réponse cellulaire comprend la phagocytose, l'encapsulation ainsi que la mélanisation.

La phagocytose :

La phagocytose est un processus cellulaire par lequel des cellules immunitaires vont ingérer des particules étrangères, telles que des débris cellulaires ou encore des agents pathogènes de petites tailles, comme les bactéries par exemple. Cette fonction immunitaire essentielle est conservée au cours de l'évolution et fut découverte par Élie Metchnikov qui reçut le prix Nobel en 1908, associé à Paul Ehrlich. Les cellules douées d'une capacité de phagocytose sont des macrophages chez les vertébrés, mais leurs noms peuvent varier chez les invertébrés. En effet, malgré leur bonne description chez les invertébrés, ces cellules phagocytaires sont décrites sous leur terme générique à savoir, hémocytes ou hémocytes phagocytaires dans la plupart des cas (Buchmann 2014).

Ces cellules sont mises en évidence chez de nombreux insectes ; chez la drosophile elles portent le nom de plasmocytes (Elrod-Erickson, et al. 2010) ; chez les moustiques, ce sont des granulocytes (Hillyer and Strand 2014) ; leur activité nécessite au préalable la reconnaissance des pathogènes par le système immunitaire (Abnave, et al. 2017). Chez le moustique, les granulocytes sont activés suite à la reconnaissance des agents pathogènes par des lectines, des PGRP, des GGBP (Yassine and Osta 2010; Gendrin, et al. 2017). Les hémocytes phagocytaires ont aussi la capacité d'activer la production de ROS pour éliminer le pathogène phagocyté (Nappi and Ottaviani 2000; Jaramillo-Gutierrez, et al. 2009). Chez les crustacés et les mollusques, des cellules présentant des capacités de phagocytose ont aussi été mises en évidence, chez l'écrevisse (guilini2007), la crevette (Jauzein, et al. 2013), la moule (Canesi, et al. 2002), l'huitre (Matozzo and Bailo 2015). Encore une fois les pathogènes sont reconnus, par le système immunitaire, par l'intermédiaire de molécules solubles ou des récepteurs fixés sur les hémocytes (LGBP, PGBP, BGBP, lectines etc.), qui déclenchera le phénomène de phagocytose ainsi que la réponse humorale, avec la production de ROS, pour éliminer le pathogène (Abnave, et al. 2017).

L'encapsulation :

L'encapsulation, est un processus multicellulaire par lequel des cellules immunitaires spécialisées vont former une capsule autour d'un pathogène ou d'un corps étranger. Au sein de cette capsule seront alors produits des effecteurs, notamment des

Introduction

ROS, dans le but de l'éliminer (Lemaitre and Hoffmann 2007). Ce processus vise des pathogènes trop imposants pour être phagocytés, tels que des organismes métazoaires parasites par exemple. Chez les invertébrés, cette fonction est assurée par un sous-ensemble d'hémocytes, souvent différents de ceux pourvus de la capacité de phagocytose.

Chez la drosophile, les hémocytes responsables de l'encapsulation sont appelées des lamellocytes; ces cellules sont cryptiques et leur différenciation est induite par le stress, notamment par la voie de signalisation JAK-STAT (Vlisidou and Wood 2015). Elles ont la capacité de s'agréger autour de pathogènes, tels que des œufs de guêpes parasitoïdes pour les éliminer (Mortimer, et al. 2013; Vlisidou and Wood 2015). Ces mêmes cellules sont aussi impliquées dans l'élimination des corps apoptotiques chez la drosophile (Vlisidou and Wood 2015). Chez les moustiques, l'encapsulation des parasites par les hémocytes fut mise en évidence dans les années 90 (Jianyong, et al. 1994) et est essentielle dans la réponse contre les plasmodiums (Ramirez, et al. 2014). Chez les crustacés, des approches *in vitro* montrent une agrégation d'hémocytes semi-granulaires appelées les semi-granulocytes, autour d'un pathogène, qui seraient responsables de son encapsulation et de son élimination (Vazquez, et al. 2009).

La mélanisation :

Chez les invertébrés, la mélanisation est une réponse immunitaire importante suite à une blessure et est visible par le noircissement du site de la plaie ou la surface du pathogène envahissant en raison de la synthèse localisée de la mélanine (Tang, et al. 2008). Les cellules cristallines sont les principaux hémocytes responsables de la réaction de mélanisation chez la drosophile (Ferrandon, et al. 2004; Lemaitre and Hoffmann 2007; Vlisidou and Wood 2015). La mélanine produite sur le site de la blessure ou de l'infection contient la progression de l'agent infectieux et facilite la fermeture de la plaie (Volz, et al. 2006). Les cellules cristallines contiennent la voie de synthèse de l'enzyme clé dans la bio-synthèse de la mélanine : la phénoloxydase (PO). L'enzyme est stockée sous forme d'inclusions cristallines et libérée lors de la rupture des cellules cristallines (Vlisidou and Wood 2015). Dans les hémocytes, elle est sous une forme inactive, la prophénoloxydase (proPO) ; lors de la reconnaissance de pathogènes, même en toute petite quantité (Cerenius and Söderhäll 2004), elle sera activée par une cascade de sérines protéases et d'autres facteurs et sera libérée dans l'hémolymphe (Vazquez, et al. 2009). La phénoloxydase (PO) catalyse la réaction enzymatique qui permettra la production de

Introduction

quinone, précurseur de la mélanine (Cerenius and Söderhäll 2004). La mélanine est un pigment brun-noir avec des propriétés biologiques diverses, telles que l'inhibition de l'activité des enzymes fongiques et bactériennes.

De nombreuses études concernant l'activité du système de la voie des PO chez les crustacés et les insectes, ont été réalisées. Chez les crustacés, sur les cellules adhérentes *in vitro*, ces protéines d'adhésion stimulent une réponse multicellulaire, telle que l'encapsulation, entravant l'activité de l'agent pathogène ; elles sont également responsables de la stimulation de la phagocytose (Vazquez, et al. 2009). Cependant, malgré plusieurs publications sur ce sujet, la contribution de l'activation de la phénoloxidase à la résistance aux infections n'a pas été établie dans des conditions *in vivo* appropriées (Leclerc, et al. 2006). Il en est de même chez la drosophile, des souches mutantes de la voie des PO sont aussi résistantes aux infections que les mouches de type sauvage, ceci nous laisse nous interroger sur la question de la fonction précise de l'activation de la voie des phénoloxidases dans la défense contre les pathogènes et les processus immunitaires (Leclerc, et al. 2006).

La réponse cellulaire de *Biomphalaria glabrata* :

Chez *Biomphalaria glabrata*, 3 types d'hémocytes sont décrits ; les blast-like cells, cellules de très petites tailles (3 μm de diamètre) de forme sphérique et possédant un ratio nucléo-cytoplasmique fort ; les granulocytes, mesurant environ 8 μm de diamètre, présentant de nombreux granules dans leur cytoplasme ; les hyalinocytes, qui sont les hémocytes le plus gros 12 μm de diamètres et présentant de nombreuses projections cytoplasmiques (Cavalcanti, et al. 2012). Les hémocytes les plus abondants sont les hyalinocytes, représentant 50% des cellules circulantes totales, suivis de prêt par les blast-like cells, correspondant à 45% des cellules et finalement les granulocytes qui sont les hémocytes les moins abondants et représentent seulement 5% de la population hémocytaire totale (Cavalcanti, et al. 2012).

Lors de la réponse immunitaire du mollusque contre des bactéries ou d'autres pathogènes de petites tailles, un phénomène de phagocytose a lieu grâce aux hémocytes circulants dans l'hémolymphe ; les intrus plus imposants, comme les parasites métazoaires, sont, quant à eux, encapsulés. Des structures de capsules, en microscopie électronique, ont été mises en évidence dès les années 70, chez *Biomphalaria glabrata* en réponse à un nématode pathogène *Angiostrongylus cantonensis* (Harris and Cheng 1974; Harris 1975), et en réponse à *Schistosoma mansoni* (Loker, et al. 1982) ; mais les types

Introduction

hémocytaires impliquées sont difficiles à observer. Chez *B. glabrata* contrairement aux observations faites chez les autres invertébrés, il semblerait que les hyalinocytes soient à la fois pourvus d'une capacité de phagocytose, mais aussi d'encapsulation (communication personnelle, observations au laboratoire) ; les granulocytes joueraient un rôle au moment de l'encapsulation, mais de manière indirecte, peut être dans la production d'espèces réactives de l'oxygène pour éliminer le parasite dans la capsule. Les cellules « blast-like cells », sont aussi retrouvées sur les pourtours de la capsule hémocytaire, sans qu'aucun rôle direct dans cette dernière n'ai pu être, pour l'instant, mis en évidence. En conclusion, le rôle de chaque type d'hémocyte dans la réponse immunitaire de *Biomphalaria glabrata* n'est pas encore finement caractérisé.

Les cellules immunitaires (hémocytes) d'un escargot sont des sentinelles qui constituent un obstacle crucial à l'infection ; toutefois la prolifération et la différenciation des hémocytes sont influencées par des facteurs exogènes spécifiques. Cependant, les mécanismes de contrôle endogène du développement des hémocytes restent à ce jour très peu connus ; mais la caractérisation fonctionnelle récente d'une granuline nommée BgGRN (Growth Factors Granulin), vient d'être réalisée, chez *Biomphalaria glabrata* (Pila, Gordy, et al. 2016). Les granulines sont des facteurs de croissance qui stimulent la prolifération des cellules immunitaires dans l'ensemble du règne animal. Chez *Biomphalaria glabrata*, la BgGRN induit la prolifération des hémocytes et conduit spécifiquement la production d'un sous-ensemble d'hémocytes adhérents qui participe à la réponse de défense anti-parasitaire (Pila, Gordy, et al. 2016).

La réponse humorale

Lors de la réponse immunitaire contre des pathogènes un certain nombre de molécules seront induites et sécrétés afin de participer à une réponse efficace contre les intrus, il s'agit de la réponse humorale. Chez les invertébrés nous retrouvons essentiellement les AMP, les ROS/RNS ou encore des molécules cytotoxiques/cytolytiques.

Les protéines et peptides anti-microbiens :

Les protéines anti-microbiennes ou peptides anti-microbiens ou « *Anti-Microbial Peptides* » (AMP) sont caractéristiques de la défense de la drosophile, et celle de la plupart des autres insectes ainsi que des mollusques, suite à une infection, ils sont synthétisés et sécrétés et s'accumulent dans l'hémolymphe où ils s'opposent à

Introduction

l'invasion de microorganismes. Bien que la synthèse des AMP soit probablement commune à tous les métazoaires, la sécrétion de ces molécules dans l'hémolymphe n'est pas un phénomène général (Lemaitre and Hoffmann 2007). Chez les insectes, la production d'AMP se fait dans le corps gras ; qui représente un organe puissant pour la synthèse et la sécrétion de peptides dans l'hémolymphe, où ils atteignent facilement leurs concentrations efficaces (Lemaitre and Hoffmann 2007). Sept classes d'AMP, ont été identifiés chez la drosophile ; la diptericine, la drosocine et l'attacine sont très efficaces contre les bactéries gram-négatifs. La défensine est active contre les bactéries gram-positifs, tandis que la drosomycine et la metchnikowine sont des agents antifongiques et spécifiques aux drosophiles. La cecropine A1 agit contre les bactéries et certains champignons (Lemaitre and Hoffmann 2007). Chez le moustique, nous retrouvons aussi plusieurs familles d'AMP, produits par la barrière épithéliale, suite à une infection (Osta, et al. 2004). Des défensines et des cecropines A sont fortement exprimés suite à un challenge bactérien chez *Aedes aegypti* (Lowenberger, et al. 1999). Chez *Anopheles gambiae*, l'étude du génome révèle des défensines, des cecropines, une attacine et une gambicine (Christophides, et al. 2002). Par ailleurs, toujours chez *Anopheles gambiae*, des activités anti-bactéries gram-positifs et anti-plasmodique ont été mises en évidence pour des défensines ainsi qu'un rôle anti-bactérien (gram-positifs et gram-négatifs) de la gambicine (Vizioli, et al. 2001). Chez les oursins, deux strongylocines et une centrocine ont été mis en évidence dans les phagocytes de *Strongylocentrotus droebachiensis* (Li, et al. 2014). Des études de co-localisation de la centrocine et des bactéries suggèrent que ce peptide soit impliqué dans la réponse contre les bactéries (Li, et al. 2014). Par ailleurs, la présence d'une autre famille d'AMP, les forteylocines, est détectée, dans les cellules vibratiles (Li, et al. 2014). Chez les crustacés aussi, des AMP ont été identifiés, ils sont au nombre de trois, la crustine, molécule à l'activité anti-bactéries gram-positifs, la panaeidine, à activités anti-bactéries gram-positifs et anti-fongique et enfin la tachycitine, dont l'activité n'a pas encore été caractérisés (Vazquez, et al. 2009).

Chez *Biomphalaria glabrata*, nous retrouvons aussi des gènes qui codent pour des protéines potentiellement anti-microbiennes impliquées dans la réponse immunitaire humorale du mollusque. L'arsenal d'AMP est très réduit chez *B. glabrata* en comparaison avec des autres invertébrés, une seule famille est présente, les macines composées de 6 gènes nommé Biomphamacines (Adema, et al. 2017). Cependant, *B.glabrata* possèdent des familles multigéniques de protéines anti-microbiennes (Adema, et al. 2017) incluant 2 achacines et les LBP/BPI (Lipopolysaccharide Binding Protein/Bactericidal Permeability

Introduction

Increasing), exprimés dans la glande albumen (Guillou, et al. 2007). Ces dernières sont sur-exprimées dans les hémocytes suite à une infection bactérienne ou d'oomycète (Baron, et al. 2013). Des études par spectrométrie de masse ont mis en évidence, que les LBP/BPI constituaient une protéine majoritaire présente dans la matrice des oeufs de *B. glabrata* (Hathaway, et al. 2010; Baron, et al. 2013). Ceci fournit la première preuve fonctionnelle qu'une LBP/BPI est impliquée dans la protection immunitaire transgénérationnelle chez des invertébrés (Baron, et al. 2013). Le MPEG1 (Macrophage Expression Gene 1) est un autre membre anti-microbien identifié chez *Biomphalaria glabrata*, il fait partie de la super famille des membrane-attaque complex/perforin (MACPF) (Bathige, et al. 2014), il est sur-exprimé dans les hémocytes suite à une infection avec un schistosome (Ittiprasert, et al. 2010).

Les espèces réactives de l'oxygène ou « Reactive Oxygen Species » (ROS) et les espèces réactives de l'azote « Nitrogen Reactive Species » (RNS) :

Les ROS, sont des espèces chimiques oxygénées, qui peuvent être exogènes ou endogènes, jouant un rôle important dans la communication entre les cellules. La production d'espèces réactives de l'oxygène (ROS) et l'expression des AMP, sont deux réponses clés pour lutter contre les infections (Buchon, et al. 2013). En plus de leur activité anti-microbienne, les ROS ont un rôle dans la réparation des tissus, la cicatrisation et l'hématopoïèse chez les drosophiles et les mammifères, ils se comportent alors comme un modulateur de signalisation. La biogénèse des ROS commence par des systèmes générateurs de superoxyde, tel que le NADPH, la nitrogène oxydase (NOX) ou la dualoxydase (DUOX). Cette famille de protéine est nommée NOX/DUOX et génère du peroxyde d'hydrogène (H₂O₂) et de l'acide hypochlorique ; tandis que la NADPH est un complexe enzymatique générant des anions superoxydes (O₂) (Bae, et al. 2010; Adema, et al. 2017). Chez les invertébrés, le rôle immunitaire des ROS a été mis en évidence chez plusieurs modèles. Chez *D. melanogaster*, la production de ROS par Duox et Nox est stimulée lors d'infection par des pathogènes (Buchon, et al. 2014). Chez les crustacés, la production de ROS, dans les hémocytes, suite à des stimulations immunitaires, a été mis en évidence chez les crevettes, *Penaeus monodon*, *Penaeus vannamei* et *Macrobrachium rosenbergii* (Song and Hsieh 1994; Muñoz, et al. 2000; Soria, et al. 2006).

Chez *Biomphalaria glabrata*, les mécanismes de destruction dépendants de l'oxygène jouent un rôle majeur dans la défense du mollusque. Les enzymes telles que la NADPH oxydase, la superoxide dismutase, la myéloperoxidase et l'oxide nitrique synthetase sont

Introduction

des composants essentiels des voies d'éliminations des parasites (Bayne, et al. 2001; Adema, et al. 2017). Des espèces réactives de l'oxygène (ROS - Reactive Oxygen Species) ont été mises en évidence chez *B. glabrata* (Hahn, et al. 2000) ; produites par les hémocytes, elles sont cruciales dans la défense du mollusque contre les schistosomes (Bayne, et al. 2001; Hahn, et al. 2001). De plus, les hémocytes produisant des molécules de peroxyde d'hydrogène (H₂O₂), participent activement à l'élimination des sporocystes (Hahn, et al. 2001).

Il existe aussi un autre type de réactifs, il s'agit des RNS « *Reactive Nitrogen Species* », qui semble aussi jouer un rôle chez les drosophiles dans la réponse anti-bactérienne (Foley and O'Farrell 2003) mais aussi chez les mollusques dans l'élimination des parasites (Adema, et al. 2017).

Les ROS et les AMP sont complémentaires, car les AMP sont essentiels pour contrôler les bactéries résistantes aux ROS.

Les molécules cytotoxiques/cytolytiques :

Parmi les molécules cytotoxiques/cytolytiques nous retrouvons les voies d'activations du complément qui initient l'assemblage d'un complexe d'attaque de membrane créant des canaux transmembranaires au travers de la couche lipidique provoquant la lyse des cellules cibles. Des molécules semblables à celles retrouvées chez les vertébrés ont été mises en évidence chez les échinodermes et semblent être impliquées principalement dans l'initiation ou l'amélioration de la phagocytose (Marchalonis and Schluter 1989; Hoffmann, et al. 1999).

Les enzymes lysosomiales présentent aussi une importance dans la réponse immunitaire des invertébrés. En effet, les lysosomes sont le principal compartiment catabolique dans les cellules eucaryotes et leur biogenèse nécessite l'intégration des voies biosynthétiques. Chez la drosophile, l'enzyme UBPY / USP8 fut mise en évidence et est nécessaire pour la biogenèse lysosomale (Jacomin, et al. 2016). Un autre exemple d'enzymes lysosomiales mise en évidence chez les invertébrés, la cathepsine B. En effet mise en évidence chez l'écrevisse, elle semblerait participer à la défense immunitaire au moment d'une infection bactérienne (Dai, et al. 2016).

Finalement, une aérolysine appartenant à la superfamille des toxines formant des pores β (β -PFT - β Pore-Forming Toxin) a été décrite chez *Biomphalaria glabrata*, appelée Biomphalysine (Galinier, et al. 2013). Ces aérolysines sont abondamment identifiées chez bactéries, mais plus rarement décrites pour des organismes eucaryotes. Malgré les

Introduction

faibles similitudes de séquence avec les aérolysines bactériennes, la biomphalysine partage une structure tridimensionnelle avec des protéines appartenant à cette superfamille ; une approche phylogénétique a permis de révéler que ces molécules avaient été héritées d'un transfert horizontal d'origine bactérien. La biomphalysine est exprimée par les hémocytes, mais n'est pas induite suite à un challenge parasitaire ; mais son activité est hautement toxique pour les sporocystes de *Schistosoma mansoni* (Galinier, et al. 2013). En effet, elle se lie à la surface du tégument parasitaire, et l'association de plusieurs protéines en un multimère permettra la formation d'un pore transmembranaire qui entraîne la lyse et l'élimination du parasite (Galinier, et al. 2013).

L'interaction immunitaire entre *Biomphalaria glabrata* et *Schistosoma mansoni*

Comme chez les autres invertébrés, l'immunité de *Biomphalaria glabrata* est complexe. En effet, ce mollusque est capable de reconnaître *Schistosoma mansoni* au moment de l'infection, puis de développer une réponse contre ce dernier. La réponse immunitaire commencera toujours par une reconnaissance des pathogènes, puis ensuite, la mise en place des mécanismes pour contrer le développement de l'intrus et l'éliminer. Chez *Biomphalaria*, entre autres, une quatrième partie dans la réponse immunitaire est observée, il s'agit du priming immunitaire ; en effet lors d'une seconde rencontre avec un même parasite, le mollusque développera une réponse immunitaire innée mémoire lui conférant une protection accrue contre ce même pathogène. La majorité des études réalisées sur la réponse immunitaire du mollusque, sont croisées avec les moyens de défense du parasite, le dialogue moléculaire entre les deux protagonistes est en conséquence au centre de nos intérêts. Dans le Chapitre 1 nous porterons un intérêt tout particulier à ce dialogue moléculaire et nous mettrons en évidence les bases moléculaires sous jacentes aux interactions immunologiques entre *Biomphalaria glabrata* et *Schistosoma mansoni* (fig. 7).

Introduction

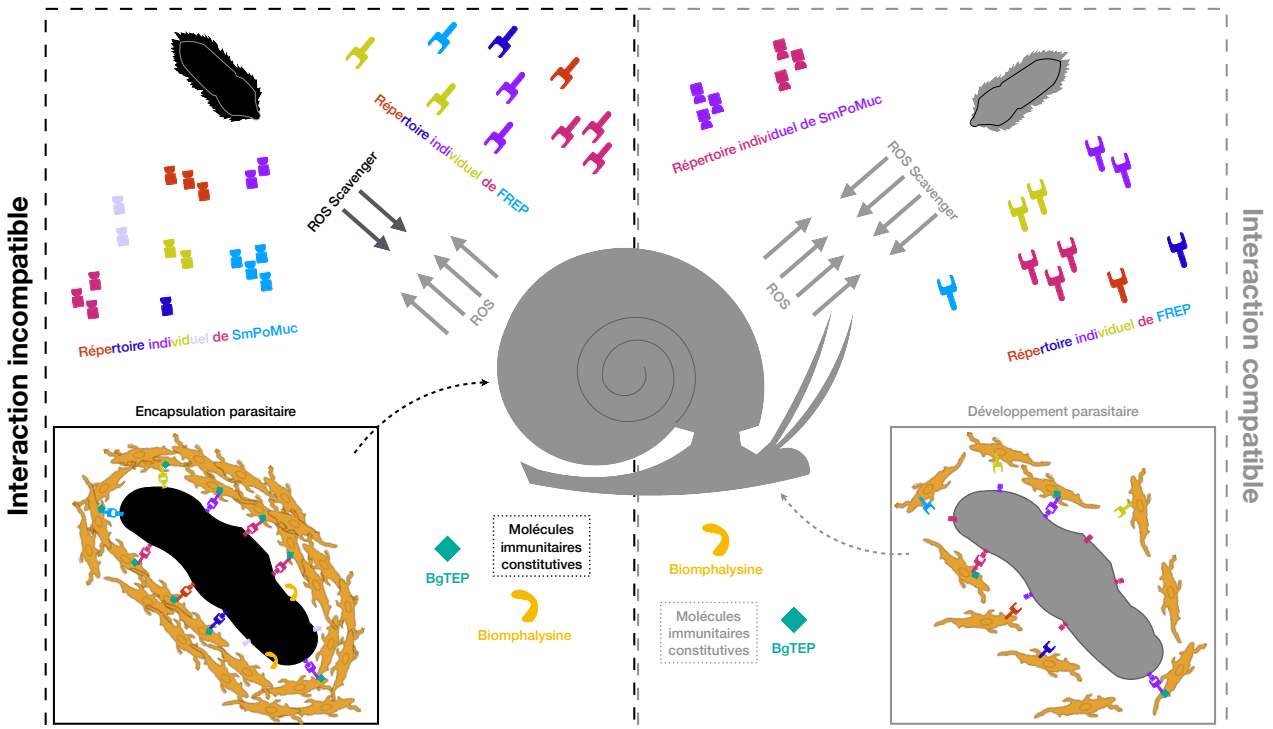


Figure 7 : Interaction immunitaire entre *Biomphalaria glabrata* et *Schistosoma mansoni*

Représentation schématique de l'interaction entre *B. glabrata* et *S. mansoni* lors d'une interaction incompatible (en noir) et compatible (en gris), sur la base d'une synthèse bibliographique des travaux menés sur le sujet avant le démarrage de la thèse.

Lors d'une infection par les parasites, le système immunitaire reconnaît cette intrusion grâce à différents récepteurs immuns. Dès le début des années 2000, les FREP ont été mises en évidence, comme des protéines hypervariables, polymorphiques et/ou diversifiées (Adema, et al. 1997; Zhang, et al. 2004; Gordy, et al. 2015). Rapidement, le rôle de FREP2 et FREP3 dans la réponse immunitaire fut démontré par des approches de knock-down (Hanington, et al. 2012; Pinaud, et al. 2016). Les mollusques présentent un répertoire individuel diversifié de FREP (Zhang, et al. 2004; Gordy, et al. 2015), suggérant que ces molécules puissent jouer un rôle dans la réponse immunitaire et la compatibilité. En parallèle, en 2008, des études de protéomique comparative, chez *Schistosoma mansoni*, ont permis de mettre en évidence des mucines très polymorphes, exprimées au stade infectant le mollusque, appelées des SmPoMuc (*Schistosoma mansoni* Polymorphic Mucins) (Roger, Mitta, et al. 2008). Les parasites présentent des répertoires individuels de SmPoMuc, que ce soit au niveau des souches ou même au niveau individuel, ce qui suggère qu'elles puissent jouer un rôle crucial dans le processus de compatibilité entre les parasites et leur hôtes (Roger, Mitta, et al. 2008). Par la suite, des expériences de Co-immunoprécipitation (CoIP) ont été réalisées au sein du laboratoire,

Introduction

qui ont mis en évidence la formation d'un complexe immunitaire entre les SmPoMuc et les FREP de *B. glabrata* (Moné, et al. 2010). Ce travail fut le premier à mettre en évidence une interaction directe entre une molécule de reconnaissance immunitaire diversifiée du mollusque et un antigène polymorphe du parasite. Au sein de ce complexe immunitaire, un troisième acteur fut identifié, une Thio-Ester-containing Protein de mollusque, la BgTEP (Moné, et al. 2010), appartenant à une famille de molécule déjà mise en évidence, chez les insectes, dans la réponse immunitaire et l'élimination des pathogènes, et jouant un rôle d'opsonine (Levashina, et al. 2001).

Une fois le parasite reconnu, une réponse cellulaire se met en place dans les tissus du mollusque. Des études histologiques montrent lors d'une infection du mollusque par un parasite incompatible, une réponse cellulaire d'encapsulation (Loker, et al. 1982) ; les hémocytes forment une capsule autour de l'intrus pour l'éliminer (fig. 7).

Dans le but d'éliminer efficacement le parasite, des mécanismes de destruction dépendants de l'oxygène sont activés et jouent un rôle majeur dans la défense du mollusque. Des espèces réactives de l'oxygène (ROS - Reactive Oxygen Species) ont été mises en évidence chez *B. glabrata* (Hahn, et al. 2000); produites par les hémocytes, elles sont cruciales dans la défense du mollusque contre les schistosomes (Bayne, et al. 2001; Hahn, et al. 2001) (fig. 7).

Quant aux schistosomes, ils se sont adaptés et ont développé des systèmes antioxydants pour contrer l'attaque du mollusque et participer à leur protection contre le stress oxydatif, comme les GST par exemple (Guillou, et al. 2007; De Moraes Mourão, et al. 2009). De plus, les parasites utilisent des ROS-scavenger pour se défendre contre les dommages oxydatifs induits par les ROS des mollusques (Moné, et al. 2010). Le mécanisme ROS / ROS-scavenger apparaît comme un processus important impliqué dans la compatibilité, et constitue un exemple de co-évolution ou d'adaptation entre ces molécules clés de l'interaction. En effet, l'étude des capacités de l'hôte et du parasite, montre un lien étroit entre les niveaux d'oxydants et d'antioxydants des deux partenaires, ce qui résulte vraisemblablement d'une adaptation locale sympatrique entre les deux protagonistes (Moné, et al. 2011).

Toujours dans le but d'une meilleure défense, le mollusque présente une arme redoutable contre les schistosomes, la biomphalysine. Cette toxine formant des pores β se lie à la membrane parasitaire et l'association de plusieurs protéines formera un pore transmembranaire qui entraîne la lyse et l'élimination du parasite (Galinier, et al. 2013).

Suite à l'infection, une sur-expression d'inhibiteurs de protéases, dans les hémocytes, est observée, ce qui suggère une réponse active contre le parasite (Lockyer, et al. 2012). Grâce aux protéases et inhibiteurs de protéases, *Schistosoma mansoni* et *Biomphalaria glabrata*, semblent s'engager dans une bataille enzymatique qui a également son importance dans les processus de la compatibilité au sein de cette interaction (Bouchut, et al. 2006; Guillou, et al. 2007; Roger, Grunau, et al. 2008; Mitta, et al. 2012; Mitta, et al. 2017).

En conclusion, l'interaction entre *B. glabrata* et *S. mansoni* semble reposer sur un système complexe (fig. 7). Basé sur le principe du « matching phenotype model », dans lequel un grand nombre de molécules immunitaires de mollusque et d'infektivité du parasite se répondent mutuellement. Ces mécanismes pourraient expliquer les phénomènes de compatibilité et pourraient résulter de phénomènes d'adaptation locale. Dans le cadre de cette thèse, nous proposons maintenant d'aller plus loin et de tenter de démontrer que les acteurs moléculaires mis en évidence et jouant un rôle clé au cœur de cette compatibilité dans ce système peuvent être généralisés à d'autres couples hôte/parasite présentant des niveaux différents de compatibilité. De plus nous avons cherché à comprendre le rôle des processus évolutifs et de l'adaptation locale dans la mise en place de cette compatibilité. Ceci a été réalisé en comparant des contextes d'interaction sympatrique et allopatrique présentant des niveaux de compatibilité similaires. Ces différentes recherches et travaux sont présentés en détail dans le chapitre 1.

Dans le deuxième chapitre de la thèse nous avons regroupé les travaux que nous avons mené afin de préciser le rôle de molécules aillant un rôle central dans cette interaction et en particulier les Thioester-containing protein, mis en évidence dans notre modèle invertébré ainsi que la complexité de la reconnaissance immunitaire du mollusques.

Enfin si la compatibilité hôte/parasite est directement liée aux caractéristiques intrinsèques des deux protagonistes en interaction ; il est intéressant de noter qu'un certain nombre de facteurs environnementaux pourraient être de nature à modifier ou changer l'issue d'une interaction la faisant basculer de compatible vers incompatible et vice versa.

Influence de facteurs environnementaux sur la compatibilité hôte / parasite

Parmi les facteurs environnementaux, nous pouvons distinguer deux catégories : (i) les facteurs abiotiques et (ii) les facteurs biotiques. Les facteurs abiotiques représentent l'ensemble des facteurs physico-chimiques de l'environnement (i.e. température, salinité des eaux, présence de polluants etc.). Les facteurs biotiques, représentent l'ensemble des interactions directes ou indirectes entre les êtres vivants d'un même écosystème. Comment tous ces facteurs environnementaux peuvent-ils influencer la compatibilité et/ou les interactions entre les hôtes et leurs pathogènes ?

Facteurs abiotiques

La majorité des études faites sur l'impact des facteurs abiotiques dans les interactions hôtes/pathogènes sont réalisées en milieu marin. En effet, l'impact de la salinité de l'eau ou encore la présence de polluant peuvent être des facteurs aggravant ayant un fort impact sur la mortalité des espèces, ce qui engendre souvent aussi un impact économique au niveau de la conchyliculture ou de l'élevage des crustacés. Plusieurs travaux recensent l'impact positif ou négatif de ces facteurs abiotiques sur l'interaction entre les hôtes et les pathogènes.

L'impact de polluants :

Chez la moule zébrée (*Dreissena polymorpha*), vivant dans les eaux douces, il semblerait que le parasitisme ne les affecte pas de la même manière selon l'environnement dans lequel elles se trouvent. En effet, les moules présentent dans des environnements contaminés chimiquement portent des parasites différents des environnements sains (Minguez, et al. 2011). De plus, selon les environnements étudiés, connus pour contenir des populations de moules parasitées, les prévalences ne sont pas les mêmes ; une teneur en polluant (i.e. nickel) plus importante dans l'eau serait corrélée avec une prévalence plus importante (Minguez, et al. 2012).

A contrario, l'équipe de Soliman, montre qu'une exposition au cadmium des *Lymnaea* post-infection par *Fasciola gigantica* entraîne une diminution de la prévalence (Soliman 2009). De plus lorsque les mollusques sont mis en contact avec ces mêmes éléments chimiques après l'infection, le nombre de mollusques n'émettant par de cercaires augmente, et l'émission cercarienne est très perturbée (les métacercaires flottantes sont

Introduction

en grandes proportions par rapport à celle fixées) (Soliman 2009). Cette fois-ci le polluant semble diminuer l'infection.

En 2003, l'impact de la pollution marine sur les interactions entre les hôtes et les virus fut mise en évidence. En effet, une augmentation des nutriments dans l'eau provenant des eaux usées peut entraîner une eutrophisation du milieu, souvent liée au développement de grands ensembles viraux (Danovaro, et al. 2003). En parallèle, les différents polluants que nous pouvons retrouver dans les océans comme les hydrocarbures, les pesticides etc. modifient l'environnement marin pouvant être responsable de profonde modification dans les interactions hôte/virus, en augmentant le potentiel d'infection virale (Danovaro, et al. 2003). L'ensemble de ces polluants peut avoir un effet synergique et ainsi augmenter les infections virales dans l'environnement marin (Danovaro, et al. 2003).

L'impact de l'augmentation de la salinité de l'eau :

Le champignon d'amphibien chytride (*Batrachochytrium dendrobatidis*) est un facteur important du déclin total des amphibiens. Des études sont menées dans le but de trouver des moyens de lutte contre ce déclin massif. Le rôle anti-fongique du chlorure de sodium ayant déjà été mis en évidence, des études menées en milieu contrôlé, contre le *B. dendrobatidis* ont été réalisées (Stockwell, et al. 2012). Les résultats montrent une intensité de parasitisme plus faible lorsque l'on augmente le taux de salinité de l'eau, sans effet négatif pour l'hôte (Stockwell, et al. 2012).

L'impact de la température :

L'influence de la température sur l'interaction hôte / parasite fut mise en évidence dans l'interaction entre *B. glabrata* et *S. mansoni*. En effet, des études menées sur l'infection du mollusque par le parasite suite à une courte acclimatation (4h) à la chaleur a montré une sensibilité accrue de mollusque présentant un phénotype de résistance à température physiologique (Ittiprasert and Knight 2012). De plus, lorsque des mollusques présentant naturellement le phénotype de résistance à *S. mansoni* sont maintenus à des températures élevées (32°C) pendant plusieurs générations, leur progéniture replacée à température physiologique (26°C) devient sensible au parasite (Knight, et al. 2015). En conclusion, les variations de température peuvent modifier la sensibilité de *B. glabrata* à l'infection par *S. mansoni*.

Introduction

Ces différents résultats sur l'influence des facteurs abiotiques sur les interactions hôtes / parasites présentent une grande complexité. En effet, selon les milieux, les hôtes, l'influence des facteurs abiotiques l'influence peut être positive ou négative sur l'interaction hôte / parasite.

Facteurs biotiques

Les facteurs biotiques peuvent également impacter l'interaction entre un hôte et son parasite. Dans ce cadre là, nous pouvons retrouver le problème de co-infection (i.e. virus; acariens etc.) qui influence et/ou favorise l'interaction et la compatibilité hôte/parasite. Un autre facteur biotique central et de plus en plus étudié ces dernières années, est le microbiote. Ce dernier correspond à l'ensemble des micro-organismes vivant en symbiose (mutualistes ou pathogènes) avec l'hôte, comprenant les bactéries, les archées, les champignons, les virus ou encore les eucaryotes protozoaire. En effet, l'influence du microbiote sur l'interaction hôte / parasite semble être particulièrement importante.

La co-infection ou l'impact d'un troisième acteur dans l'interaction:

Lors d'une interaction entre les hôtes et les parasites, un troisième organisme vivant peut venir contribuer ou modifier à cette interaction.

Au début des années 2000, des études révèlent une très forte prévalence virale dans les populations d'abeilles à travers la France. En effet, non seulement la prévalence est forte mais en plus la diversité virale l'est également, au total 15 virus différents, pour la plupart non pathogènes ou faiblement, sont identifiés dans les colonies à travers la France (Tentcheva, et al. 2004). En 2004, une étude confirme que l'acarien *Varroa destructor*, porteur de virus d'abeille, joue un rôle dans la transmission de ces différentes virus au sein des colonies d'abeilles (Tentcheva, et al. 2004). Dans ce contexte là, l'acarien joue un rôle important dans l'interaction hôte / parasite.

Une autre type d'interaction entre un hôte, un parasite et un troisième acteur profite de nombreuses études, il s'agit des co-infections d'insectes par des guêpes parasitoïdes et des virus. Les Bracovirus sont un genre de virus appartenant à la famille des Polydnavirus, composés exclusivement de virus d'insecte (Moreau, et al. 2009). Les particules virales se répliquent à l'intérieur des ovaires des guêpes parasitoïdes et sont injectés dans les insectes au moment de la pondaison de la guêpe. Par la suite ces virus sont nécessaires au développement des larves des guêpes parasitoïdes (Chevignon, et

Introduction

al. 2014; Drezen, et al. 2014). Les gènes de virulences du virus permettront un contrôle de l'immunité et du développement de l'hôte (Herniou, et al. 2013).

En 2015, une relation identique fut identifiée cette fois-ci chez la coccinelle. En effet, un nouveau virus à ARN, *Dinocampus coccinellae Paralysis Virus* (DcPV), est mis en évidence chez la coccinelle et joue un rôle important dans l'interaction entre cette dernière (*Coleomegilla maculata*) et la guêpe parasitoïde (*Dinocampus coccinellae*) (Dheilly, Maure, et al. 2015). Le virus est stocké dans l'oviducte des guêpes parasitoïdes femelles puis dans les larves et est transmis à la coccinelle au moment de la ponte de la larve de guêpe par la femelle parasitoïde. Le virus se réplique alors dans le système nerveux de la coccinelle aboutissant à des symptômes de paralysie (Dheilly, Maure, et al. 2015). Il s'agit d'une modification comportementale de la coccinelle par le virus, qui contribue à un meilleur développement de la larve de guêpe à l'intérieur des tissus de l'hôte (Dheilly, Maure, et al. 2015). Dans ce modèle, le virus joue un rôle important dans le succès de développement du parasite à l'intérieur de son hôte et donc dans sa compatibilité.

Finalement, chez *Biomphalaria glabrata* lors de la co-infection avec *Schistosoma mansoni* et *Echinostoma liei*, les cercaires de *S. mansoni* présentent une très forte diminution de leur infectiosité chez la souris (Jourdane and Mounkassa 1986). De plus, les mollusques *B. glabrata* naturelles résistants à *S. mansoni* deviennent sensibles à ce dernier lorsqu'ils sont préalablement exposés à *E. paraensei* (Garcia, et al. 2010).

Le microbiote :

L'accumulation d'études sur le microbiote pour une grande diversité d'organismes, allant de l'homme aux invertébrés, a révélé son importance tant au niveau de la fitness (ou valeur sélective) de l'hôte que dans son rôle dans de nombreuses fonctions physiologiques comprenant la reproduction, le développement, ou encore l'immunité, etc (Blacher, et al. 2017; Igartua, et al. 2017). Les perturbations du microbiote peuvent affecter l'acclimatation de l'hôte et par conséquent sa survie. Le microbiote est ainsi actuellement au cœur de nombreuses réflexions et débats sur le fonctionnement, l'homéostasie des organismes ou encore leur évolution.

Lorsque l'on considère non plus l'organisme seul mais l'organisme et son microbiote comme une seule entité, nous devons aussi prendre en compte l'impact de ce microbiote dans les interactions auxquelles l'hôte sera confronté tout au long de sa vie, comme par

Introduction

exemple une infection parasitaire. On parle alors d'une relation tripartie entre l'hôte, son microbiote et le pathogène.

De par son implication dans de nombreuses maladies vectorielles, le moustique fait l'objet de nombreuses études sur les interactions possibles entre son microbiote et les pathogènes qu'il transmet, que ce soit des protozoaires, pour la malaria ou des virus, comme la dengue, zika ou chikungunya. Depuis quelques années, l'impact du microbiote sur le contrôle de la transmission de ces agents pathogènes commence à être révélé.

Dés le début des années 90, des expériences menées sur la relation entre les bactéries et les parasites infectant le moustique ont été initiées, et ont en premier lieu nécessité de caractériser le microbiote bactérien afin d'étudier son impact dans cette étroite relation hôte-parasite.

De nombreuses études ont reportées l'association d'espèces de bactéries gram-négatifs, du tube digestif de moustiques de laboratoire ou de terrain, avec une activité inhibitrice du développement des parasites *Plasmodium sp.* dans l'intestin de leur hôte définitif (Dong, et al. 2009). En 1993, l'injection de différentes espèces bactériennes gram-négatifs montrent une diminution du développement de *Plasmodium falciparum* à l'intérieur d'*Anopheles stephensi* qui n'est pas visible lors d'injection de bactéries gram-positifs (Pumpuni, et al. 1993). Des études menées en Afrique centrale sur les intestins de différentes espèces d'anophèles montrent que les femelles qui abritent des bactéries gram-positifs sont plus susceptibles d'être infectées par des sporozoïtes comparativement à celles qui ne possèdent aucune bactérie cultivable ou des bactéries gram-négatifs, dans leurs intestins. Il semblerait que la variation de la prévalence de divers types de bactéries chez les moustiques puisse influencer le développement du parasite et par conséquent la prévalence des sporozoïtes correspondants (Straif 1998). La co-infection d'un moustique au moment de son repas sanguin, avec le parasite et des bactéries gram-négatifs, affecte négativement la transmission parasitaire ; ce qui sous-entend que la composition bactérienne globale de l'intestin du moustique pourrait influencer la transmission du parasite (Gonzalez-Ceron, et al. 2003).

Plus tard, en 2011, une étude sur les populations de moustiques sauvages met en évidence une corrélation entre l'identification d'enterobactéries chez l'anophèle et une résistance naturelle de 99 % des moustiques au parasite *Plasmodium* (Cirimotich, et al. 2011). Le microbiote intestinal du moustique ne joue pas un rôle uniquement contre *Plasmodium*. En effet lors d'une infection virale par l'arbovirus *o'nyong-nyong virus*, la présence de la flore bactérienne intestinale est requise pour une infectivité complète chez

Introduction

les anophèles, contrairement à l'infection par le plasmodium, où cette fois-ci la présence de la flore bactérienne intestinale est nécessaire pour jouer un rôle protecteur (Carissimo, et al. 2015).

Par ailleurs, des études sur l'interaction tripartite entre l'hôte, son microbiote et les pathogènes associés ont permis de mettre en évidence un lien entre le microbiote et l'immunité des invertébrés. Dans certains cas, le microbiote joue un rôle de modulateur ; c'est le cas chez *Aedes sp.*, pour qui l'infection par le virus de la dengue est modulée par une stimulation basale de la voie Toll par le microbiote intestinale (Xi, et al. 2008). Toujours dans l'interaction entre *Aedes sp.* et le virus de la dengue, cette fois-ci il est montré une réelle interaction tripartite ; dans un premier temps, le microbiote intestinal du moustique influence l'infection virale, par stimulation basale de la voie Toll, puis à son tour l'infection virale, influe sur la réponse antibactérienne des moustiques, qui affecte alors le microbiote intestinal (Ramirez, et al. 2012).

Ceci devrait nous permettre de nous interroger sur le rôle des communautés microbiennes associées aux mollusques dans l'expression des phénotypes de compatibilité. Ce travail constituera une approche préliminaire visant à mieux comprendre l'interaction entre le mollusque, son microbiote et le parasite.

Chez la plupart des organismes, il est tout d'abord nécessaire de réaliser un inventaire des espèces composant le microbiote, de caractériser leurs spécificités, ainsi que de déterminer leurs stabilités au cours des générations. A la suite de ces études, il est possible d'identifier une fraction du microbiote stable entre les individus ainsi qu'au cours du temps que l'on appelle le Core-microbiote. Par la suite, il est alors envisageable d'aborder leur dynamique suite à des perturbations environnementales (température, modification du milieu de vie, pollution, etc.), abiotiques (modification du pH, etc.) ou biotiques (parasitisme, infection, etc.). Dans ce contexte, nous regardons alors les perturbations globales du microbiote dans les individus ainsi que celle du Core-microbiote, correspondant à la fraction commune à tous les individus. Pour cela, différentes approches sont possibles, des méthodes de biologie traditionnelles (études de la composition du microbiote par culture), ou bien des méthodes d'analyses moléculaires à haut débit, tant au niveau taxonomique que fonctionnel (métatranscriptomique, métabarcoding). Cette connaissance du microbiote permettra alors de déterminer

Introduction

l'importance de ce dernier dans le fonctionnement des organismes, ainsi que ses interactions avec d'autres organismes, tels que des pathogènes.

Dans notre modèle, nous proposons une caractérisation du microbiote viral ainsi que bactérien dans le but d'avoir une meilleure connaissance des organismes vivants en symbiose avec *B. glabrata*. Par la suite nous proposons une étude des modifications de la composition bactérienne du microbiote suite à l'infection. Cette fois-ci nous explorons l'impact de l'interaction hôte / parasite sur les communautés microbiennes associées à l'hôte. Ces travaux sont pour leur part détaillés dans le chapitre 3 du mémoire de thèse.

Chapitre 1

Chapitre I : Du Polymorphisme de Compatibilité à l'Adaptation Locale entre *Biomphalaria* et *Schistosoma*.

Introduction

Les interactions hôtes / parasites sont des systèmes biologiques dynamiques dans lesquels des mécanismes de défense de l'hôte font face aux mécanismes d'infectivité du parasite (Combes 2000). Les pressions sélectives dans ces systèmes sont le résultat de l'opposition continue, des intérêts divergents de chacun des deux partenaires (Combes 2000). Ce sont ces pressions de sélections entre les individus, qui sont responsables du caractère continue des changements adaptatifs, on parle alors de co-évolution (Combes 2000). La co-évolution entre l'hôte et le parasite est soutenue par un processus dit de "courses aux armements" (Van Valen 1974), supposant des changements adaptatifs continus chez chacun des deux partenaires afin de répondre aux changements adaptatifs réciproques.

Cette dynamique co-évolutive peut alors avoir comme conséquence une mosaïque de niveau d'infectivité entre hôte et parasite décrite sous le concept de polymorphisme de compatibilité. Le polymorphisme de compatibilité est basé sur un modèle de "matching-phenotype" dans lequel la compatibilité est testée indépendamment pour chaque miracidium et chaque hôte potentiel en populations naturelles (Théron, et al. 1997; Theron, et al. 2014).

Dès les années 70, des expériences de croisements montrent que des facteurs de compatibilité existent entre les mollusques et les parasites (Basch 1976). Un mollusque exposé individuellement à plusieurs parasites peut présenter différents phénotypes à travers les différents tissus. Des parasites peuvent être retrouvés en développement et d'autres encapsulés (Basch 1976). Ceci suggère alors que l'activation de la réponse cellulaire ou non réside dans la reconnaissance ou la non-reconnaissance des sporocystes (Basch 1976). De nombreuses expériences d'infections ont été réalisées entre des isolats sympatriques et allopatriques de souches de *B. glabrata* et *S. mansoni*, et les résultats ont révélé un haut degré de polymorphisme de compatibilité au sein et entre les populations (Richards and Shade 1987; Theron, et al. 2014).

Chapitre 1

Les précédents rapports montrent clairement que le succès ou l'échec de l'infection de *B. glabrata* par *S. mansoni* reflète une interaction complexe entre les stratégies de défense de l'hôte et d'infectivité du parasite.

Depuis des années, nous nous intéressons à la compréhension du polymorphisme de compatibilité, en étudiant les bases moléculaires des interactions compatibles et incompatibles entre *B. glabrata* et *S. mansoni*. Dans ce cadre là, nous utilisons des approches intégratives qui combinent une diversité de méthodes pour regarder les différents niveaux moléculaires potentiellement impliqués dans l'interaction hôte / parasite ainsi que dans la complexité de ces systèmes biologiques.

Le but étant de comprendre les différences moléculaires dans les interactions immunologiques entre les deux partenaires afin de pouvoir mettre en évidence les bases moléculaires de la résistance du mollusque. Pour cela, nous utilisons des mollusques brésiliens (BgBRE) infectés soit par leur parasite sympatrique brésilien (SmBRE), l'interaction est dite compatible, soit par un parasite allopatrique guadeloupéen (SmGH2), l'interaction étant alors incompatible. Ces deux interactions ont permis de mettre en évidence le premier complexe immunitaire entre des lectines diversifiées de *Biomphalaria glabrata*, les FREP et des mucines polymorphes de *Schistosoma mansoni*, les SmPoMuc. Dans le cadre d'une revue, nous nous proposons de mettre en évidence l'approche intégrative "multi-omics" qui nous a permis de mettre en évidence et de caractériser ces deux répertoires polymorphes et/ou diversifiés de molécules : les FREP et les SmPoMuc et leur rôle comme support du polymorphisme de compatibilité.

La majorité des études sur le polymorphisme de compatibilité ayant été réalisées uniquement sur deux souches de parasites compatible et incompatible vis-à-vis d'une même souche de mollusque, un manque d'information existait sur le rôle de ces déterminants moléculaires de la compatibilité dans d'autres couples hôte / parasite présentant des niveaux de compatibilités variables. Nous avons alors cherché à combler ce vide dans ces études sur le polymorphisme de compatibilité. Pour cela, nous étudions un panel de quatre souches de parasites et quatre souches de mollusques présentant des phénotypes de compatibilité variés afin d'évaluer les potentielles différences, dans les mécanismes de compatibilité, entre les populations, mais également afin de confirmer le rôle des FREP et des SmPoMuc dans ces interactions.

Finalement, lorsque l'on étudie le polymorphisme de compatibilité entre *B. glabrata* et *S. mansoni*, les souches utilisées sont soit sympatriques et compatibles, soit allopatriques et incompatibles, mais dans le large panel de souches dont nous disposons il existe aussi des souches allopatriques et compatibles. Nous nous proposons donc d'analyser, au

Chapitre 1

niveau moléculaire deux interactions immunologiques compatibles, une sympatrique (BgBRE vs SmBRE), où l'on s'attend à de la co-évolution ou des processus d'adaptation locale, et une allopatrique (BgBRE vs SmVEN) constituant une primo-rencontre entre l'hôte et le parasite. Le but étant de comparer s'il existe ou non des mécanismes immunologiques différents entre deux interactions qui présentent un même phénotype compatible, mais dont l'une est potentiellement sujette à de l'adaptation locale et l'autre pas. Pour cela nous réaliserons une approche duale comparative au niveau moléculaire (Dual RNAseq) à la fois menée sur le mollusque *Biomphalaria glabrata* mais également sur les stades intramolluscaux du parasite. Cette étude sera complétée par une analyse approfondie de la réponse immunitaire cellulaire du mollusque dans ce même contexte évolutif contrasté.

1. L'intérêts des omics dans la mise en évidence du dialogue moléculaire entre *Biomphalaria glabrata* et *Schistosoma mansoni*

Publication : Integrated multi-omic analyses in *Biomphalaria-Schistosoma* dialogue reveal the immunobiological significance of FREP-SmPoMuc interaction.

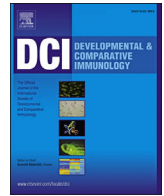
PORTET Anaïs, PINAUD Silvain, TETREAU Guillaume, GALINIER Richard, COSSEAU Céline, DUVAL David, GRUNAU Christoph, MITTA Guillaume and GOURBAL Benjamin

*Article publié en 2017 dans *Developmental and Comparative Immunology*



Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

Integrated multi-omic analyses in *Biomphalaria-Schistosoma* dialogue reveal the immunobiological significance of FREP-SmPoMuc interaction

Anaïs Portet¹, Silvain Pinaud¹, Guillaume Tetreau, Richard Galinier, Céline Cosseau, David Duval, Christoph Grunau, Guillaume Mitta, Benjamin Gourbal*

^a Univ. Perpignan Via Domitia, IHPE UMR 5244, IFREMER, Univ. Montpellier, F-66860 Perpignan, France

ARTICLE INFO

Article history:

Received 23 November 2016
 Received in revised form
 23 February 2017
 Accepted 26 February 2017
 Available online xxx

Keywords:

Compatibility polymorphism
Schistosoma mansoni
Biomphalaria glabrata
 FREPs
 SmPoMucs

ABSTRACT

The fresh water snail *Biomphalaria glabrata* is one of the vectors of the trematode pathogen *Schistosoma mansoni*, which is one of the agents responsible of human schistosomiasis. In this host–parasite interaction, co-evolutionary dynamic results into an infectivity mosaic known as compatibility polymorphism. Integrative approaches including large scale molecular approaches have been conducted in recent years to improve our understanding of the mechanisms underlying compatibility.

This review presents the combination of integrated Multi-Omic approaches leading to the discovery of two repertoires of polymorphic and/or diversified interacting molecules: the parasite antigens *S. mansoni* polymorphic mucins (SmPoMucs) and the *B. glabrata* immune receptors fibrinogen-related proteins (FREPs). We argue that their interactions may be major components for defining the compatible/incompatible status of a specific snail/schistosome combination.

© 2017 Elsevier Ltd. All rights reserved.

1. Introduction

Host–parasite interactions are dynamic biological systems in which the host's defense mechanisms face the parasite's infectivity mechanisms (Combes, 2000; Howard and Jack, 2007). The co-evolution of hosts and parasites involves huge reciprocal selective pressures on both partners. Therefore, in natural environment, all living organisms have developed defense systems capable of recognizing and activating efficient effectors to eliminate most of the pathogens encountered during their life span.

In invertebrate host/parasite interactions, invariable germ-line-encoded or somatically diversified pathogen recognition receptors (PRRs) that recognize a set of pathogen associated molecular patterns (PAMPs) appear to be the ultimate outcome of this molecular arms race to limit pathogen invasion (Medzhitov and Janeway, 1997). These polymorphic and/or diversified receptors have been identified in echinoderms (SRCR or Sp185/333 of sea urchin (Ghosh et al., 2010; Pancer, 2000; Pancer et al., 1999), insects (Dscam of

Drosophila melanogaster and *Anopheles gambiae* (Dong et al., 2006; Watson et al., 2005), crustaceans (Dscam of *Daphnia* (Brites et al., 2008) or *Litopenaeus vannamei* (Hung et al., 2013), and molluscs (FREPs of *Biomphalaria glabrata* (Zhang et al., 2004)). These studies indicated that these putative immune receptor variants vary considerably between individuals, yielding an enlarged repertoire of putative recognition molecules. We can speculate that large and individual repertoires of immune receptors might confer the immune recognition specificity of invertebrate immune systems and explain in part, the issue of host–pathogen compatibility.

The present chapter explores the molecular interactions between *Schistosoma mansoni* and the snail *Biomphalaria glabrata* that provides a model of choice in evolutionary biology for investigating host–parasite co-evolutionary dynamics and the mechanisms of invertebrate innate immune response (Lockyer et al., 2008; Mitta et al., 2012; Mone et al., 2011; Pinaud et al., 2016; Zhang et al., 2004).

The genus *Schistosoma* contains around twenty species, three of which, *S. haematobium*, *S. japonicum*, and *S. mansoni* are the principal agents of human schistosomiasis that represents the second most prevalent parasitic disease in the world affecting 200 million people and killing 200,000 persons every year (Chitsulo et al., 2004). Adult worms mate in a vertebrate host venous system.

* Corresponding author. UMR5244 IHPE, 58 avenue Paul Alduy, 66860 Perpignan, France.

E-mail address: benjamin.gourbal@univ-perp.fr (B. Gourbal).

¹ Co-first authors.

They produce eggs that reach the aquatic environment by passing through the vertebrate intestinal or bladder wall. In water, eggs hatch and release a miracidium that specifically infects fresh water snail species. Schistosomes, like all trematode species, needs molluscs as intermediate hosts to carry out part of their life cycle before infecting humans. Inside snail tissues, the miracidium turns into primary sporocyst (Sp1) that gives sequentially a secondary sporocyst (Sp2) and then cercaria by asexual multiplication. After leaving the snails, cercariae actively look for a definitive vertebrate host to infect (Yoshino et al., 2016).

In South America, *B. glabrata* snails have important medical and epidemiological impacts due to their role as the main vector of *S. mansoni*, the agent of intestinal Schistosomiasis. Consequently, a better understanding of the molecular interactions and the compatibility (susceptibility/resistance status) between *B. glabrata* and *S. mansoni* would contribute to the discovery of new ways to prevent and/or control Schistosomiasis diseases in the field (Tennesen et al., 2015b).

A high level of specificity exists between the *Schistosoma* miracidia and the snail intermediate hosts. This specificity could be explained by the suitability/unsuitability between the physiological and biochemical states of the host and the parasite. However, the main factor supporting this specificity is the host immune system and the ability of the parasite to cope with this immunity and/or to circumvent it (Sapp and Loker, 2000; Van Der Knaap and Loker, 1990). Thus, during specific interactions, either the host wins and the parasite is eliminated or the parasite wins and infects the host. This aspect of host/parasite interactions is described under the spelling compatibility polymorphism (Mitta et al., 2012) (see Fig. 5). Interestingly, investigation of the fate of miracidium parasites after penetration in the same host snail revealed that the infected snail can contain both developing primary sporocysts and encapsulated sporocysts located side by side within its tissues (Theron et al., 1997, 2014). The phenotype (infective vs. uninfected) of a parasite is expressed as a function of the genotype of the particular host that it enters. Likewise, the phenotype (susceptible vs. unsusceptible) of a host is expressed as a function of the parasite genotype it harbours (Theron et al., 2014). Compatibility polymorphism is thus based on a matching-phenotype model in which the compatibility is tested independently for each entering miracidium and each exposed snail (Theron et al., 1997, 2014). Numerous studies using isolates or strains of *Biomphalaria* snails from diverse geographic localities that have been exposed to sympatric or allopatric strains of *S. mansoni* (see Fig. 5) have emphasized the high compatibility polymorphism that characterizes this host-parasite matching-phenotype (Basch, 1976; Morand et al., 1996; Richards, 1975; Richards and Shade, 1987; Theron et al., 2014).

To go further in the understanding of this matching-phenotype, we must decipher the mechanisms through which snails and schistosomes interact. Integrative approaches are needed to combine a diversity of methodologies required to address the different levels of the molecular hierarchy potentially involved in host-pathogen compatibility and unravel the complexity of such biological systems (Fig. 1). The recent development of comparative genomic, transcriptomic, proteomic and epigenomic approaches, referred as multi-omic analyses, paved the way to a better understanding of the underlying molecular processes of snail/schistosome compatibility (Fig. 1). The molecular mechanisms underlying the high level of compatibility polymorphism observed in these interactions are based on the formation of immune complexes between *B. glabrata* fibrinogen related proteins (FREPs) and the *S. mansoni* polymorphic mucins (*SmPoMucs*), which were previously demonstrated as the most promising candidates involved in snail/schistosome compatibility (Dheilly et al., 2015; Mitta et al., 2012; Moné et al., 2010). On one hand, *SmPoMucs* are mucin-like

molecules displaying a domain containing a variable number of tandem repeats (VNTR). Mucins are known to be involved in host-parasite interplay (Buscaglia et al., 2006; Hicks et al., 2000; Rathore et al., 2005; Theodoropoulos et al., 2001). *SmPoMucs* could act as pathogen associated molecular patterns (PAMPs) and would be the initiator of snail host immune recognition (Moné et al., 2010). On the other hand, fibrinogen-related proteins (FREPs) constitute a highly diversified family of soluble immune pathogen recognition receptors (PRRs) from *B. glabrata* (Adema et al., 1997; Mitta et al., 2012; Moné et al., 2010). FREPs combine one or two N-terminal immunoglobulin superfamily (IgSF) domains with a C-terminal fibrinogen-related (FBG) domain and participate in binding trematode parasites (Moné et al., 2010; Zhang and Loker, 2004; Zhang et al., 2008a).

In this review, the roles played by FREPs and *SmPoMucs* in compatibility polymorphism has been compiled from integrated “Multi-Omic” approaches conducted on different snail and schistosome combinations displaying different compatibility phenotypes.

2. What have we learned from genomic approaches on FREPs and *SmPoMucs*?

Genetic studies conducted in the *S. mansoni/B. glabrata* model demonstrated that compatibility was heritable and could be selected in the laboratory either for susceptibility/resistance of the snail or for infectivity of the parasite (Davies et al., 2001; Richards, 1975; Richards et al., 1992; Richards and Shade, 1987; Webster and Davies, 2001), although the underlying genetic and molecular mechanisms were largely unknown. Compatibility may reside in a concordance of genetically determined phenotypes in snail and schistosome (Basch, 1975, 1976), a reduced set of parasite phenotypes in a population generally match with solely a fraction of the phenotypes present in the host strain (Théron and Coustau, 2005).

The first genome assembly and annotation of *S. mansoni* was published in 2009 (Berriman et al., 2009). It is composed of approximately 363 megabases in which 11,809 putative genes encoding 13,197 transcripts were identified. The functions of only a small percentage of these genes have been characterized so far. Moreover, the assembly was not perfect considering that 40% of the genome was repetitive and that the annotation mostly focused on possible therapeutic target genes against adult worms. This genomic resource has contributed to functional studies (Protasio et al., 2013), and was particularly helpful for investigating the mechanism of resistance to specific drugs (Valentim et al., 2013). Nevertheless, it has never been used to identify which genomic regions were under selection for the interplay with its intermediate host snail.

Additional efforts have been made in the characterization of gene loci in the *B. glabrata* genome associated with resistance or compatibility to *S. mansoni* infection. A first study using random amplified polymorphic DNA (RAPD) approach on several *B. glabrata* strains displaying a range of compatibility toward the same parasite isolate led to the identification of two heritable markers related to the resistance phenotype of adult snails (Knight et al., 1999). As these RAPD markers were located in repetitive genomic regions in the *B. glabrata* genome, further attempts to characterize the associated genes were not successful.

However, more recently a restriction site associated DNA sequencing (RADseq) study was conducted using two laboratory lines of snails that have been selected for *S. mansoni* resistance by experimental evolution (Tennesen et al., 2015b). RAD genotyping revealed in both lines a genomic region linked to resistance to the parasite. This region comprised 12 putative noncoding and 15 coding genes, among which 7 presented similarities for single-pass

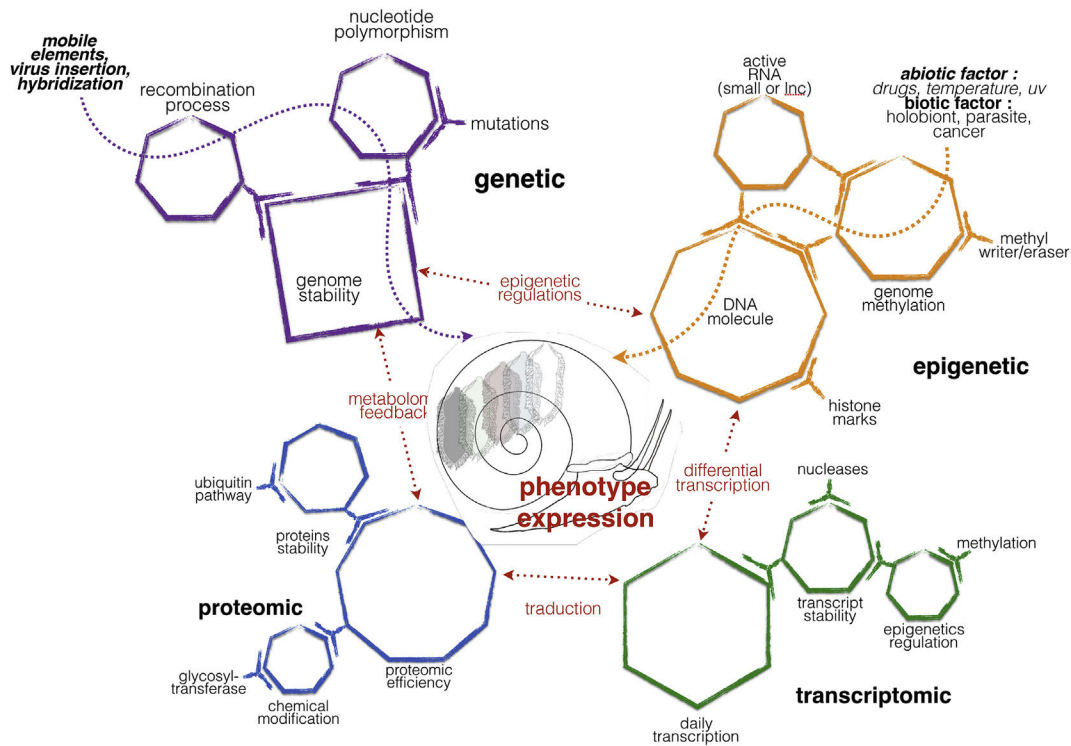


Fig. 1. Integrative multi-Omics approaches of host-parasite interaction.

transmembrane proteins. Proteins from this family are implicated in different processes like cell migration, adhesion, growth, and typically act as receptors for extracellular signals (Tennesen et al., 2015b). The authors notably underlined their potential role in the immune system such as B- and T-cell receptors, Fc receptors, major histocompatibility complex (MHC) receptors and Toll-like receptors, which are all membrane-bound receptors recognizing foreign molecules (Tennesen et al., 2015b). Complementary RNA-seq analysis revealed that the compatibility phenotype of the snail (susceptible or resistant) was not correlated to the level of gene expression but rather to differences in allele sequences (Tennesen et al., 2015a, 2015b).

Several molecules have been identified and implicated for immunity of *Biomphalaria glabrata* against *Schistosoma mansoni* (for review, see (Coustau et al., 2015)). However, few have been characterized at the genetic or genomic level, including one particular allele of the copper-zinc superoxide dismutase (SOD1) that is associated with snail resistance to *S. mansoni* infection (Blouin et al., 2013; Goodall et al., 2006) and of course the *SmPoMuc* and FREP molecules described thereafter.

2.1. The *Schistosoma mansoni* polymorphic mucins (*SmPoMuc*s)

The only parasite molecules characterized at the genomic level and implicated in the interaction with the intermediate host snail are the *S. mansoni* Polymorphic Mucins (*SmPoMuc*s) (for review, see (Gourbal et al., 2015)). These molecules were shown to be highly polymorphic at both population and individual level. In a population each miracidium expresses a unique combination of *SmPoMuc*s derived from a limited set of genes. *SmPoMuc*s are encoded by at least 6–9 multi-gene family members (Roger et al., 2008b). These genes are composed of 15 exons: the first two exons of the 5' gene region are considered as the variable region of

SmPoMuc genes, while the 13 other exons are considered as the conserved 3' region. Indeed, southern blot experiments revealed that *SmPoMuc*s contained a variable number of repeated exon 2 that never exceeded 20 copies. The different genes were classified in four paralogous sequence groups according to their sequence coding for the conserved 3'-terminal part of the gene. All members of group 4 were considered as pseudogenes. Fluorescent in situ hybridization (FISH) allowed locating *SmPoMuc*s genes in four distinct genomic regions of chromosomes 3 and 4. This genomic clustering associated with the presence of pseudogenes strongly supports that gene duplications would be at the basis of *SmPoMuc* gene diversification.

In *SmPoMuc* genes, Exon 2 (27 nucleotides) and its flanking introns are organized in 1 kb repeats, each separated by microsatellites. The high level of similarity of these genomic repeats between all members of *SmPoMuc* multigene family results in the frequent recombination and homogenization of the *SmPoMuc*s genes. Diversification of *SmPoMuc* molecules is driven by complex genomic mechanisms. Calculation of synonymous to non-synonymous substitutions ratios (K_S/K_N) and Tajima's neutrality test indicated that purifying selection acts on *SmPoMuc* genes (Roger et al., 2008b). In these genes, genomic repeats are subjected to concerted evolution and intron sequences are more homogenized than exons that evolve under purifying selection (Roger et al., 2008b). This results in combinatory events observed at the genomic level, probably linked to ectopic recombination and exon exchange between clusters.

The *SmPoMuc*s genes were not present in the current *S. mansoni* genome annotation but can be found by similarity searches. However, the current assembly (v5) around these loci contains potential errors since similarities between genes of this family as well as the genomic repeats prevented their correct assembly into the genome.

2.2. The fibrinogen-related protein (FREP) genes

Several studies have focused on *B. glabrata* fibrinogen-related proteins (FREPs) (for a review see (Gordy et al., 2015)) that have been shown *in vitro* to interact with the parasite molecules, *SmPoMuc* (Moné et al., 2010).

FREPs are composed of a signal peptide (SP), target signal for extracellular secretion, immediately followed by an immunoglobulin superfamily domain (IgSF), which is finally connected by a length-variable interceding region (ICR) to a fibrinogen domain (FBG). Moreover two classes of FREPs are described, single-IgSF FREPs and tandem-IgSF FREPs, in which IgSF domain are arranged in tandem and separated by a short connecting region (Hanington and Zhang, 2011; Zhang et al., 2001). The two IgSF domains of the tandem-IgSF FREP are quite different in sequence and are not just a repeat of a unique domain. The two IgSF domains of tandem-IgSF FREP are more similar to each other than to the IgSF domain of a single-IgSF FREP (Dheilly et al., 2015).

Fourteen FREP families have been described up to date, mainly from *B. glabrata* M-line strain (Adema et al., 1997; Zhang and Loker, 2003; Zhang et al., 2008a). Families 2, 4 and 14 belong to single-IgSF FREP, while families 3, 7, 12 and 13 belong to tandem-IgSF FREP. The other FREP families couldn't be clearly assigned to one of these groups, because solely one IgSF or FBG domain has been described for these genes. At the genomic level complete gene sequences are available for only 4 FREP families (2, 3, 4 and 7). The FREP 2 and 4 (single-IgSF) are encoded by 4 exons and 3 introns (Léonard et al., 2001; Zhang et al., 2008a), and FREP 3 and 7 (tandem-IgSF) are encoded by 6 exons and 5 introns. It has been shown that introns from single-IgSF FREPs are composed of repeats that varied in length and number, and that they presented low similarity between FREP2 and FREP4 (Léonard et al., 2001). The FBG region is the most conserved domain, while IgSF and ICR are the most variable domains (Dheilly et al., 2015; Léonard et al., 2001; Zhang et al., 2001; Zhang and Loker, 2004).

To characterize the diversity of FREPs, several approaches have been conducted. Southern blot analysis and PCR with degenerated primers on FBG domains revealed their abundance in *B. glabrata* genome and suggest the diversity of FREPs at this level (Zhang and Loker, 2004). Moreover, other studies have highlighted the capacity of somatic diversification by point mutation, gene conversion and ectopic recombination at the genomic level in all FREP families studied so far. Such diversification events have been reported for IgSF, ICR and FBG domains (Dheilly et al., 2015; Moné et al., 2010; Zhang et al., 2001, 2004). Altogether, these results suggest a high potential of diversification of FREP molecules. The assembly and annotation of the *B. glabrata* genome will provide a powerful expected tool to describe and understand the mechanisms responsible for diversity of FREPs at the genomic level.

Such diversity of FREP/*SmPoMuc* molecules provides support for the matching phenotype hypothesis and might partly explain the compatibility polymorphism observed between schistosomes and *Biomphalaria* strains.

3. What have we learned from transcriptomic approaches on FREPs and *SmPoMuc*?

Transcription embodies the first step of gene expression in cells. The absence or presence of specific transcripts at a precise time and/or specific localization in cells might gives insights on activated or repressed genes in specific physiological or molecular pathways. With this objective, many techniques have emerged since the 1980s to massively and easily explore the transcriptomic compartment, from suppression subtractive hybridization (SSH) and microarray to massive RNA sequencing (RNAseq).

The history of the discovery and investigation of FREPs and *SmPoMuc* follows the improvement of next generation sequencing (NGS) methods and benefited from several methods until nowadays. In 2003, Marathon cDNA libraries brought the first information about sequence diversity of FREPs genes family. It also provided evidence for alternative splicing abilities of FREPs in *Biomphalaria* in response to parasite infection (Zhang and Loker, 2003).

Alternative splicing is described as a transcriptomic process to increase the production of host diversified recognition molecules and enhance host's ability to recognize pathogens. This provided the first insights toward the idea of a polymorphism and/or diversification in the FREPs family. In *SmPoMuc*, a similar pattern of alternative splicing, in addition to aberrant splicing or *trans*-splicing, led to inclusion or exclusion of some parts of the genes that ultimately produce a high level of polymorphism from a restricted number of genes (Fig. 2), allowing to increase the diversity of proteins that are the potential targets recognized by the host immune system (Roger et al., 2008b). Concerning *Biomphalaria* factors, reverse transcription quantitative PCR (RT-qPCR) first revealed the differential expression of FREP family genes in *Biomphalaria* following infection with two species of highly infective parasites (*S. mansoni* and *Echinostoma paraensei*) (Hertel et al., 2005). This result gave support for putative roles of FREP2 and FREP4 in *Biomphalaria* defense. Functional invalidations were rapidly developed in *Biomphalaria* system to explore the involvement of specific genes in compatibility phenotypes. The first RNAi (interference RNA) gene knockdown developed in *Biomphalaria* strain involved the injection of double-stranded RNA (dsRNA) into the snail hemolymph or their addition to co-cultures of *Biomphalaria* embryonic cell line (Bge cells) and parasites (Jiang et al., 2006). This technique opens the way to gene inactivation of FREPs. Rapidly, the related molecules in *Schistosoma* have also been explored. The first transcriptomic analysis of *SmPoMuc* revealed that mRNAs encoding mucin-like proteins were detected at three different developmental stages (*i.e.*, egg, miracidium and primary sporocyst Sp1), particularly at a high-level at the miracidium stage of the parasite (Roger et al., 2008a). Mucins may be involved in the penetration of miracidia into the snail and help during the transition from the free-living miracidium stage to the first intramolluscal stage Sp1 (Theodoropoulos et al., 2001). Comparison between strains of parasites revealed that less-recognized compatible strain (*SmbRE*) expressed 3-fold less *SmPoMuc* than the best-recognized incompatible one (*SmGH2*) (Galinier et al., 2017; Roger et al., 2008a). These observations suggest that mucins can be the target of the recognition by the snail and that diversity of these putative antigenic proteins can increase parasite capacity to evade the host immune pathogen recognition receptors (PRRs). Proteins containing fibrinogen C-terminal domain have been shown to be triggered in response to compatible and incompatible *Schistosoma* infection but also following an infection with irradiated-miracidium (Ittiprasert et al., 2010). Based upon microarray experiments, the variation in FREP gene transcription was analyzed and revealed the over-expression of FREP2, 3, 4 and 6, the under-expression of FREP1, 5, 7, 10, 12 and divergent expression of FREP11 between 12 h and 32 days following *Schistosoma* infection (Hanington et al., 2010). This study provided the first evidence that FREPs constitute a complex immune-related family in which some members are over-expressed while other are under-expressed following the same infection response. The specificity of parasite detection appears to be more efficient when the host possesses a diversified repertoire of differentially-selected PRRs that can be used in response to different pathogens. After microarrays, massive RNA sequencing (RNAseq) with Illumina/Solexa technologies have generated a lot of new information associated

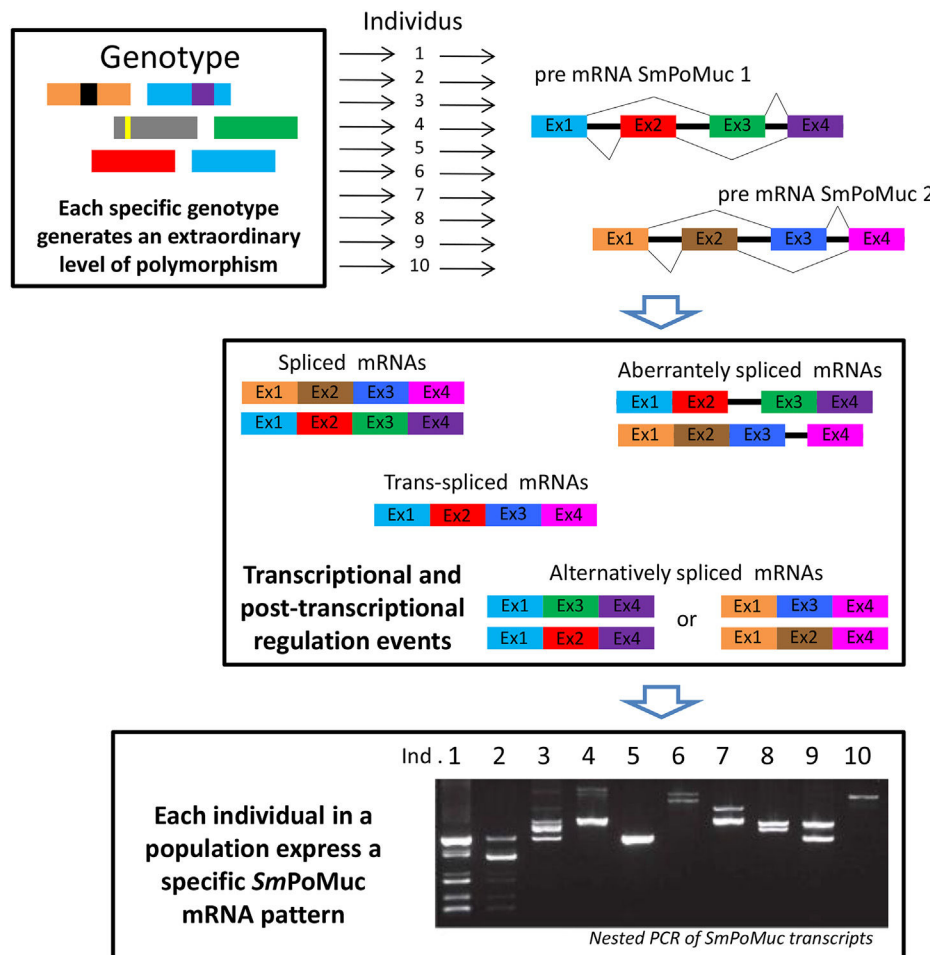


Fig. 2. Transcriptional *SmPoMuc* polymorphism defines a specific *SmPoMuc* pattern for each individual in a population of *S. mansoni* parasites. Hypothetical exon/intron structures of *SmPoMuc*s were used here to describe the transcriptional processes involved in the genesis of *SmPoMuc* diversity or polymorphism.

with the deeper sequencing resolution. By investigating *B. glabrata* FREPs diversity from a *de novo* generated transcriptome, Dheilly and collaborators (Dheilly et al., 2015) revealed that within each FREP family member, a significant nucleotide variability was observed in transcripts resulting in polymorphism in the predicted amino-acid protein sequence. These authors showed that the FREP molecules did not show the highest level of transcript variants among lectin family members and also that, FREPs were not the more expressed in naïve snails. This means that putative other immune-related recognition receptor molecules within the large lectin family (including Ig-like fold, C-Type lectin, Leucin-rich-repeat, Selectin, Galectin) may increase immune recognition capabilities in *Biomphalaria*. Several Variable Immunoglobulins (VIGs), CREPs (C-Type lectin related protein) and GREPs (Galectin related protein) sharing IgSF domain with FREPs were also evidence. This indicates the existence of recombination or *trans*-slicing events between highly-variable molecules belonging to different lectin families. This demonstrates the strong evolutionary pressures playing on those closely-related molecules, which share similar putative immune-related features, which are able to generate additional polymorphism and diversity.

Recent RNAseq approach confirmed the role of FREPs during a shift from a cellular to a humoral immune response in the innate immune memory of *Biomphalaria* (Pinaud et al., 2016). Despite an absence of up-regulation of FREP genes following sympatric

infection, a global over-representation of FREPs was observed following immune homologous challenge. Knockdown of FREP2, 3 and 4 genes revealed that the 100% acquired resistant snails became partially susceptible (15%) after a reinfection with the same parasite and confirmed the key role of FREPs among lectin families, while highlighting that additional factors might complement FREP function in *Biomphalaria* innate immune memory.

4. What have we learned from epigenomic approaches on FREPs and *SmPoMuc*s?

Heritable phenotypic diversity has traditionally been attributed to genetic diversity. We know today that this view of heritability is incomplete and must include other diversity-generating mechanisms that can be heritable such as those regulated by epigenetic elements. The epigenetic changes refer to a set of molecular processes that can affect gene expression by non-coding RNA, through methylation of nucleic acids in DNA, chemical modification of histones and re-localization of a locus inside different nucleus territories (Keung et al., 2015). These epigenetic factors have been shown to be under the influence of the environment and their modifications can have consequences on the chromatin compaction and therefore may affect gene expression. In this sense, epigenetic modifications can result in new phenotypes that might be adaptive. The epigenotype is mitotically and to some degree

meiotically heritable, but unlike in the genotype, changes in the epigenotype are generally reversible (Keung et al., 2015). Therefore, the epigenetic information system (EIS, epigenetics) can provide heritable, novel phenotypes that do not rely on variation in DNA sequence (Geoghegan and Spencer, 2012). If this EIS influences the capacity to generate different phenotypes, both the better adapted phenotype and the capacity to generate this phenotype will be selected and conserved in the next generation (Gomez-Diaz et al., 2012). This hypothesis has been investigated in the context of the arm race hypothesis that occurs during the interaction between *S. mansoni* and its intermediate host *B. glabrata*. The availability of the genome for both partners (Berriman et al., 2009) (Bg genome submitted: see Vector Base website (<https://www.vectorbase.org/>)) and the recent advances in genome-wide analysis have allowed the first description of different bearers of epigenetic information in different contexts. Histones are highly conserved proteins and commercial drugs and antibodies are usable to target these proteins and study their modifications in diverse species. Chromatin immunoprecipitation (ChIP) analysis targeting these modified histones has been applied to *S. mansoni* and allowed the visualization of the position of modified histones *in vivo* in different environmental contexts and at different developmental stages of this parasite (Picard et al., 2016; Roquis et al., 2015). Several studies have demonstrated how histone modifications in *S. mansoni* are engaged to generate phenotypic plasticity during the interaction with its intermediate host. *SmPoMuc* are highly conserved genes in their coding region and upstream putative promoter regions. Despite this high degree of conservation observed between compatible and incompatible strains of *S. mansoni*, their patterns of expression strongly differ between these two strains (Roger et al., 2008a). ChIP analysis revealed that different modified histones are present in the upstream regulatory regions of the *SmPoMuc* genes which could be responsible for an epigenetic-based regulation of their expression (Perrin et al., 2013). Other studies further showed that treatment of the parasite with drugs that target histone acetylation led to an increased compatibility with the snail (Fneich et al., 2016). The snail environment has also been demonstrated to be an epigenome modifier itself as infection of allopatric and sympatric snails with genetically identical *S. mansoni* resulted in the generation of different epigenotypes in each genetically identical parasite (Roquis et al., 2016). Furthermore, *S. mansoni* is a typical organism with a tRNA methyltransferase DNMT2 for which DNA methylation is really low and has been controversial (Raddatz et al., 2013). DNA methylation can be studied by a large panel of techniques such as bisulfite conversion (BS) assay that allows studying cytosine methylation at the single base resolution (Grunau et al., 2001), methylation-sensitive amplification polymorphism (MSAP) (Reyna-Lopez et al., 1997) and immunological and cytochemical approaches based on the use of conserved antibodies. By combining some of these different methodologies, Geyer et al. (2011) studied methyl-5-cytosine (m5C) in *S. mansoni* and linked the presence of the cytosine methylation machinery to plathyhelminth oviposition processes (Geyer et al., 2011). Finally, different works reported stage and sex-specific expression of several non-coding RNAs, which indicate that they might be involved in parasite development. On the side of the intermediate host, *B. glabrata* epigenome has also been shown to be under the influence of the environment (Knight et al., 2016). DNA methylation and locus topography have been shown to be modified in response to *S. mansoni* infection (Arican-Goktas et al., 2014; Knight et al., 2016). A methylome of *B. glabrata* has been generated by BS-Seq analysis (Cosseau et al. unpublished data and Fig. 3). This snail displays a typical invertebrate mosaic-like DNA methylation pattern, with highly methylated regions, predominantly in the CpG context, interspaced with poorly methylated regions. Unlike plants and mammals, the role of DNA

methylation for regulation of gene expression is controversial in invertebrates and other bearers of epigenetic information such as small RNA, histone modifications and locus position in chromosome territories certainly deserve further attention to elucidate the involvement of epigenetic regulation events on the observed patterns of gene expression. FREP genes are typical phenotypic variants for which we anticipate an epigenetic-based gene regulation. Such a regulation is expected in the case of the generation of phenotypic diversity followed by natural selection upon environmental changes (Cortes et al., 2012). FREPs genes in *Biomphalaria glabrata* are mainly localized in low methylated regions (Fig. 3). The absence of DNA methylation in these regions has been hypothesized to contribute to stochastic transcriptional opportunities (Gavery and Roberts, 2014), which is typically what is expected to explain part of the transcriptional diversification observed in highly diversified FREP gene families. These FREP genes are therefore candidates for which valuable tools such as ChIP-assays against modified histones will be worth pursuing. This will certainly open new perspectives and improve the knowledge of the sophisticated molecular pathways involved in the host parasite interplay.

5. What have we learned from proteomic approaches on FREPs and *SmPoMucs*?

Proteomic approaches allow investigating and characterizing features of immune-related actors by focusing on the last form of the gene expression process, *i.e.* protein that can explain the phenotype. Although genomic, transcriptomic and epigenetic approaches can inform on the sequence diversity at the gene and transcript level and on gene expression regulation, only proteomic approaches can provide information about the molecular interaction between proteins from hosts and pathogens and give insights into their function. This can come from characterization by gel-based approaches (SDS-PAGE, 2D-PAGE) or gel-free approaches (Label-Free protein sequencing) of entire proteomes coupled with tandem mass spectrometry, or by analyzing post-translational modifications of proteins, protein localization in cells, organs or tissues and/or by analyzing protein/protein interactions. All these approaches have been used in the *S. mansoni/B. glabrata* interaction to identify parasite antigens and the potential corresponding pathogen recognition receptors in the host's hemolymph.

Following snail infection by *S. mansoni*, incompatible miracidia are recognized, encapsulated and killed in few hours, while compatible miracidia remain unaffected. This observation suggests constitutive antigenic differences between those strains of parasite. Thus, a global comparative proteomic approach has been conducted to characterize these antigenic differences (Roger et al., 2008a, 2008c). The main difference identified corresponded to *SmPoMucs* that exhibited a pronounced qualitative and quantitative polymorphism between compatible and incompatible parasite strains (Roger et al., 2008c). *SmPoMuc* were further investigated (Roger et al., 2008a, 2008c) and the data obtained showed that these proteins are: (i) only expressed at larval stages interacting with the mollusc, (ii) located in the apical gland of miracidia and sporocysts, (iii) secreted and released in excretory-secretory products and finally (iv) displayed a high degree of polymorphism at the protein primary structure level and (v) are highly glycosylated. The analysis of the global glycosylation status of *SmPoMucs*, for both strains through chemical deglycosylation experiments, highlighted higher glycosylation levels for variants of the incompatible strain as compared to compatible strain (Roger et al., 2008a). All of these characteristics make *SmPoMucs* key candidates of the compatibility polymorphism and matching phenotype process, being potential pathogen antigens recognized by the host recognition receptors.

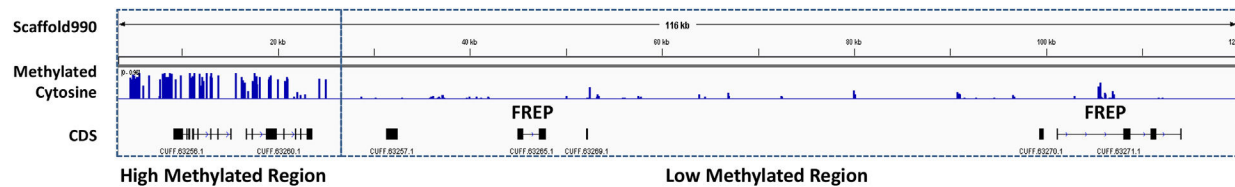


Fig. 3. Genome extract of the *B. glabrata* genome assembly: LGUN_random_Scaffold990:3335-119,948. Methylated cytosines are represented by blue bars. The height of the bar represents the percentage of methylation obtained for the methylated cytosines. The highest percentage of methylation observed in this window is 96%. CDS are represented according to a previously published transcriptome (Dheilly et al., 2015). Two FREPs have been annotated in this window (from position 44,955 to 47,325 and from position 99,422 to 114,087) (Richard Galinier, personal communication). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Mucins have indeed already been identified as important actors of the host-parasite interaction (Theodoropoulos et al., 2001), FREPs were originally discovered as circulating glycoproteins present in the plasma of *B. glabrata* and able to agglutinate rabbit erythrocytes (Boswell and Bayne, 1984). This agglutination was shown to be inhibited when carbohydrates were pre-incubated with plasma before the addition of rabbit red blood cells, suggesting the key role of sugar in this interaction. Further studies demonstrated that an agglutination reaction also occurred when *Biomphalaria glabrata* plasma was exposed to sporocyst of trematode parasites species (*S. mansoni* and *Echinostoma paraensei*) (Couch et al., 1990; Loker et al., 1984, 1994). Furthermore, the intensity of the reaction independent of the snail strain used regarding the infection (naïve or infected) and its resistance (susceptible or resistant) toward the parasite considered (Bayne et al., 1986; Couch et al., 1990; Loker and Hertel, 1987). These agglutination factors were finally demonstrated to precipitate *E. paraensei* secretory/excretory products, which later turned out to contain FREPs (Adema et al., 1997; Zhang et al., 2001).

To shed light on the molecules from the host that specifically recognize parasite proteins and therefore trigger the appropriate immune response, an interactome approach has been performed on the *Biomphalaria-Schistosoma* host-pathogen system. In addition to several enzyme inhibitors, hormones and metalloenzymes, two families of lectins from *B. glabrata* were identified as able to interact with *Schistosoma* proteins: several galactose binding lectin-like proteins and FREPs (Moné et al., 2010). Moreover, many different proteins from the parasite interacting with host proteins were also identified including three glycoprotein families that are integral membrane protein Sm23, secretory glycoprotein k5 and SmPoMuc (Moné et al., 2010). To go further, co-immunoprecipitations were mandatory to demonstrate which snail protein(s) bind to SmPoMucs. CO-IP assays were thus conducted using proteins from *Biomphalaria* hemolymph, parasite extract containing SmPoMuc and antibody raised against C-terminal part of SmPoMucs. This revealed three proteins directly interacting with SmPoMuc include an alpha amylase, a thioester-containing protein (TEP) and a specific protein from the FREP family, FREP2 (Moné et al., 2010). It was hypothesized that TEP would interact with the complex primarily formed by FREP2 and SmPoMuc but the mechanisms and the binding sites involved in such an immune complex are currently unknown (Gordy et al., 2015; Moné et al., 2010).

Finally, in *B. glabrata*, it has been hypothesized that these PRR molecules could serve as complementary or collaborative recognition factors that interact with each other to form complexes (Adema and Loker, 2015). Among them, FREPs have been shown by western blot analysis to occur in *Biomphalaria* hemolymph as multimers of high molecular weight although the mechanism responsible for this multimerization and its importance in the role of FREP-based pathogen recognition has yet to be investigated (Adema et al., 1997; Gordy et al., 2015; Zhang et al., 2008b).

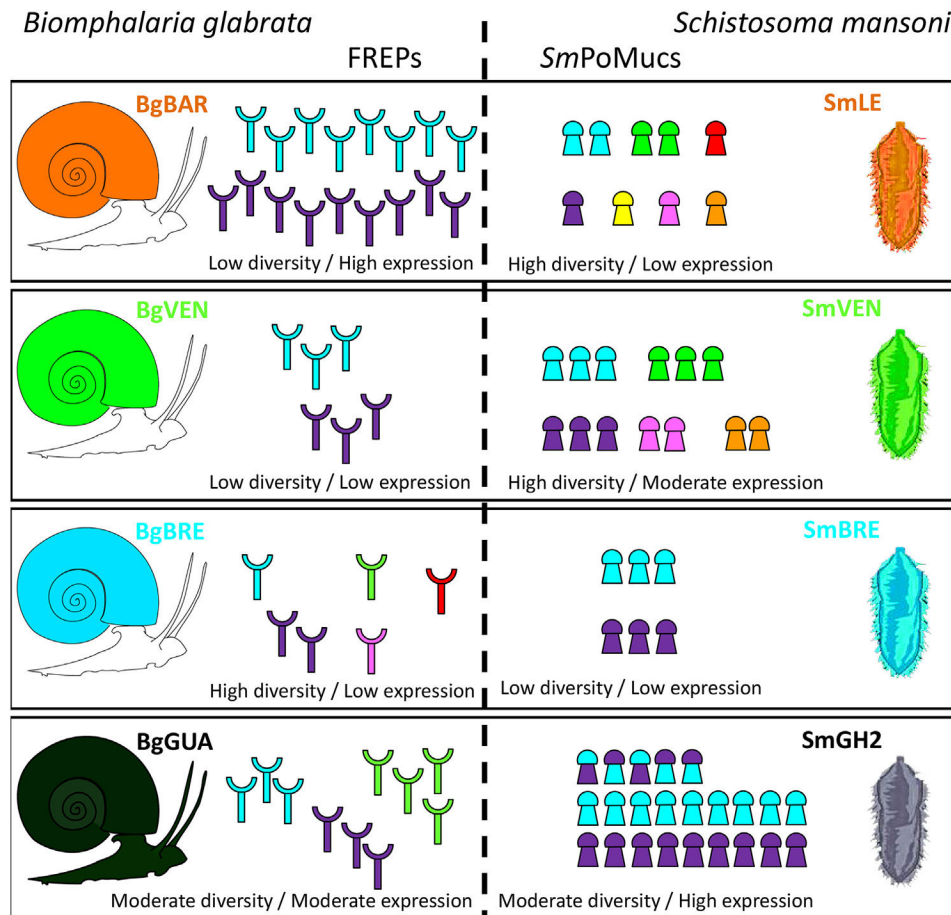
Nevertheless, such multimerization could be an additional process for increasing the mollusc pathogen recognition repertoire, by generating a wide range of multimers each with a different level of parasite antigen specificity. As such, it could increase the potential for specific recognition of parasite molecules, allowing a high capacity of immune system to recognize non-self and to trigger specific anti-pathogen innate immune responses (Adema et al., 1997; Schulenburg et al., 2007).

FREPs diversification would have been the evolutionary response of host to maintain its recognition capacity and could be engaged with hemocytes to mediate phagocytosis after parasite recognition (Hanington and Zhang, 2011; Peterson et al., 2009). These results highlight how, even at the protein level, the interaction between FREPs and SmPoMuc could be at the basis of the process of compatibility between different strains of *Schistosoma* and *Biomphalaria* (Coustau et al., 2015; Mitta et al., 2012).

6. A multi-omic integrative view of FREPs/SmPoMucs compatibility polymorphism

The molecular mechanisms underlying the high level of immune specificity observed in these interactions are based on the formation of complexes between a specific set of highly polymorphic or diversified immune recognition molecules named the fibrinogen-related proteins (FREPs) from *B. glabrata* and polymorphic parasite's glycosylated mucin molecules, named *Schistosoma mansoni* polymorphic mucins (SmPoMucs) (Dheilly et al., 2015; Moné et al., 2010). Results obtained from "Omic" approaches on these two candidates were summarized in the Fig. 4 for different sympatric snail and Schistosome combinations displaying different levels of compatibility (see legend of Fig. 4 for details on *S. mansoni* and *B. glabrata* strain origins and phenotypes).

We found that the polymorphism and expression levels of FREPs and SmPoMucs would be linked to the compatibility level between *S. mansoni* and *B. glabrata* (Galinié et al., 2017). In BgBAR/SmLE interaction, snail hosts presented a low diversity but a high level of expression of FREPs while the parasite expressed low levels of highly diversified SmPoMucs (Fig. 4). In this context, the increased diversification combined with the decrease expression levels of the SmPoMuc glycoproteins could allow the parasite to escape from being recognized by FREP immune receptors. The same strategy could also be described for the BgVEN/SmVEN interaction, but as the host has a low expression of FREPs, the recognition selective pressures on the parasite were reduced and thus a moderate expression of SmPoMucs has been maintained. In this case, the parasite invests more in SmPoMuc diversification than in reduction of expression to circumvent recognition. A totally different strategy could be observed in BgBRE/SmBRE interaction. In this case, the snail host presents a high diversity and low expression of FREPs and the parasite a low diversity and low expression of SmPoMucs. We could hypothesize that it could be difficult for the parasite to



Expected / Observed compatibility phenotypes

	SmLE	SmVEN	SmBRE	SmGH2
BgBAR	C / C P= 96% I = 3.7	M / M P= 55% I = 3.9	IC / IC P= 12% I = 2.3	IC / IC P= 0% I = 0.0
BgVEN	C / C P= 100% I = 7.4	C / C P= 100% I = 7.1	C / C P= 80% I = 5.5	M / M P= 44% I = 4.7
BgBRE	C / C P= 100% I = 8.4	C / C P= 100% I = 8.2	C / C P= 100% I = 7.1	M / IC P= 4% I = 1.0
BgGUA	C / C P= 100% I = 7.4	C / C P= 100% I = 6.5	M / C P= 83% I = 3.0	M / M P= 60% I = 2.6

Fig. 4. Diversity and expression levels of FREPs and SmPoMucs in four sympatric host/parasite combinations.

Host and parasite were designated *BgBAR/SmLE* (for Belo Horizonte, Brazil), *BgVEN/SmVEN* (for Guaraca, Venezuela), *BgBRE/SmBRE* (for Recife, Brazil), and *BgGUA/SmGH2* (for le Lamentin, Guadeloupe) (Theron et al., 2014). The number of FREPs and SmPoMucs symbols represents the level of expression. The color of FREPs and SmPoMucs symbols represents the level of diversity. Mixed colors in SmGH2 represent the SmPoMucs with intermingled repeats which are a specificity of this Guadeloupean parasite strain.

In each table cell was indicated in the left corner the expected/observed compatibility phenotype (C: compatibility; M: moderate compatibility; IC: incompatibility) and in the right corner the prevalence (P, percentage of snail infected) and intensity values (I, number of parasites per infected snails) for all sympatric and allopatric combinations following experimental infection with 20 miracidia (Theron et al., 2014). Expected compatibilities were estimated based on the hypothesis proposed from the observations of sympatric compatibility phenotypes (see part 5. for details). Highlighted table cells correspond to host/parasite interactions in which expected and observed phenotype data are not in concordance. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

diversify the SmPoMucs more than the host FREP molecules (*BgBRE* snails exhibited the highest FREP diversity) and thus parasite would reduce SmPoMuc expression to limit recognition. Finally, in *BgGUA/SmGH2* interaction, intermediate patterns can be observed, with moderate diversity/moderate expression of host FREPs and moderate diversity/high expression of parasite SmPoMucs. In this case, SmPoMucs were highly expressed and could be easily recognized by FREPs, which could explain the low compatibility observed in this host-parasite couple. On one hand, these results seemed to

demonstrate that a high diversity and a low expression of SmPoMucs are required to enhance parasite infectivity. On the other hand, the diversity of FREPs seems not to be correlated to the compatibility level as it could be expected from an ongoing arms race between FREPs and SmPoMucs. Indeed, *BgBRE* and *BgGUA* (highly compatible snail strains) displayed the higher numbers of variants by comparison with *BgVEN* and *BgBAR* (low compatible snail strains). The explaining factor seems to be the high level of expression of FREPs that is required to enhance snail resistance

- **Compatibility:** a pair of specific host and parasite genotypes/phenotypes in which an individual parasite infects an individual host and completes the part of its life cycle appropriate to the host.
- **Incompatibility:** a pair of specific host and parasite genotypes/phenotypes in which an individual parasite did not infects an individual host and fails to complete the part of its life cycle appropriate to the host.
- **Sympatric interaction:** host and parasite species coexist in the same geographic area. They experiment frequent encounters one another.
- **Allopatric interaction:** host and parasite species originate from different geographic area. They never experiment encounters one another.

Fig. 5. Key definitions related to snail-schistosome interactions.

toward *S. mansoni*. Allopatric interactions would also confirm these hypotheses; for example, *SmVEN* parasite succeed in infecting *BgBAR* but solely at a prevalence of 55% because *SmPoMuc* diversification is high but expression remains quite high and *BgBAR* FREPs would manage to form complexes with *SmPoMucs*. In *SmGH2*, the moderate diversity and high expression of *SmPoMucs* appeared to be a deleterious pattern that could perhaps explain the high incompatibility phenotype of this strain (prevalence never exceeds 60%).

Based on these hypotheses, the expected compatibilities between all potential combination of the four strains of hosts and parasites were estimated and compared to the experimentally observed compatibility phenotypes (see table in Fig. 4, experimental prevalence and intensity values are indicated). Interestingly, most of the expected and observed phenotypes were in agreement. Only two cases for which expected phenotypes did not fit with observed phenotypes were identified: (i) *SmBRE* in *BgGUA* expected to have a moderate compatibility because of the moderate expression of FREPs from *BgGUA* that are expected to recognize a large part of *SmBRE* parasites but is actually compatible (see Table Fig. 4); (ii) *SmGH2* in *BgBRE* expected to have a moderate compatibility because of the low expression of FREPs in *BgBRE* and the high expression of *SmPoMucs* in *SmGH2*, and is actually incompatible (see Table Fig. 4).

This discrepancy between expected and observed phenotypes would be expected. Indeed, if recognition by FREPs seemed to explain most of the compatibility, we know, based on knockdown experiments (Hanington et al., 2012; Pinaud et al., 2016), that FREPs were not the unique determinant of compatibility and that additional factors were involved in *Biomphalaria* immune response. The activation of signalling pathways following recognition leading to hemocytes-dependent phagocytosis or encapsulation and to the release of humoral factors have to be considered.

Finally, the results described above on *B. glabrata/S. mansoni* interactions, challenged our view of the role or function of FREP diversification. These results lead us to a totally new unexpected hypothesis: we argue that quantity rather than diversity of FREPs would benefit the overall recognition and elimination of a specific *S. mansoni* pathogen. Local adaptation or co-evolution would indeed favour the selection of a specific subset of FREPs that would be expressed at a high level to recognize a specific subset of *SmPoMucs* expressed by the parasite.

Nevertheless, it remains unclear to what extent FREP diversity benefits immune function of *B. glabrata* (Adema, 2015). Polymorphism and/or diversity of FREP immune receptor variants yield an enlarged repertoire of putative recognition molecules between individuals. This diversification provides a mechanism to increase host's recognition ability, and as such, the potential for specific recognition of pathogens. Indeed, *Biomphalaria glabrata* snails are confronted with an environment filled with complex changing communities of microorganisms and potential pathogens (bacteria,

fungus, yeasts, trematodes, nematodes, etc ...). Therefore, it can be expected that snails have to co-evolve with these pathogen communities and should possess sophisticated recognition systems for dealing with them. FREP diversification would be part of this enhanced immune recognition capabilities used to recognize and eliminate most of these pathogens.

7. And now, what next?

To further understand the interaction between *S. mansoni* and *B. glabrata* different lines of research can be considered.

6.1.) The interaction between FREPs and *SmPoMucs* is part of an immune complex involving different partners, like the thio-ester containing protein (BgTEP) and perhaps other molecules that remain to be characterized (Gourbal et al., 2015; Mone et al., 2010). Identification of such molecules will necessitate co-immunoprecipitation or pull-down experiments with recombinant FREP or TEP incubated with parasite extracts and snail plasma. Another question to solve is to identify the nature of the interacting domains and how molecules interact with one another. To answer this question, approaches such as those described above could be conducted using targeted mutagenesis or truncated recombinant proteins.

6.2.) To explore the function and role of highly-variable molecules like FREPs and *SmPoMucs*, transcriptomic approaches from RT-qPCR to NGS have suffered from technical limitations, notably the lack of genomic resources in non-model species (Dheilly et al., 2014). However, emerging technologies such as single-cell RNA sequencing will provide technical breakthroughs for this field of research. We can expect that individual diversity and expression of both FREPs and *SmPoMuc* will be unraveled using this technology. For example, sequencing the hemocyte transcriptome for the same individual before and after pathogen infection might give insights on pathogen recognition receptor diversification in an invertebrate model.

6.3.) Analysis of transcriptomic data from different strains of *B. glabrata* (Dheilly et al., 2015) strongly suggests the existence of other FREP subfamilies and consequently additional genes that have to be clarified with the use of the future *B. glabrata* genome annotation (Bg genome available at Vector Base website (<https://www.vectorbase.org/>)).

6.4.) Whole-genome re-sequencing of parasite populations using PacBio and/or illumina genomic sequencing would be a powerful tool to circumvent the limits of actual genome assemblies for multi-gene families containing genomic repeats. The highly-contiguous *de novo* assemblies using PacBio sequencing can close gaps in current reference assemblies and longer reads would be useful to sequence through extended repetitive regions (Rhoads and Au, 2015). Whole-genome re-sequencing might permit to explore how patterns of genetic variation change across the genome and detect, based on population genetic tests, the genes

under directional and balancing selection (Crellen and Iantorno, 2015; Rhoads and Au, 2015). Genetic approach by quantitative trait locus (QTL) mapping or extreme QTL (X-QTL) mapping using *S. mansoni* genome data would also be useful to identify genomic regions that were under selection for the interplay with its intermediate snail host.

6.5.) Methods for the functional characterization of genes of interest (i.e., invalidate and/or restore gene functions to analyze the corresponding phenotype modifications: functional genomic) are still lacking in schistosomes and snails. Except gene knockdown by RNA interference (RNAi) that is successfully used in both animal models, knockout or stable transgenic systems have to be developed now. Development of tools to introduce DNA constructs into adult and larval schistosome stages to express reporter genes have been tentatively developed using particle bombardment, electroporation or virus-based infection strategies. Unfortunately, until now non stable transformations were obtained (Beckmann and Grevelding, 2012; Crellen and Iantorno, 2015; Kines et al., 2008, 2010; Mann et al., 2008; Rhoads and Au, 2015). Such developments in *Biomphalaria* still lag behind. Finally, gene knockout using the CRISPR-Cas genome-editing technique (Komor et al., 2016) provides the ability to edit genomes within living cells or organisms representing a major advance for both host and parasite models.

The existence of polymorphic and/or diversified putative host immune receptors and parasite antigens that vary considerably in pathogen and snail populations no longer has to be demonstrated. However, understanding the function of such polymorphic or diversified molecules in invertebrates will be now a new challenge to solve and will deserve further investigations.

Funding

This work was supported by the French National Agency for Research (ANR) [grant ANR-13-JSV7-0009] Invimory to BG.

Acknowledgements

We thank Ms. Anne Rognon and Ms. Nathalie Arancibia for their work and diligence in helping to produce part of the data described herein.

References

Adema, C.M., 2015. Fibrinogen-related proteins (FREPs) in mollusks. *Results Probl. Cell Differ.* 57, 111–129.

Adema, C.M., Hertel, L.A., Miller, R.D., Loker, E.S., 1997. A family of fibrinogen-related proteins that precipitates parasite-derived molecules is produced by an invertebrate after infection. *Proc. Natl. Acad. Sci. U. S. A.* 94, 8691–8696.

Adema, C.M., Loker, E.S., 2015. Digenean-gastropod host associations inform on aspects of specific immunity in snails. *Dev. Comp. Immunol.* 48, 275–283.

Arican-Goktas, H.D., Ittiprasert, W., Bridger, J.M., Knight, M., 2014. Differential spatial repositioning of activated genes in *Biomphalaria glabrata* snails infected with *Schistosoma mansoni*. *PLoS Negl. Trop. Dis.* 8, e3013.

Basch, P.F., 1975. An interpretation of snail-trematode infection rates: specificity based on concordance of compatible phenotypes. *Int. J. Parasitol.* 5, 449–452.

Basch, P.F., 1976. Intermediate host specificity in *Schistosoma mansoni*. *Exp. Parasitol.* 39, 150–169.

Bayne, C.J., Loker, E.S., Yui, M.A., 1986. Interactions between the plasma proteins of *Biomphalaria glabrata* (Gastropoda) and the sporocyst tegument of *Schistosoma mansoni* (Trematoda). *Parasitology* 92 (Pt 3), 653–664.

Beckmann, S., Grevelding, C.G., 2012. Paving the way for transgenic schistosomes. *Parasitology* 139, 651–668.

Berriman, M., Haas, B.J., LoVerde, P.T., Wilson, R.A., Dillon, G.P., Cerqueira, G.C., Mashiyama, S.T., Al-Lazikani, B., Andrade, L.F., Ashton, P.D., Aslett, M.A., Bartholomeu, D.C., Blandin, G., Caffrey, C.R., Coghlan, A., Coulson, R., Day, T.A., Delcher, A., DeMarco, R., Djikeng, A., Eyre, T., Gamble, J.A., Ghedin, E., Gu, Y., Hertz-Fowler, C., Hirai, H., Hirai, Y., Houston, R., Ivans, A., Johnston, D.A., Lacerda, D., Macedo, C.D., McVeigh, P., Ning, Z., Oliveira, G., Overington, J.P., Parkhill, J., Perceca, M., Pierce, R.J., Protasio, A.V., Quail, M.A., Rajandream, M.A., Rogers, J., Sajid, M., Salzberg, S.L., Stanke, M., Tivey, A.R., White, O.,

Williams, D.L., Wortman, J., Wu, W., Zamanian, M., Zerlotini, A., Fraser-Liggett, C.M., Barrell, B.G., El-Sayed, N.M., 2009. The genome of the blood fluke *Schistosoma mansoni*. *Nature* 460, 352–358.

Blouin, M.S., Bonner, K.M., Cooper, B., Amarasinghe, V., O'Donnell, R.P., Bayne, C.J., 2013. Three genes involved in the oxidative burst are closely linked in the genome of the snail, *Biomphalaria glabrata*. *Int. J. Parasitol.* 43, 51–55.

Boswell, C.A., Bayne, C.J., 1984. Isolation, characterization and functional assessment of a hemagglutinin from the plasma of *Biomphalaria glabrata*, intermediate host of *Schistosoma mansoni*. *Dev. Comp. Immunol.* 8, 559–568.

Brites, D., McTaggart, S., Morris, K., Anderson, J., Thomas, K., Colson, I., Fabbro, T., Little, T.J., Ebert, D., Du Pasquier, L., 2008. The Dscam homologue of the crustacean *Daphnia* is diversified by alternative splicing like in insects. *Mol. Biol. Evol.* 25, 1429–1439.

Buscaglia, C.A., Campo, V.A., Frasc, A.C., Di Noia, J.M., 2006. Trypanosoma cruzi surface mucins: host-dependent coat diversity. *Nat. Rev. Microbiol.* 4, 229–236.

Chitsulo, L., Loverde, P., Engels, D., 2004. Schistosomiasis. *Nat. Rev. Microbiol.* 2, 12–13.

Combes, C., 2000. Selective pressure in host-parasite systems. *J. Soc. Biol.* 194, 19–23.

Cortes, A., Crowley, V.M., Vaquero, A., Voss, T.S., 2012. A view on the role of epigenetics in the biology of malaria parasites. *PLoS Pathog.* 8, e1002943.

Couch, L., Hertel, L.A., Loker, E.S., 1990. Humoral response of the snail *Biomphalaria glabrata* to trematode infection: observations on a circulating hemagglutinin. *J. Exp. Zool.* 255, 340–349.

Coustau, C., Gourbal, B., Duval, D., Yoshino, T.P., Adema, C.M., Mitta, G., 2015. Advances in gastropod immunity from the study of the interaction between the snail *Biomphalaria glabrata* and its parasites: a review of research progress over the last decade. *Fish Shellfish Immunol.* 46, 5–16.

Crellen, T., Iantorno, S., 2015. A switch in time. *Nat. Rev. Microbiol.* 13, 190.

Davies, C.M., Webster, J.P., Woolhous, M.E., 2001. Trade-offs in the evolution of virulence in an indirectly transmitted macroparasite. *Proceedings* 268, 251–257.

Dheilly, N.M., Adema, C., Raftos, D.A., Gourbal, B., Grunau, C., Du Pasquier, L., 2014. No more non-model species: the promise of next generation sequencing for comparative immunology. *Dev. Comp. Immunol.* 45, 56–66.

Dheilly, N.M., Duval, D., Mouahid, G., Emans, R., Allienne, J.F., Galinier, R., Genthon, C., Dubois, E., Du Pasquier, L., Adema, C.M., Grunau, C., Mitta, G., Gourbal, B., 2015. A family of variable immunoglobulin and lectin domain containing molecules in the snail *Biomphalaria glabrata*. *Dev. Comp. Immunol.* 48, 234–243.

Dong, Y., Taylor, H.E., Dimopoulos, G., 2006. AgDscam, a hypervariable immunoglobulin domain-containing receptor of the Anopheles gambiae innate immune system. *PLoS Biol.* 4, e229.

Fneich, S., Theron, A., Cosseau, C., Rognon, A., Aliaga, B., Buard, J., Duval, D., Arancibia, N., Boissier, J., Roquis, D., Mitta, G., Grunau, C., 2016. Epigenetic origin of adaptive phenotypic variants in the human blood fluke *Schistosoma mansoni*. *Epigenetics Chromatin* 9, 27.

Galiniere, R., Roger, E., Mone, Y., Duval, D., Portet, A., Pinaud, S., Chaparro, C., Grunau, C., Genthon, C., Dubois, E., Rognon, A., Arancibia, N., Dejean, B., Theron, A., Gourbal, B., Mitta, G., 2017. A multistrain approach to studying the mechanisms underlying compatibility in the interaction between *Biomphalaria glabrata* and *Schistosoma mansoni*. *PLoS Negl. Trop. Dis.* <http://dx.doi.org/10.1371/journal.pntd.0005398>.

Gavery, M.R., Roberts, S.B., 2014. A context dependent role for DNA methylation in bivalves. *Brief. Funct. Genom.* 13, 217–222.

Geoghegan, J.L., Spencer, H.G., 2012. Population-epigenetic models of selection. *Theor. Popul. Biol.* 81, 232–242.

Geyer, K.K., Rodriguez Lopez, C.M., Chalmers, I.W., Munshi, S.E., Truscott, M., Heald, J., Wilkinson, M.J., Hoffmann, K.F., 2011. Cytosine methylation regulates oviposition in the pathogenic blood fluke *Schistosoma mansoni*. *Nat. Commun.* 2, 424.

Ghosh, J., Buckley, K.M., Nair, S.V., Raftos, D.A., Miller, C., Majeske, A.J., Hibino, T., Rast, J.P., Roth, M., Smith, L.C., 2010. Sp185/333: a novel family of genes and proteins involved in the purple sea urchin immune response. *Dev. Comp. Immunol.* 34, 235–245.

Gomez-Diaz, E., Jorda, M., Peinado, M.A., Rivero, A., 2012. Epigenetics of host-pathogen interactions: the road ahead and the road behind. *PLoS Pathog.* 8, e1003007.

Goodall, C.P., Bender, R.C., Brooks, J.K., Bayne, C.J., 2006. *Biomphalaria glabrata* cytosolic copper/zinc superoxide dismutase (SOD1) gene: association of SOD1 alleles with resistance/susceptibility to *Schistosoma mansoni*. *Mol. Biochem. Parasitol.* 147, 207–210.

Gordy, M.A., Pila, E.A., Hanington, P.C., 2015. The role of fibrinogen-related proteins in the gastropod immune response. *Fish Shellfish Immunol.* 46, 39–49.

Gourbal, B., Theron, A., Grunau, C., Duval, D., Mitta, G., 2015. Polymorphic mucin-like proteins in *Schistosoma mansoni*, a variable antigen and a key component of the compatibility between the schistosome and its snail host. *Results Probl. Cell Differ.* 57, 91–108.

Grunau, C., Clark, S.J., Rosenthal, A., 2001. Bisulfite genomic sequencing: systematic investigation of critical experimental parameters. *Nucl. Acids Res.* 29, E65.

Hanington, P.C., Forsy, M.A., Loker, E.S., 2012. A somatically diversified defense factor, FREP3, is a determinant of snail resistance to schistosome infection. *PLoS Negl. Trop. Dis.* 6, e1591.

Hanington, P.C., Lun, C.M., Adema, C.M., Loker, E.S., 2010. Time series analysis of the transcriptional responses of *Biomphalaria glabrata* throughout the course of

- intramolluscan development of *Schistosoma mansoni* and *Echinostoma paraensei*. *Int. J. Parasitol.* 40, 819–831.
- Hanington, P.C., Zhang, S.M., 2011. The primary role of fibrinogen-related proteins in invertebrates is defense, not coagulation. *J. Innate Immun.* 3, 17–27.
- Hertel, L.A., Adema, C.M., Loker, E.S., 2005. Differential expression of FREP genes in two strains of *Biomphalaria glabrata* following exposure to the digenetic trematodes *Schistosoma mansoni* and *Echinostoma paraensei*. *Dev. Comp. Immunol.* 29, 295–303.
- Hicks, S.J., Theodoropoulos, G., Carrington, S.D., Corfield, A.P., 2000. The role of mucins in host–parasite interactions. Part I—protozoan parasites. *Parasitol. Today* 16, 476–481.
- Howard, J.C., Jack, R.S., 2007. Evolution of immunity and pathogens. *Eur. J. Immunol.* 37, 1721–1723.
- Hung, H.Y., Ng, T.H., Lin, J.H., Chiang, Y.A., Chuang, Y.C., Wang, H.C., 2013. Properties of *Litopenaeus vannamei* Dscam (LvDscam) isoforms related to specific pathogen recognition. *Fish Shellfish Immunol.* 35, 1272–1281.
- Ittiprasert, W., Miller, A., Myers, J., Nene, V., El-Sayed, N.M., Knight, M., 2010. Identification of immediate response genes dominantly expressed in juvenile resistant and susceptible *Biomphalaria glabrata* snails upon exposure to *Schistosoma mansoni*. *Mol. Biochem. Parasitol.* 169, 27–39.
- Jiang, Y., Loker, E.S., Zhang, S.M., 2006. In vivo and in vitro knockdown of FREP2 gene expression in the snail *Biomphalaria glabrata* using RNA interference. *Dev. Comp. Immunol.* 30, 855–866.
- Keung, A.J., Joung, J.K., Khalil, A.S., Collins, J.J., 2015. Chromatin regulation at the frontier of synthetic biology. *Nat. Rev. Genet.* 16, 159–171.
- Kines, K.J., Morales, M.E., Mann, V.H., Gobert, G.N., Brindley, P.J., 2008. Integration of reporter transgenes into *Schistosoma mansoni* chromosomes mediated by pseudotyped murine leukemia virus. *FASEB J. Official Publ. Fed. Am. Soc. Exp. Biol.* 22, 2936–2948.
- Kines, K.J., Rinaldi, G., Okatcha, T.I., Morales, M.E., Mann, V.H., Tort, J.F., Brindley, P.J., 2010. Electroporation facilitates introduction of reporter transgenes and virions into schistosome eggs. *PLoS Negl. Trop. Dis.* 4, e593.
- Knight, M., Ittiprasert, W., Arican-Goktas, H.D., Bridger, J.M., 2016. Epigenetic modulation, stress and plasticity in susceptibility of the snail host, *Biomphalaria glabrata*, to *Schistosoma mansoni* infection. *Int. J. Parasitol.* 46, 389–394.
- Knight, M., Miller, A.N., Patterson, C.N., Rowe, C.G., Michaels, G., Carr, D., Richards, C.S., Lewis, F.A., 1999. The identification of markers segregating with resistance to *Schistosoma mansoni* infection in the snail *Biomphalaria glabrata*. *Proc. Natl. Acad. Sci. U. S. A.* 96, 1510–1515.
- Komor, A.C., Badran, A.H., Liu, D.R., 2017. CRISPR-based technologies for the manipulation of eukaryotic genomes. *Cell* 168 (1–2), 20–36.
- Léonard, P.M., Adema, C.M., Zhang, S.-M., Loker, E.S., 2001. Structure of two FREP genes that combine IgSF and fibrinogen domains, with comments on diversity of the FREP gene family in the snail *Biomphalaria glabrata*. *Gen* 269, 155–165.
- Lockyer, A.E., Spinks, J., Kane, R.A., Hoffmann, K.F., Fitzpatrick, J.M., Rollinson, D., Noble, L.R., Jones, C.S., 2008. *Biomphalaria glabrata* transcriptome: cDNA microarray profiling identifies resistant- and susceptible-specific gene expression in haemocytes from snail strains exposed to *Schistosoma mansoni*. *BMC Genom.* 9, 634.
- Loker, E.S., Couch, L., Hertel, L.A., 1994. Elevated agglutination titres in plasma of *Biomphalaria glabrata* exposed to *Echinostoma paraensei*: characterization and functional relevance of a trematode-induced response. *Parasitology* 108 (Pt 1), 17–26.
- Loker, E.S., Hertel, L.A., 1987. Alterations in *Biomphalaria glabrata* plasma induced by infection with the digenetic trematode *Echinostoma paraensei*. *J. Parasitol.* 73, 503–513.
- Loker, E.S., Yui, M.A., Bayne, C.J., 1984. *Schistosoma mansoni*: agglutination of sporocysts, and formation of gels on miracidia transforming in plasma of *Biomphalaria glabrata*. *Exp. Parasitol.* 58, 56–62.
- Mann, V.H., Morales, M.E., Kines, K.J., Brindley, P.J., 2008. Transgenesis of schistosomes: approaches employing mobile genetic elements. *Parasitology* 135, 141–153.
- Medzhitov, R., Janeway Jr., C.A., 1997. Innate immunity: the virtues of a nonclonal system of recognition. *Cell* 91, 295–298.
- Mitta, G., Adema, C.M., Gourbal, B., Loker, E.S., Theron, A., 2012. Compatibility polymorphism in snail/schistosome interactions: from field to theory to molecular mechanisms. *Dev. Comp. Immunol.* 37, 1–8.
- Mone, Y., Gourbal, B., Duval, D., Du Pasquier, L., Kieffer-Jaquinod, S., Mitta, G., 2010. A large repertoire of parasite epitopes matched by a large repertoire of host immune receptors in an invertebrate host/parasite model. *PLoS Negl. Trop. Dis.* 4, e813.
- Mone, Y., Gourbal, B., Duval, D., Du Pasquier, L., Kieffer-Jaquinod, S., Mitta, G., 2010. A large repertoire of parasite epitopes matched by a large repertoire of host immune receptors in an invertebrate host/parasite model. *PLoS Negl. Trop. Dis.* 4, e813.
- Mone, Y., Ribou, A.C., Cosseu, C., Duval, D., Theron, A., Mitta, G., Gourbal, B., 2011. An example of molecular co-evolution: reactive oxygen species (ROS) and ROS scavenger levels in *Schistosoma mansoni*/*Biomphalaria glabrata* interactions. *Int. J. Parasitol.* 41, 721–730.
- Morand, S., Manning, S.D., Woolhouse, M.E., 1996. Parasite–host coevolution and geographic patterns of parasite infectivity and host susceptibility. *Proceedings* 263, 119–128.
- Pancer, Z., 2000. Dynamic expression of multiple scavenger receptor cysteine-rich genes in coelomocytes of the purple sea urchin. *Proc. Natl. Acad. Sci. U. S. A.* 97, 13156–13161.
- Pancer, Z., Rast, J.P., Davidson, E.H., 1999. Origins of immunity: transcription factors and homologues of effector genes of the vertebrate immune system expressed in sea urchin coelomocytes. *Immunogenetics* 49, 773–786.
- Perrin, C., Lepesant, J.M., Roger, E., Duval, D., Fneich, S., Thuillier, V., Allienne, J.F., Mitta, G., Grunau, C., Cosseu, C., 2013. *Schistosoma mansoni* mucin gene (SmPoMuc) expression: epigenetic control to shape adaptation to a new host. *PLoS Pathog.* 9, e1003571.
- Peterson, N.A., Hokke, C.H., Deelder, A.M., Yoshino, T.P., 2009. Glycotope analysis in miracidia and primary sporocysts of *Schistosoma mansoni*: differential expression during the miracidium-to-sporocyst transformation. *Int. J. Parasitol.* 39, 1331–1344.
- Picard, M.A., Boissier, J., Roquis, D., Grunau, C., Allienne, J.F., Duval, D., Toulza, E., Arancibia, N., Caffrey, C.R., Long, T., Nidelet, S., Rohmer, M., Cosseu, C., 2016. Sex-biased transcriptome of *Schistosoma mansoni*: host–parasite interaction, genetic determinants and epigenetic regulators are associated with sexual differentiation. *PLoS Negl. Trop. Dis.* 10, e0004930.
- Pinaud, S., Portela, J., Duval, D., Nowacki, F.C., Olive, M.A., Allienne, J.F., Galinier, R., Dheilly, N.M., Kieffer-Jaquinod, S., Mitta, G., Theron, A., Gourbal, B., 2016. A shift from cellular to humoral responses contributes to innate immune memory in the vector snail *Biomphalaria glabrata*. *PLoS Pathog.* 12, e1005361.
- Protasio, A.V., Dunne, D.W., Berriman, M., 2013. Comparative study of transcriptome profiles of mechanical- and skin-transformed *Schistosoma mansoni* schistosomes. *PLoS Negl. Trop. Dis.* 7, e2091.
- Raddatz, G., Guzzardo, P.M., Olova, N., Fantappie, M.R., Rampp, M., Schaefer, M., Reik, W., Hannon, G.J., Lyko, F., 2013. Dnmt2-dependent methylomes lack defined DNA methylation patterns. *Proc. Natl. Acad. Sci. U. S. A.* 110, 8627–8631.
- Rathore, D., Nagarkatti, R., Jani, D., Chattopadhyay, R., de la Vega, P., Kumar, S., McCutchan, T.F., 2005. An immunologically cryptic epitope of Plasmodium falciparum circumsporozoite protein facilitates liver cell recognition and induces protective antibodies that block liver cell invasion. *J. Biol. Chem.* 280, 20524–20529.
- Reyna-Lopez, G.E., Simpson, J., Ruiz-Herrera, J., 1997. Differences in DNA methylation patterns are detectable during the dimorphic transition of fungi by amplification of restriction polymorphisms. *Mol. General Genet.* MGG 253, 703–710.
- Rhoads, A., Au, K.F., 2015. PacBio sequencing and its applications. *Genom. Proteom. Bioinforma.* 13, 278–289.
- Richards, C.S., 1975. Genetic factors in susceptibility of *Biomphalaria glabrata* for different strains of *Schistosoma mansoni*. *Parasitology* 70, 231–241.
- Richards, C.S., Knight, M., Lewis, F.A., 1992. Genetics of *Biomphalaria glabrata* and its effect on the outcome of *Schistosoma mansoni* infection. *Parasitol. Today* 8, 171–174.
- Richards, C.S., Shade, P.C., 1987. The genetic variation of compatibility in *Biomphalaria glabrata* and *Schistosoma mansoni*. *J. Parasitol.* 73, 1146–1151.
- Roger, E., Gourbal, B., Grunau, C., Pierce, R.J., Galinier, R., Mitta, G., 2008a. Expression analysis of highly polymorphic mucin proteins (Sm PoMuc) from the parasite *Schistosoma mansoni*. *Mol. Biochem. Parasitol.* 157, 217–227.
- Roger, E., Grunau, C., Pierce, R.J., Hirai, H., Gourbal, B., Galinier, R., Emans, R., Cesari, I.M., Cosseu, C., Mitta, G., 2008b. Controlled chaos of polymorphic mucins in a metazoan parasite (*Schistosoma mansoni*) interacting with its invertebrate host (*Biomphalaria glabrata*). *PLoS Negl. Trop. Dis.* 2, e330.
- Roger, E., Mitta, G., Moné, Y., Bouchut, A., Rognon, A., Grunau, C., Boissier, J., Theron, A., Gourbal, B.E., 2008c. Molecular determinants of compatibility polymorphism in the *Biomphalaria glabrata*/*Schistosoma mansoni* model: new candidates identified by a global comparative proteomics approach. *Mol. Biochem. Parasitol.* 157, 205–216.
- Roquis, D., Lepesant, J.M., Picard, M.A., Freitag, M., Parrinello, H., Groth, M., Emans, R., Cosseu, C., Grunau, C., 2015. The epigenome of *Schistosoma mansoni* provides insight about how cercariae voice transcription until infection. *PLoS Negl. Trop. Dis.* 9, e0003853.
- Roquis, D., Rognon, A., Chaparro, C., Boissier, J., Arancibia, N., Cosseu, C., Parrinello, H., Grunau, C., 2016. Frequency and mitotic heritability of epimutations in *Schistosoma mansoni*. *Mol. Ecol.* 25, 1741–1758.
- Sapp, K.K., Loker, E.S., 2000. A comparative study of mechanisms underlying digenetic–snail specificity: in vitro interactions between hemocytes and digenetic larvae. *J. Parasitol.* 86, 1020–1029.
- Schulenburg, H., Boehnisch, C., Michiels, N.K., 2007. How do invertebrates generate a highly specific innate immune response? *Mol. Immunol.* 44, 3338–3344.
- Tennessen, J.A., Bonner, K.M., Bollmann, S.R., Johnstun, J.A., Yeh, J.Y., Marine, M., Tavalire, H.F., Bayne, C.J., Blouin, M.S., 2015a. Genome-wide scan and test of candidate genes in the snail *Biomphalaria glabrata* reveal new locus influencing resistance to *Schistosoma mansoni*. *PLoS Negl. Trop. Dis.* 9, e0004077.
- Tennessen, J.A., Theron, A., Marine, M., Yeh, J.Y., Rognon, A., Blouin, M.S., 2015b. Hyperdiverse gene cluster in snail host conveys resistance to human schistosome parasites. *PLoS Genet.* 11, e1005067.
- Theodoropoulos, G., Hicks, S.J., Corfield, A.P., Miller, B.G., Carrington, S.D., 2001. The role of mucins in host–parasite interactions: Part II - helminth parasites. *Trends Parasitol.* 17, 130–135.
- Théron, A., Coustau, C., 2005. Are *Biomphalaria* snails resistant to *Schistosoma mansoni*? *J. Helminthol.* 79, 187–191.
- Theron, A., Pages, J.R., Rognon, A., 1997. *Schistosoma mansoni*: distribution patterns of miracidia among *Biomphalaria glabrata* snail as related to host susceptibility and sporocyst regulatory processes. *Exp. Parasitol.* 85, 1–9.
- Theron, A., Rognon, A., Gourbal, B., Mitta, G., 2014. Multi-parasite host susceptibility and multi-host parasite infectivity: a new approach of the *Biomphalaria*

- glabrata*/*Schistosoma mansoni* compatibility polymorphism. *Infect. Genet. Evol.* 26, 80–88.
- Valentim, C.L., Cioli, D., Chevalier, F.D., Cao, X., Taylor, A.B., Holloway, S.P., Pica-Mattocchia, L., Guidi, A., Basso, A., Tsai, I.J., Berriman, M., Carvalho-Queiroz, C., Almeida, M., Aguilar, H., Frantz, D.E., Hart, P.J., LoVerde, P.T., Anderson, T.J., 2013. Genetic and molecular basis of drug resistance and species-specific drug action in schistosome parasites. *Science* 342, 1385–1389.
- Van Der Knaap, W.P.W., Loker, E.S., 1990. Immune mechanisms in trematod-snail interactions. *Parasitol. Today* 6, 175–182.
- Watson, F.L., Puttmann-Holgado, R., Thomas, F., Lamar, D.L., Hughes, M., Kondo, M., Rebel, V.I., Schmucker, D., 2005. Extensive diversity of Ig-superfamily proteins in the immune system of insects. *Science* 309, 1874–1878.
- Webster, J.P., Davies, C.M., 2001. Coevolution and compatibility in the snail-schistosome system. *Parasitology* 123 (Suppl. 1), S41–S56.
- Yoshino, T.P., Gourbal, B., Theron, A., 2016. Chapter 7: schistosoma sporocysts. In: Jamieson, Barrie G.M. (Ed.), *Schistosoma: Biology, Pathology and Control*. CRC Press, Taylor & Francis Group, p. 523.
- Zhang, S.M., Adema, C.M., Kepler, T.B., Loker, E.S., 2004. Diversification of Ig superfamily genes in an invertebrate. *Sci. New York N. Y.* 305, 251–254.
- Zhang, S.M., Leonard, P.M., Adema, C.M., Loker, E.S., 2001. Parasite-responsive IgSF members in the snail *Biomphalaria glabrata*: characterization of novel genes with tandemly arranged IgSF domains and a fibrinogen domain. *Immunogenetics* 53, 684–694.
- Zhang, S.M., Loker, E.S., 2003. The FREP gene family in the snail *Biomphalaria glabrata*: additional members, and evidence consistent with alternative splicing and FREP retrosequences. *Fibrinogen-related proteins. Dev. Comp. Immunol.* 27, 175–187.
- Zhang, S.M., Loker, E.S., 2004. Representation of an immune responsive gene family encoding fibrinogen-related proteins in the freshwater mollusc *Biomphalaria glabrata*, an intermediate host for *Schistosoma mansoni*. *Gene* 341, 255–266.
- Zhang, S.M., Nian, H., Zeng, Y., DeJong, R.J., 2008a. Fibrinogen-bearing protein genes in the snail *Biomphalaria glabrata*: characterization of two novel genes and expression studies during ontogenesis and trematode infection. *Dev. Comp. Immunol.* 32, 1119–1130.
- Zhang, S.M., Zeng, Y., Loker, E.S., 2008b. Expression profiling and binding properties of fibrinogen-related proteins (FREPs), plasma proteins from the schistosome snail host *Biomphalaria glabrata*. *Innate Immun.* 14, 175–189.

2. L'étude du complexe immunitaire FREP/SmPoMuc

Publication : A multistrain approach to studying the mechanisms underlying compatibility in the interaction between *Biomphalaria glabrata* and *Schistosoma mansoni*.

GALINIER Richard, ROGER Emmanuel, MONÈ Yves, DUVAL David, PORTET Anaïs, PINAUD Silvain, CHAPARRO Cristian, GRUNAU Christoph, GENTHON Clémence, DUBOIS Emeric, ROGNON Anne, ARANCIBIA Nathalie, DEJEAN Bernard, THÈRON André, GOURBAL Benjamin and MITTA Guillaume

*Article publié en 2017 dans PLOS Neglected Tropical Diseases

RESEARCH ARTICLE

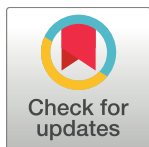
A multistrain approach to studying the mechanisms underlying compatibility in the interaction between *Biomphalaria glabrata* and *Schistosoma mansoni*

Richard Galinier¹✉, Emmanuel Roger¹✉, Yves Moné¹, David Duval¹, Anaïs Portet¹, Silvain Pinaud¹, Cristian Chaparro¹, Christoph Grunau¹, Clémence Genthon², Emeric Dubois², Anne Rognon¹, Nathalie Arancibia¹, Bernard Dejean¹, André Théron¹, Benjamin Gourbal^{1*}, Guillaume Mitta^{1*}

1 Univ. Perpignan Via Domitia, IHPE UMR 5244, CNRS, IFREMER, Univ. Montpellier, Perpignan, France, **2** MGX-Montpellier GenomiX, Montpellier Genomics and Bioinformatics Facility, Montpellier, France

✉ These authors contributed equally to this work.

* benjamin.gourbal@univ-perp.fr (BG); mitta@univ-perp.fr (GM)



OPEN ACCESS

Citation: Galinier R, Roger E, Moné Y, Duval D, Portet A, Pinaud S, et al. (2017) A multistrain approach to studying the mechanisms underlying compatibility in the interaction between *Biomphalaria glabrata* and *Schistosoma mansoni*. PLoS Negl Trop Dis 11(3): e0005398. doi:10.1371/journal.pntd.0005398

Editor: Cinzia Cantacessi, University of Cambridge, UNITED KINGDOM

Received: October 21, 2016

Accepted: February 7, 2017

Published: March 2, 2017

Copyright: © 2017 Galinier et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: The obtained *SmPoMuc* sequences were deposited in GenBank under the following accession numbers: KX645102-KX645377 for *SmLE*, KX645378-KX645624 for *SmVEN*, EU676447-EU676459/EU676503-EU676530/EU676572-EU676583/EU676595-EU676625 for *SmBRE*, and EU676460-EU676502/EU676531-EU676571/EU676584-EU676594/EU676556-EU676626 for *SmGH2*. The obtained FREP sequences were deposited in

Abstract

In recent decades, numerous studies have sought to better understand the mechanisms underlying the compatibility between *Biomphalaria glabrata* and *Schistosoma mansoni*. The developments of comparative transcriptomics, comparative genomics, interactomics and more targeted approaches have enabled researchers to identify a series of candidate genes. However, no molecular comparative work has yet been performed on multiple populations displaying different levels of compatibility. Here, we seek to fill this gap in the literature. We focused on *B. glabrata* FREPs and *S. mansoni* *SmPoMucs*, which were previously demonstrated to be involved in snail/schistosome compatibility. We studied the expression and polymorphisms of these factors in combinations of snail and schistosome isolates that display different levels of compatibility. We found that the polymorphism and expression levels of FREPs and *SmPoMucs* could be linked to the compatibility level of *S. mansoni*. These data and our complementary results obtained by RNA-seq of samples from various snail strains indicate that the mechanism of compatibility is much more complex than previously thought, and that it is likely to be highly variable within and between populations. This complexity must be taken into account if we hope to identify the molecular pathways that are most likely to be good targets for strategies aimed at blocking transmission of the parasite through the snail intermediate host.

Author summary

Schistosomiasis is the second most widespread human tropical parasitic disease after malaria. It is caused by flatworms of the genus *Schistosoma*, and poses a considerable threat for human health in numerous Asian, African and South American countries. The World Health Organization has set the goal of eradicating schistosomiasis by 2025.

GenBank under the following accession numbers: KY024239 to KY024307.

Funding: BG acknowledges support from ANR JCJC INVIMORY (ANR-13-JSV7-0009). GM acknowledges support from ANR Schistophepigen (ANR-07-BLAN-0119). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

However, no vaccine is available, and we currently have only one drug (praziquantel) that can effectively and efficiently treat the disease. As treatment by mass drug administration would enhance the risk of drug resistance in schistosome parasites, complementary strategies to fight this parasitic disease are urgently needed. Freshwater snails of the *Biomphalaria* genus act as intermediate hosts in the transmission of the schistosome species. Thus, learning more about the mechanisms of the interaction between these snails and the schistosomes could critically facilitate the identification of potential new candidate molecules that may be targeted to prevent schistosome transmission in the field.

Introduction

Schistosomes are the causative agents of schistosomiasis, which is one of the most important neglected human tropical diseases in the world. Schistosomes infect over 200 million people worldwide, causing both acute and chronic debilitating diseases [1,2]. There is no effective vaccine against schistosomes, and the treatment of schistosomiasis still relies on a single drug: praziquantel [3]. Praziquantel resistance can be easily selected experimentally [4], and some human populations subjected to mass treatment now show evidence of reduced drug susceptibility [5]. Thus, we need alternate control strategies. Toward this end, researchers have sought to block disease transmission at the level of the snail that acts as the intermediate host. However, if we hope to identify target genes that may be used to develop new strategies aimed at disrupting the transmission of schistosomiasis, we must decipher the mechanisms through which snails and schistosomes interact. Over the past four decades, numerous investigators have sought to understand these mechanisms by focusing on the interaction between *Biomphalaria glabrata* and *Schistosoma mansoni*, which was chosen as a model system.

The genetic determinism of the compatibility between *B. glabrata* and *S. mansoni* was clearly demonstrated by the C.S. Richards group in the 1970s [6,7]. Since then, several research groups have investigated the underlying molecular determinants using different laboratory strains of snails and schistosomes. Genetic studies of crosses between snail lines displaying compatible and incompatible phenotypes have revealed some candidate loci, including a gene cluster containing a super oxide dismutase (SOD)-encoding gene [8–10] and a genomic region containing genes putatively involved in parasite recognition [11]. Various transcriptomic comparisons have also been performed on other compatible and incompatible strains of snails and schistosomes [12–16]. These studies uncovered a series of candidate genes involved in recognition, effector, and signaling pathways that could contribute to the compatibility process (see [17] for a recent review). Taken together, the previous reports clearly show that the success or failure of *S. mansoni* in infecting *B. glabrata* reflects a complex interplay between the host's defense mechanisms and the parasite's infective strategies. Little is known about the molecular variability playing of these molecular determinants underlying the compatibility; only one work has studied and shown the differential allelic expression of a SOD gene in different individuals of the predominantly resistant 13-16-R1 strain of *B. glabrata* [10]. The objective of the present work is to fill this gap by studying the molecular determinants of compatibility in different populations with varied compatibility phenotypes, in order to evaluate potential between-population differences in the compatibility mechanisms. To achieve this aim, we focused on molecular determinants known to be involved in snail/schistosome compatibility, and studied their expressions and polymorphisms in host and parasite isolates that differ in their compatibilities. We first studied the *SmPoMucs* (polymorphic mucins from *S. mansoni*), which were initially identified by a comparative proteomic analysis of two strains of *S. mansoni*

that differed in their compatibility towards the same mollusk strain [18]. *SmPoMucs* share the general features of mucins, including a N-terminal domain containing a variable number of tandem repeats and a conserved C-terminal domain [19]. These proteins are expressed only by larval-stage parasites during interactions with the snail intermediate host; they are produced and located in the apical gland of miracidia and sporocysts, and are characterized by high levels of glycosylation and polymorphism [19,20]. A detailed analysis of intra- and inter-strain *SmPoMuc* polymorphisms revealed that the diversification of these proteins has been driven by a complex cascade of mechanisms involving recombination between genes of the multigene *SmPoMuc* family (10 genes), epigenetic control of transcription, post-transcriptional regulation events, and post-translational modifications [20–22]. This yields a remarkably high degree of diversification from a limited set of genes, enabling each individual parasite to express a specific and unique pattern of *SmPoMucs* [20]. Functionally, these proteins are thought to play roles in the very early steps of infection [23]. Based on the above findings, it has been proposed that *SmPoMucs* could be crucial antigens in the snail-schistosome compatibility process. Then, we developed co-immunoprecipitation (CoIP) experiments that enabled us to identify putative *SmPoMuc*-interacting immune receptors of the snail [24]. We found that *SmPoMucs* form molecular complexes with the fibrinogen-related proteins (FREPs) of *B. glabrata* [25]. FREPs are highly polymorphic, with somatic diversification generating unique repertoires in individual *B. glabrata* [26]. Thus, we considered these proteins to be good candidates as molecular determinants on the snail side of the compatibility between *B. glabrata* and *S. mansoni*. This importance of FREPs in the compatibility process was confirmed by specific knockdown of FREP 3 in *B. glabrata* BS-90 snails, which are totally resistant to a specific laboratory strain of *S. mansoni* [27]. The knockdown snails lost 21.4% of their resistance to *S. mansoni* infection, suggesting that FREP 3 participates in recognition but is not the sole determinant.

As FREP immune receptors and their *SmPoMuc* antigens are clearly involved in the compatibility process, we herein focused on these molecular determinants of the recognition process between the host and the parasite.

We first characterized the compatibilities among all sympatric and allopatric combinations of four strains of *S. mansoni* (two from Brazil, one from Venezuela, and one from Guadeloupe Island) and four strains of *B. glabrata* (from the same locations) from South America and the Caribbean area. We then used targeted approach to analyze the expressions of *SmPoMucs* in Schistosomes and global transcriptomic approach on FREPs between the four strains of *B. glabrata*. The global transcriptomic analysis of snail strains also revealed large transcriptional differences, especially for the *B. glabrata* strain that showed the least compatibility when confronted with the studied schistosome strains. Global transcriptomic differences were observed among numerous genes involved in the different phases of the immune response. Based on our findings, we propose that the compatibility between *B. glabrata* and *S. mansoni* depends on a multistep process that involves both recognition and effector/anti-effectors systems.

Results

A multistrain approach for assessing compatibility phenotypes

As the objective of the present work was to evaluate the putative link between the expression patterns of *SmPoMucs* and FREPs and the compatibility between snails and schistosomes, we first established the compatibility levels of four strains of *B. glabrata* (*BgBAR*, *BgVEN*, *BgBRE*, and *BgGUA*) when confronted with four strains of *S. mansoni* (*SmLE*, *SmVEN*, *SmbRE*, and *SmGH2*). Compatibility was tested for all sympatric and allopatric combinations.

All strain combinations displayed different compatibility levels (Fig 1). *SmLE* displayed the highest compatibility, showing a 96–100% prevalence of infection when confronted with the

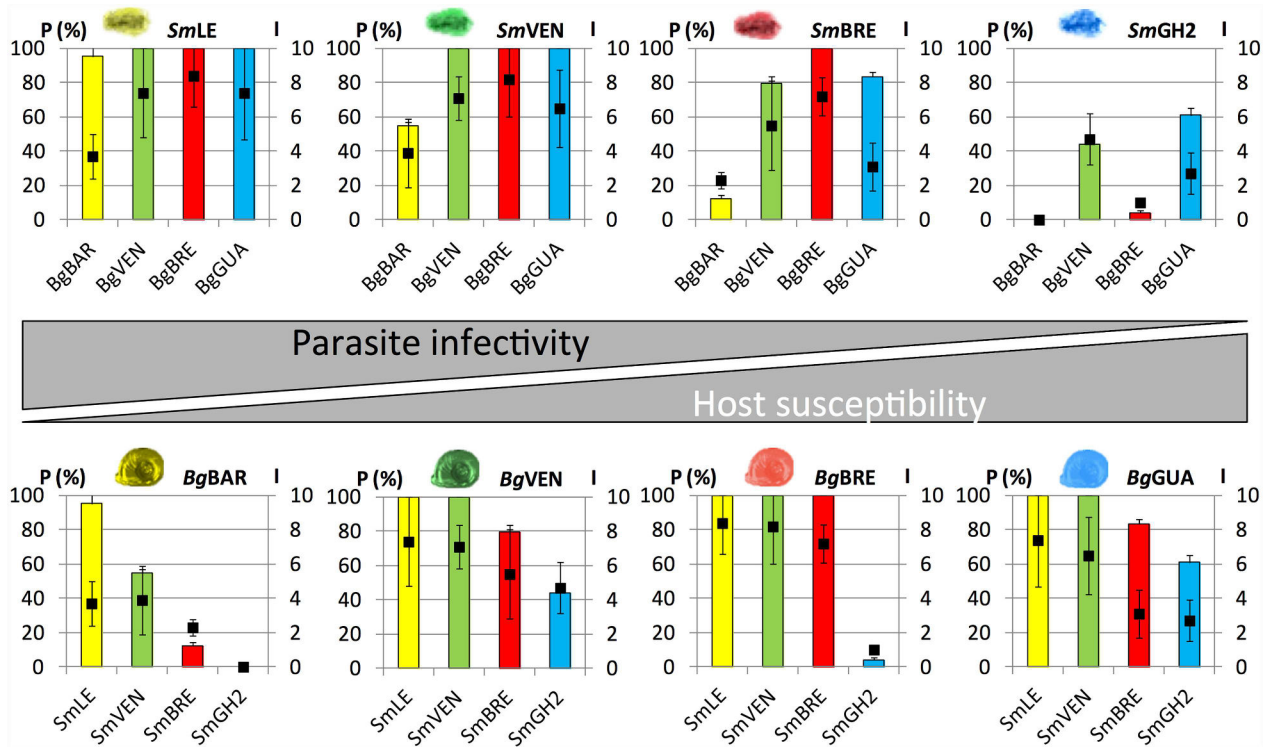


Fig 1. Compatibility trials between different strains of parasites and snails. Each pairwise combination of the studied strains of schistosomes (*SmLE*, *SmVEN*, *SmBRE*, and *SmGH2*) and snails (*BgBAR*, *BgVEN*, *BgBRE*, and *BgGUA*) were tested for compatibility. The upper graphs present the prevalence (P in %) and intensity (I) of infection for each *S. mansoni* in the different *B. glabrata* host strains. The lower graphs present the prevalence and intensity of infection for each *B. glabrata* strain when confronted by the four *S. mansoni* strains. Prevalence values are represented by colored histograms, while intensity values are indicated by black squares. The sympatric snails and schistosomes bear the same color. The presented data represent the mean values obtained for three independent experiments, the data obtained by (Theron et al, 2014) and two other experiments performed in 2010 and 2012. Error bars represent the average absolute deviation of the mean.

doi:10.1371/journal.pntd.0005398.g001

four *B. glabrata* strains. *SmGH2* showed the least compatibility, exhibiting prevalences of 0–44% for allopatric combinations and only 60% when confronted with its sympatric mollusk. *SmVEN* was highly compatible (100%) with its sympatric mollusk strain and with *BgBRE* and *BgGUA*, but it was less efficient when infecting the *BgBAR* snail strain (55%). *SmBRE* displayed an intermediate compatibility phenotype; it showed 100% prevalence for its sympatric mollusk *BgBRE*, and its compatibility ranged from 12% to 83% when confronted by the other mollusk strains. Our results for the intensity of infection followed similar trends, ranging from 1 to 8.4 parasites per infected snails (Fig 1). The infective capacities of the four parasite strains exhibited a gradient of compatibility in the following descending order: *SmLE*, *SmVEN*, *SmBRE*, and *SmGH2*.

Considering the mollusk strains, *BgBAR* was the least compatible; it exhibited prevalences of 0–55% when confronted with allopatric parasite strains and reached 96% when infected with its sympatric *SmLE*, but the intensity of infection never exceeded 3.9 (even for *SmLE*). *BgVEN*, *BgBRE*, and *BgGUA* showed very similar compatibility patterns, exhibiting complete compatibility (prevalence, 100%) when exposed to *SmLE* and *SmVEN*, but less compatibility with *SmGH2*. *BgGUA* and *BgVEN* displayed very similar compatibility patterns, showing slightly more infectivity when confronted with *SmBRE* than *SmGH2*. *BgBRE* was totally compatible with its sympatric schistosome, but displayed a very low compatibility with *SmGH2* (4%). The intensities were generally high, largely between 4.7 and 8.4. Our results indicate that

BgBAR is the least compatible host strain, whereas *BgVEN*, *BgBRE*, and *BgGUA* are much more compatible and display quite similar compatibility phenotypes.

SmPoMuc polymorphism analysis

We previously showed that the *SmPoMucs* are encoded by a multi-gene family of 10 members that can be divided into four paralogous sequence groups (groups 1–4) [20]. Fig 2A shows the *SmPoMuc* cDNA structure shared by the different groups. Their 5' regions comprise a variable number of tandem repeats corresponding to repetitions of exon 2, whereas the 3' regions (exons 3 to 15) differ in their sequences, enabling the *SmPoMucs* to be divided into groups 1 through 4. At the transcript level, only groups 1, 2 and 3.1 have been detected to date [20]. *SmPoMucs* of groups 1 and 2 contain the same type of exon 2 (r2, 27 nucleotides) that is repeated in the transcript structure, while those of group 3 contain a different type of repeated exon 2. Most group 3 *SmPoMucs* have r1 as their repeated sequence, except for the subgroup denoted group 3.1 (r1-r2), which displays both types of exon 2 (r1 and r2) in the gene structure. These recombined genes are present in the genomes of different schistosome strains, but appear to be expressed only in *SmGH2* (see also [19,20]).

To analyze *SmPoMuc* transcript polymorphism, RNA was extracted from 11 individual sporocysts of each strain (*SmBRE*, *SmVEN*, *SmLE*, and *SmGH2*) and subjected to nested RT-PCR. Consensus primers (see Fig 2A for the positions of the utilized primers) were used to amplify the complete coding sequence of all *SmPoMucs* in each individual. Fig 2B shows the *SmPoMuc* banding patterns obtained on agarose gels.

We first examined inter-strain variability. Consistent with the results of previous studies performed using the same method [20], the *SmPoMuc* banding patterns were highly different across the analyzed strains. To analyze this polymorphism at the protein level, we analyzed *SmPoMucs* by Western blotting. Proteins from 5000 individuals of each *S. mansoni* strain were resolved and detected with an anti-*SmPoMuc* antibody directed against a conserved region of the *SmPoMucs* [24]. As shown in Fig 2C, the *SmPoMuc* protein patterns differed across *SmLE*, *SmVEN*, *SmBRE*, and *SmGH2*, each isolate expressed a specific *SmPoMuc* profile.

With respect to intra-strain comparisons, the polymorphism was found to be very high at the transcript level. The *SmBRE* and *SmGH2* banding patterns were obtained from a previous work [21] and the banding patterns of *SmVEN* and *SmLE* was obtained in the present work. For the 4 *S. mansoni* isolates, no two individuals display the same amplification profile (Fig 2B). To more precisely characterize these patterns, we sequenced the amplicons obtained from each individual of the four *S. mansoni* strains. The results are shown in S1 Table. All individuals expressed multiple variants; some expressed only variants belonging to a single group of *SmPoMucs*, while others expressed variants from two or three groups. Within each group, the *SmPoMuc* transcript polymorphisms reflected: (i) a variable number of tandem repeats (from 1 to 100) in the 5' region; (ii) the occurrence of alternative and aberrant splicing in the 3' region; and (iii) nucleic acid substitutions (synonymous or non-synonymous) in the 5' and 3' regions. These different mechanisms were previously reported [19,20]. We did not observe any clear link between the presence of a given variant and the compatibility level of the different strains, with one exception. A sub-group of variants belonging to *SmPoMuc* group 3.1, named group 3.1(r1-r2), was more abundant in the less compatible *SmGH2* strain than in the other strains. This variant was found in six individuals of *SmGH2*, but in only one individual each of *SmBRE* and *SmVEN* and no individual of *SmLE*.

Interestingly, *SmLE* and *SmVEN*, which were the most infective strains, displayed a larger number of *SmPoMuc* variants (276 and 247, respectively) than the less infective *SmBRE* and

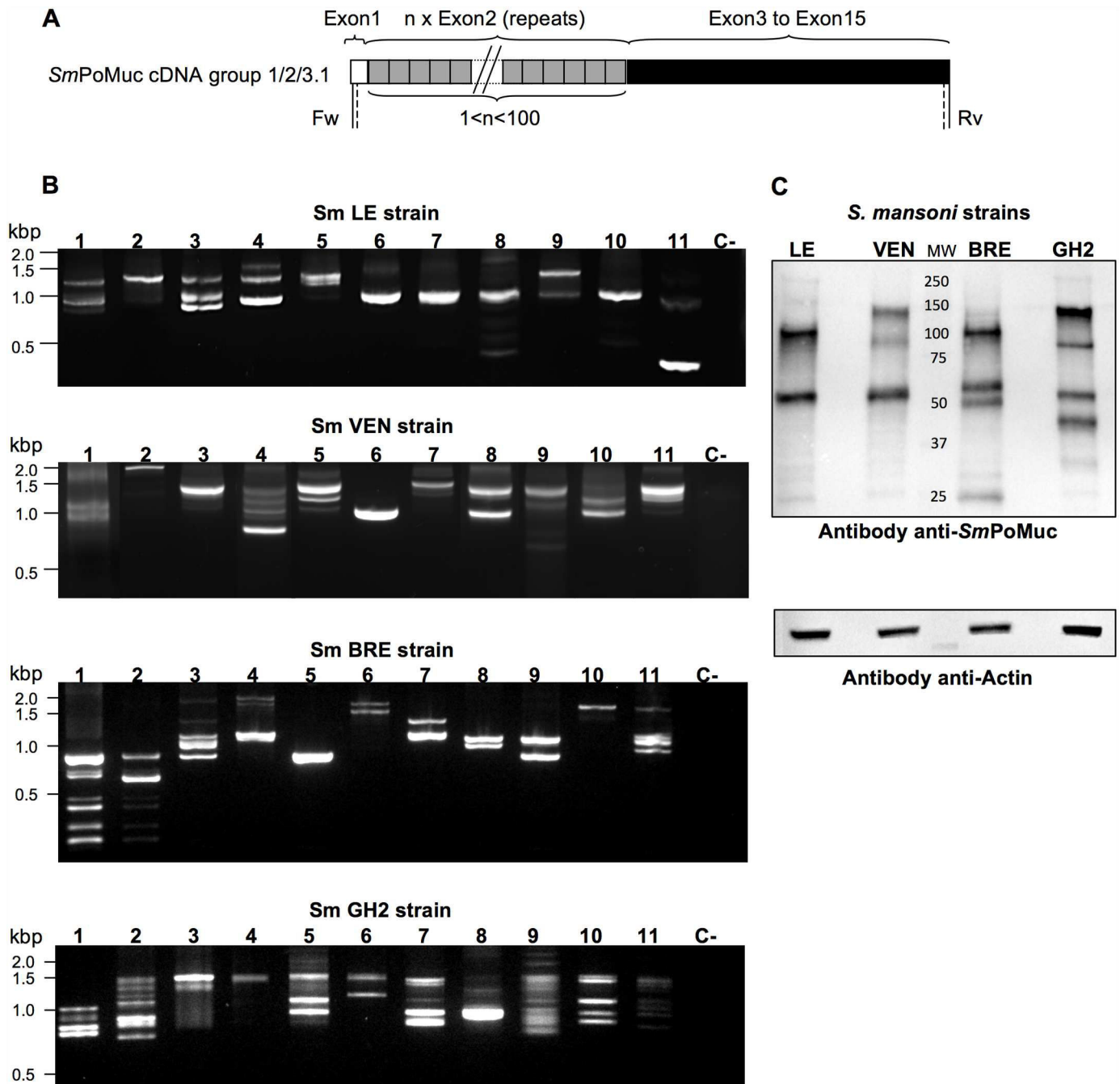


Fig 2. Diverse *SmPoMucs* are expressed by the different strains of *S. mansoni*. (A) *SmPoMuc* cDNA structure. The 5' region is characterized by a variable number of tandem repeats. Two different types of repeats (r1 and r2) were mainly identified in previous studies [19,20]. *SmPoMuc* cDNA structure is shared by previously published *SmBRE*, and *SmGH2* strains [19,20] and the new ones, *SmLE* and *SmVEN*. The 3' region (exon 3 to exon 15) harbors sequence differences that enable the proteins to be classified into the only three groups (groups 1, 2, and 3.1) known to be expressed [19,20]. (B) Transcription patterns of *SmPoMucs* in 11 individuals of each schistosome strain. Nested RT-PCR amplicons were obtained from individual sporocysts (1 to 11) of *SmLE*, *SmVEN*, *SmBRE*, and *SmGH2*, and resolved by agarose gel electrophoresis. PCR was performed using consensus primers that amplified the complete coding sequence of all *SmPoMuc* transcripts (located in exon 1 and exon 15; vertical and dashed lines represent the positions of the primers used for PCR and nested PCR, respectively). "C-" denotes the negative control. *SmBRE* and *SmGH2* nested PCR results were from [21] (C) Inter-strain polymorphism at the protein level. Protein extracts (8 μ g) were obtained from sporocysts of each *S. mansoni* strain, and Western blot analysis was performed using anti-*SmPoMuc* polyclonal antibodies. Actin was detected as a control.

doi:10.1371/journal.pntd.0005398.g002

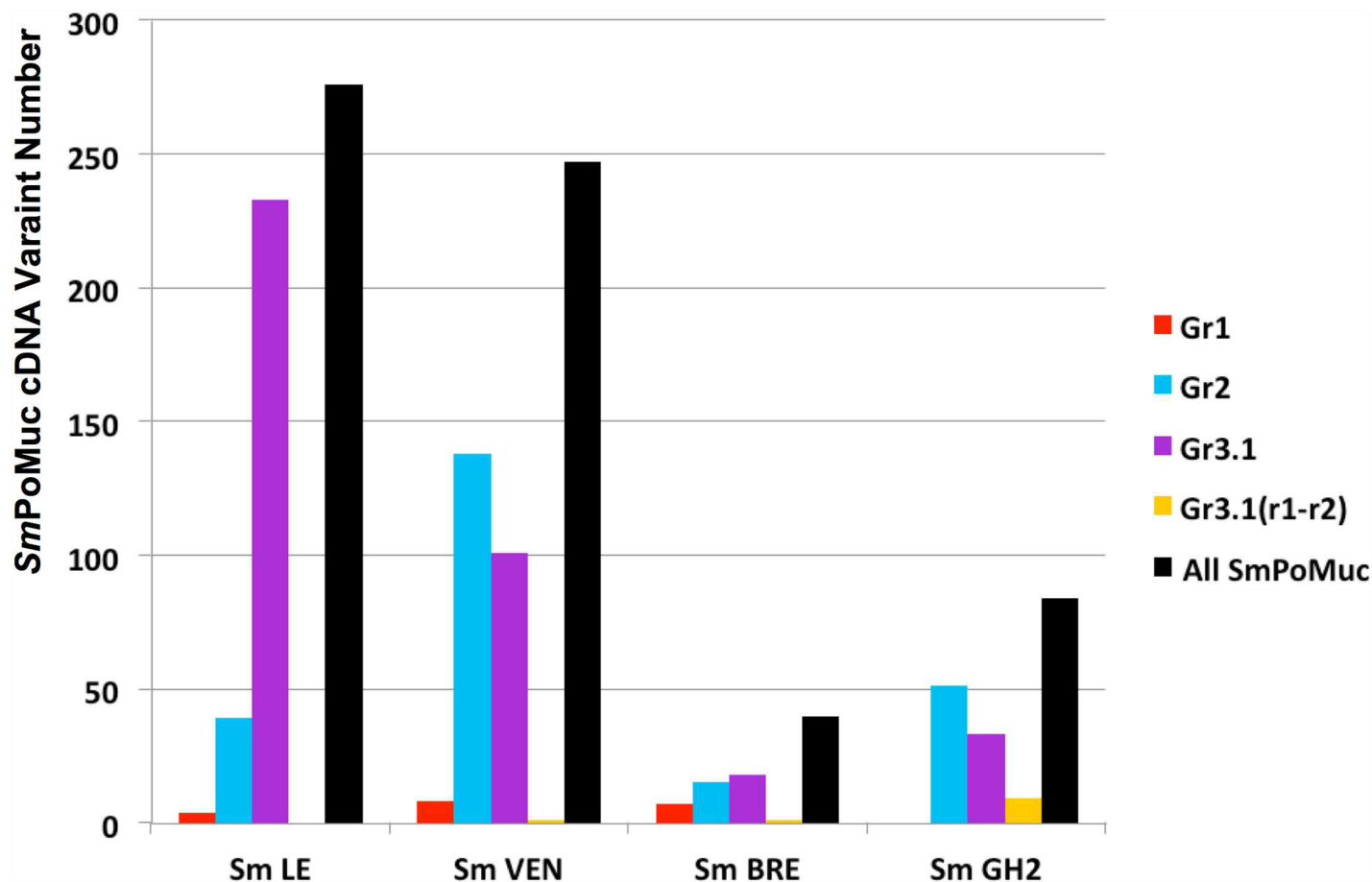


Fig 3. *SmPoMuc* variant numbers in the different *S. mansoni* strains. Nested RT-PCR for *SmPoMuc*s was performed on 11 individual sporocysts for each *S. mansoni* strain (*SmLE*, *SmVEN*, *SmBRE*, and *SmGH2*), and the obtained fragments were cloned and analyzed by Sanger sequencing (see S1 Table). The numbers of *SmPoMuc* cDNA variants obtained for group 1 (red), group 2 (blue), group 3.1 (purple), group 3.1 (r1-r2) (yellow), and all groups (black) are indicated.

doi:10.1371/journal.pntd.0005398.g003

SmGH2 strains (40 and 84, respectively). It is interesting to note that all of the 647 variants sequenced in the present study were different. These results are shown in Fig 3.

*SmPoMuc*s are differentially expressed between *S. mansoni* strains

The expression levels of *SmPoMuc* transcripts were assessed for each *S. mansoni* strain by RT-Q-PCR. Primers E11allgrFw and E14allgrRv were universal to all *SmPoMuc* genes, whereas the other utilized primers (see Fig 4) allowed us to quantify the transcripts corresponding to the group 1, group 2, group 3.1, and group 3.1 (r1-r2) *SmPoMuc* genes. The positions of the utilized primers are indicated in Fig 4A. Relative expression ratios were calculated using α -tubulin as a reference gene (Fig 4B).

Our results revealed that the levels of *SmPoMuc* transcripts of all groups differed across the four schistosome strains. *SmLE* and *SmBRE* displayed the lowest levels of *SmPoMuc* transcripts, and *SmVEN* and *SmGH2* expressed around 2 and 2.5 fold more *SmPoMuc* transcripts, respectively (Mann-Whitney test: LE vs VEN $P = 0.0033$; LE vs GH2 $P = 0.0079$; BRE vs VEN $P = 0.0021$; BRE vs GH2 $P = 0.0025$). With respect to the different *SmPoMuc* groups, the transcription patterns were quite similar between the strains: group 1 was weakly expressed in all

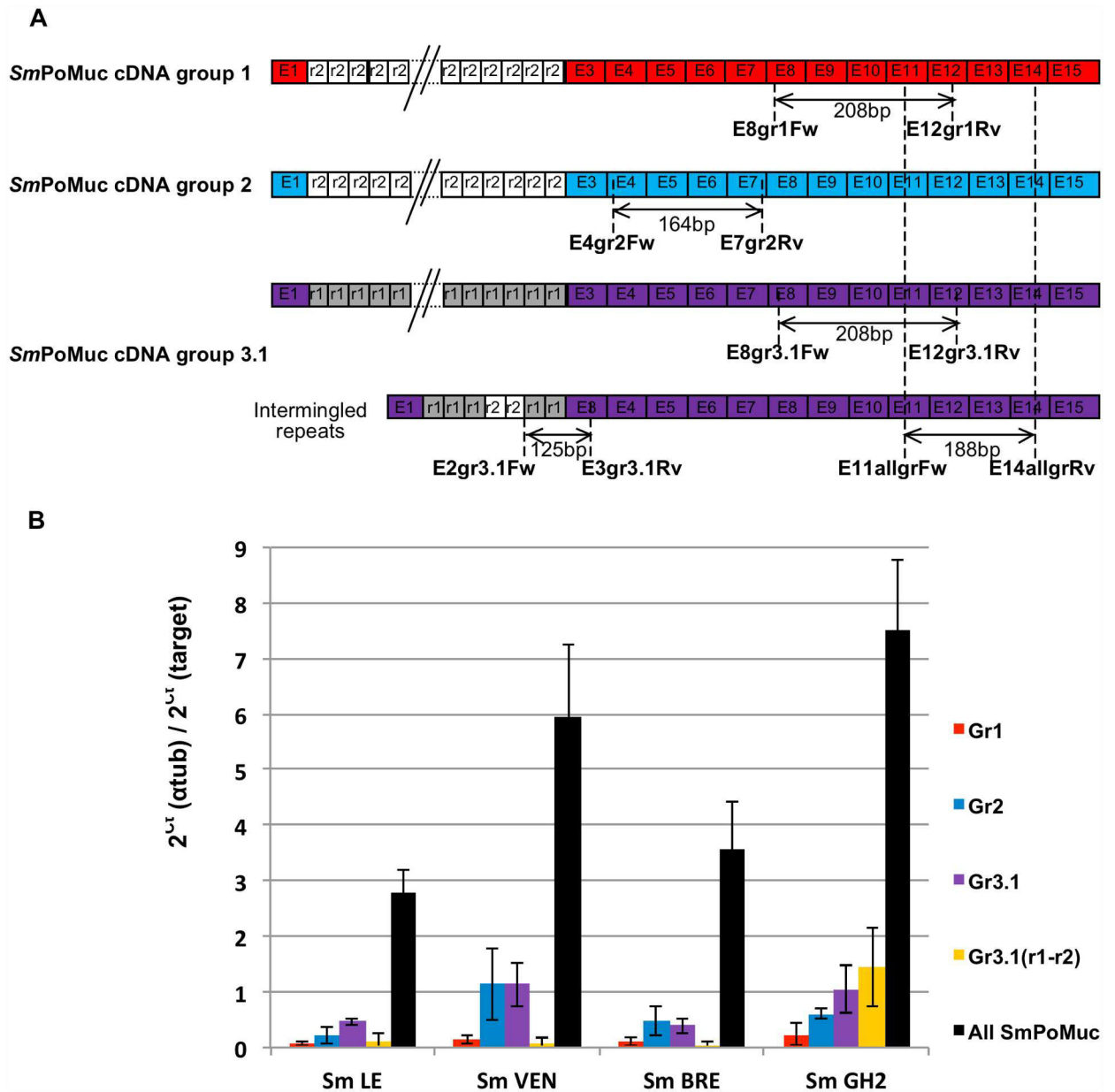


Fig 4. *SmPoMuc* expression between *S. mansoni* strains. (A) Quantitative PCR was performed with primers that targeted all *SmPoMuc* transcripts or discriminated among groups 1, 2, 3.1, and 3.1(r1-r2). The primer positions and amplicon sizes are indicated. (B) RNA was extracted from pooled sporocysts of each *S. mansoni* strain (*SmLE*, *SmVEN*, *SmBRE*, and *SmGH2*). The results are presented as the mean Ct values normalized with respect to the α -tubulin gene, and were obtained from three biological replicates.

doi:10.1371/journal.pntd.0005398.g004

cases, whereas group 2 and 3.1 transcripts were expressed at higher levels (2- to 15 fold more, depending on the group and strain). The group 3.1 (r1-r2) *SmPoMuc* transcripts were highly expressed only in strain *SmGH2* (Mann-Whitney test: GH2 vs LE $P = 0.0079$; GH2 vs BRE $P = 0.0025$; GH2 vs VEN $P = 0.0009$).

Interestingly, the sum of the group-level expression data did not correspond to the *SmPoMuc* expression revealed by the *SmPoMuc* universal primers. This may reflect that some of the spliced variants of *SmPoMuc*s revealed in a previous study [20] are amplified only with

universal primers. As no specific primer could be designed to specifically amplify these spliced variants, we were unable to test this hypothesis.

RNA-seq-based analysis of the four *B. glabrata* strains

Until now, most of the experiments done on compatibility between Schistosomes and *Biomphalaria* snails were conducted using targeted Quantitative PCR or micro-array approaches to identified differentially represented transcripts following infection. In the present paper a more global and powerful approach was conducted to identify the differentially regulated transcripts or differential level of constitutive expression between snail strains. This global approach will also ensure a gene discovery effort without foreseeing the molecules involved compared to targeted approaches. To investigate such differences, four *B. glabrata* strains were used. The global transcript representation was analyzed by RNAseq and correlated with their compatibility phenotypes. *BgBAR* was the less compatible strain, while *BgBRE*, *BgVEN* and *BgGUA* present higher compatible phenotypes that were mostly similar (Fig 1). To compare the native immune potentials of the four *Bg* strains, we compared the biological replicates, *BgBRE1* and *BgBRE2*, to the other *Bg* strains. *BgBRE* strain was selected for duplicate sequencing because it is the strain for which we have the largest number of individuals in the laboratory. To identify the differentially represented transcripts in the transcriptomes of the different strains, we used the DEseq2 software comparing the duplicate of *BgBRE* strain to the others transcriptomes. Of the 117,269 transcripts of the *BgBRE* transcriptome, 6555 (5.6%) were found to be differentially expressed between *BgBRE* and the three other *B. glabrata* strains. Fig 5A and 5B present the numbers of *BgBAR*, *BgVEN*, and *BgGUA* transcripts that were significantly over- and under-represented, respectively. *BgBAR* showed the highest number of differentially expressed transcripts (1257 over-represented and 3462 under-represented). In contrast, *BgVEN* and *BgGUA* showed differential expression (both over- and under-representation) of 2983 and 1613 transcripts, respectively. Of the 6555 modulated transcripts identified in the present study, 72%, 45%, and 25% were from *BgBAR*, *BgVEN*, and *BgGUA*, respectively.

Based on our observations that *BgBAR* differed the most from the other strains in both its molecular phenotype and its compatibility phenotype, we decided to analyze the putative functions that were enriched among its differentially expressed genes. We carried out a Gene Ontology (GO) enrichment analysis on the differentially expressed transcripts that were uniquely over-represented (865 transcripts, Fig 5C) and under-represented (2059 transcripts, Fig 5D) in *BgBAR* relative to *BgBRE*. The over-represented transcripts of *BgBAR* corresponded to 85 biological processes representing 44 categories. Interestingly, eight categories (red arrows in Fig 5C) that contained 15% of the transcripts were grouped together by the REVIGO software into the biological process of “response to biotic and abiotic stress.” One additional category (3% of the transcripts) corresponded to the biological process of “reactive oxygen species metabolism.” These two biological processes are clearly of interest in our study context. Indeed reactive oxygen species (ROS) have been widely shown to be involved in snail/parasite compatibility [8,28,29]. Reactive oxygen species (ROS) produced by the hemocytes of *B. glabrata* are known to play a crucial role in killing *S. mansoni* [30,31]. This process could be related to the level of H₂O₂ expressed by resistant snails associated with a higher expression of SOD genes (copper/zinc superoxide dismutase) [8,32].

The under-represented transcripts of *BgBAR* fell into 18 categories. Among them, only the “defense response” (3% of the transcripts) seems likely to be related to immune function.

Considering the previous results presented above, we decided to deepen our investigation of immune functions in the different *Bg* strains. We first identified genes bearing immune-relevant domains and examined their proportions in the studied strains. We referred to a

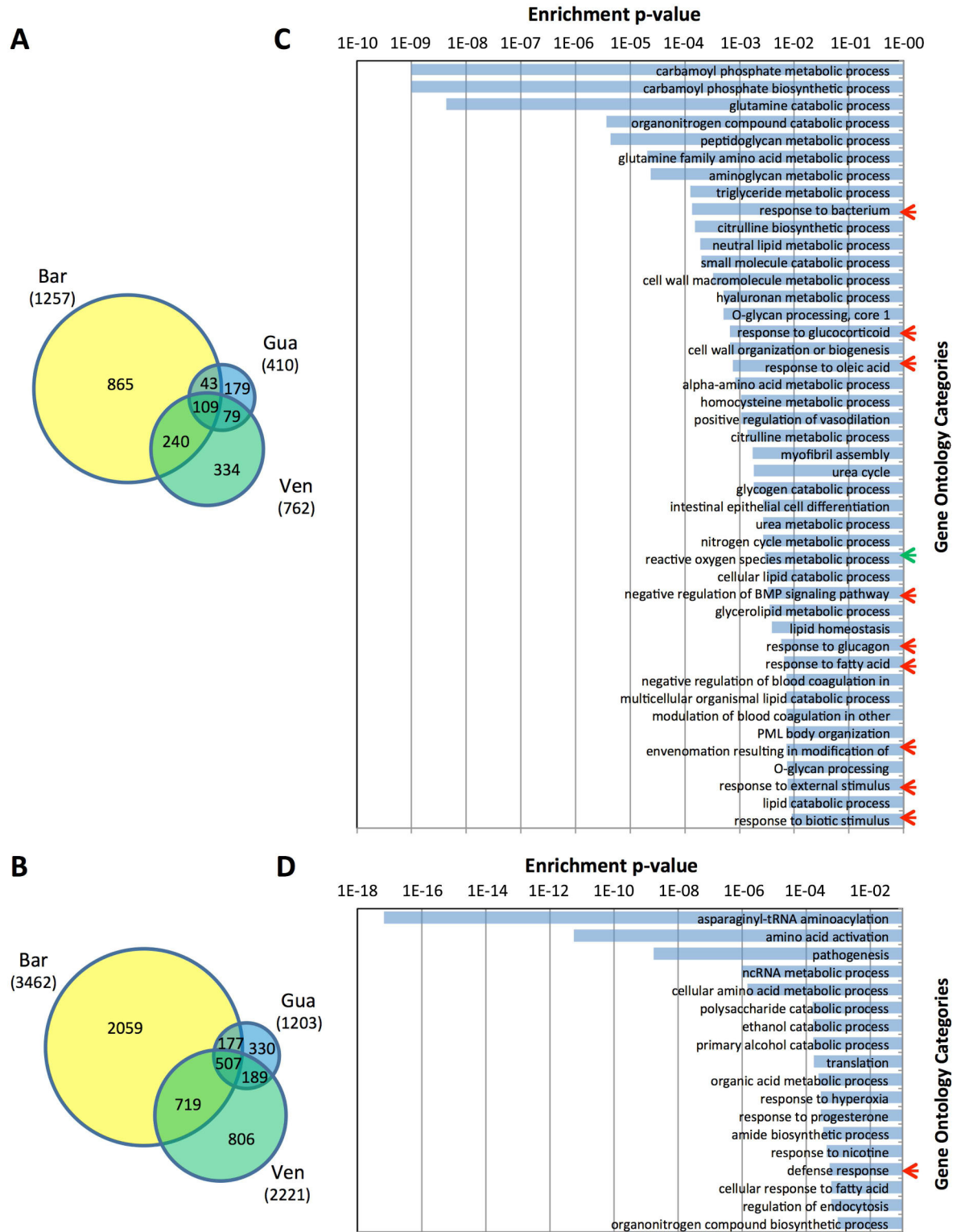


Fig 5. RNA-seq of the different *B. glabrata* strains, and enrichment analysis. Venn diagram presenting the transcripts found to be over-represented (A) and under-represented (B) in the transcriptomes of *BgBAR*, *BgVEN*, and *BgGUA* versus *BgBRE*. Gene Ontology (GO) enrichment analysis of genes that were over-represented (C) and under-represented (D) in the transcriptome of *BgBAR*. Red arrows indicate biological processes belonging to the “response to biotic and abiotic stress” group, while the green arrow shows an immune-response-related biological process.

doi:10.1371/journal.pntd.0005398.g005

previous study in which the whole transcriptome of *BgBRE* was screened for the presence of immune-relevant domains using Interproscan [33]. Using these data, we determined the proportion of the relevant transcripts that were differentially expressed in the strains studied herein. The results are shown in Fig 6A. Given the focus of the present paper and our interest in FREPs, we found it interesting that 3% and 10% of all transcripts bearing an “Ig-fold” domain were over and under-represented, respectively, in *BgBAR*. This particular domain is shared by a family of variable immunoglobulin and lectin domain-containing proteins [33]; notably, this family includes numerous FREPs. A large proportion of the transcripts corresponding to proteins containing other recognition domains, as well as immune signaling molecules and effectors, were also differentially represented between *BgBRE* and the other three strains. As expected, *BgBAR* had higher proportions of differentially expressed genes for most of the categories of putative immune-relevant genes.

To study the immune function of the different snail strains more deeply, we constituted a *B. glabrata* immunome comprising 122 transcripts identified after immune challenge of *B. glabrata* or by comparative “omic” analyses of snail strains displaying different compatibility phenotypes towards trematodes (S2 Table) [11,17,34]. When we examined these immune-relevant molecules in *BgBAR*, *BgGUA*, and *BgVEN* versus *BgBRE* (Fig 6B), we identified 37 differentially expressed transcripts. Nine of them were over-represented and 28 were under-represented: of the latter group, 14 were specifically under-represented in *BgBAR*. Again, *BgBAR* is the most different among the strains; the nine over-represented transcripts displayed their highest representation in *BgBAR*, while 20 of the 28 under-represented genes showed their lowest expression levels in this strain. The nine over-represented immune-relevant transcripts included the following: two FREP-encoding transcripts; a transcript containing a Aerolysin domain belonging to the epsilon toxin ETX/Bacillus mosquitocidal toxin MTX2 superfamily previously characterized as a biomphalysin-like protein shown to kill *S. mansoni* sporocysts [35]; and a transcript encoding an achacin-like, which suggests that *BgBAR* may have an enhanced ability to respond to antimicrobial stress [34,36]. The under-represented transcripts included the following: several transcripts corresponding to FREP family members; transcripts encoding different molecules of the CREP family, whose members are composed of immunoglobulin domain(s) followed by a C-type lectin domain [33]; and transcripts encoding several factors involved in extracellular matrix remodeling and cell migration (e.g., sco-spondin, ADAMTS, and VEGF receptor).

Characterization and expression analysis of FREPs in the four *B. glabrata* strains

As several FREPs (available in GenBank databases) were revealed in the above described transcriptomic analysis, we decided to exhaustively study all transcripts in the transcriptomes of *BgBAR*, *BgVEN*, *BgBRE*, and *BgGUA* that could belong to FREP family members. We selected transcripts corresponding to proteins that contain immunoglobulin (IPR013783) and fibrinogen (IPR002181) domains using the Blast2Go and/or Interproscan software packages. This selection process yielded 258 transcripts. Most of them were not full-length, which meant they could potentially represent molecules that contain fibrinogen or immunoglobulin domains but do not follow the classical organization of FREPs [1 or 2 IgSF domain(s) associate with a fibrinogen domain]. As our objective was to study FREPs-encoding transcripts, we retained all transcripts that contained contiguous immunoglobulin superfamily (IgSF) and fibrinogen (FBG) domains (full-length or partial). In total, we retained 69 FREP transcripts.

The 69 retained transcripts were translated, and the corresponding amino acid sequences were aligned with the *Bg* FREP protein sequences contained within GenBank. None was an

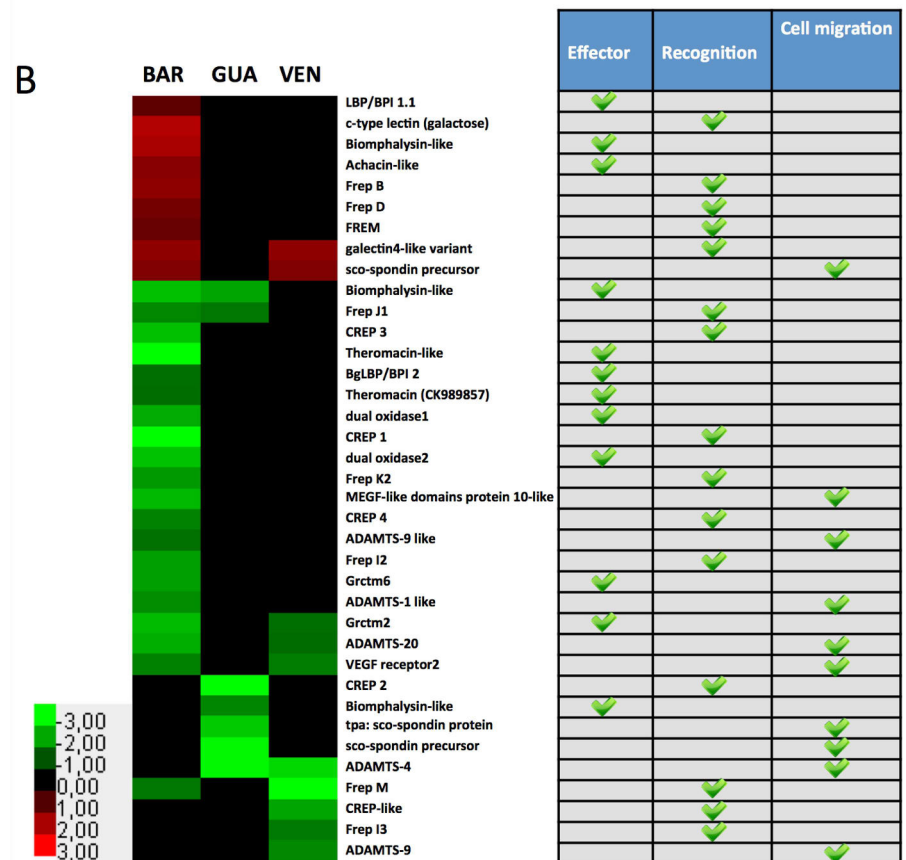
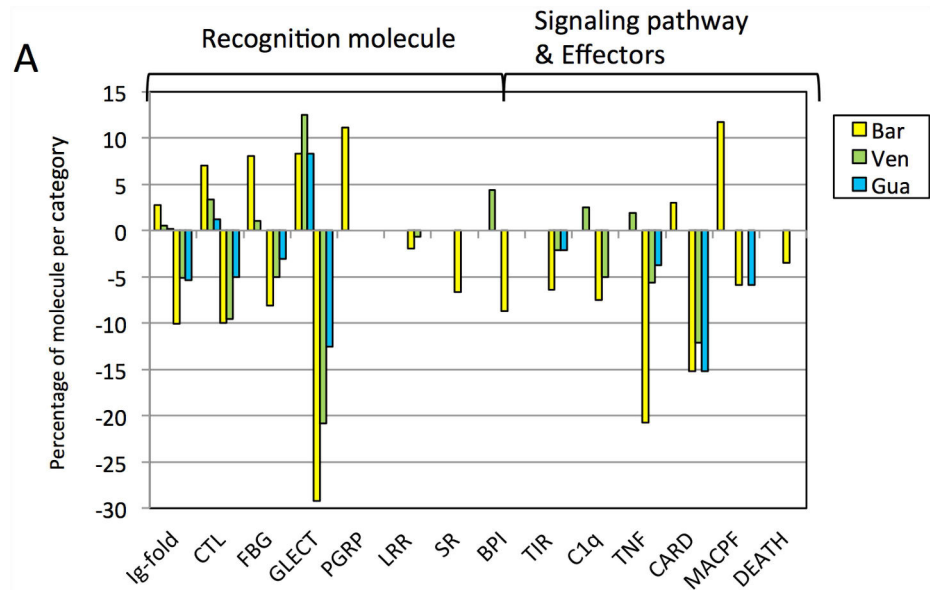


Fig 6. Analysis of immune-relevant genes differentially represented between *BgBAR*, *BgVEN*, *BgGUA* and *BgBRE* reference transcriptome. (A) Histograms showing the proportion of genes bearing immune-relevant protein domains that were differentially represented (DESeq 2 analysis) in *BgBAR* (yellow), *BgVEN* (green) and *BgGUA* (blue) versus *BgBRE*. The monitored immune-relevant genes include immune recognition molecules, immune signaling proteins, and immune effectors. Within these categories, the genes were subdivided by shared protein domains (defined by Interproscan analysis of predicted protein

sequences). Ig-fold: immunoglobulin-like fold, IPR013783. CTL: C-type lectin, IPR001304. FBG: fibrinogen-related domains, IPR002181. GLECT: galectin, IPR001079. PGRP: animal peptidoglycan protein homolog, IPR006619. LRR: leucine-rich repeat, IPR001611. SR: scavenger receptor cysteine-rich, IPR017448. BPI: lipopolysaccharide-binding protein, IPR001124 and IPR017942. TIR: toll-interleukin 1-receptor, IPR000157. C1q: complement component C1q domain, IPR001073. TNF: tumor necrosis factor family, IPR006052. CARD: caspase recruitment domain, IPR001315. MACPF: membrane-attack complex/perforin, IPR020864. DEATH: DEATH domain, found in proteins involved in cell death, IPR000488. (B) Genes previously described to have an immune function in *B. glabrata* species. The heat map represents the log₂ fold change values for transcripts that are differentially represented between the snail strains. The table beside the heat map classifies the corresponding genes into three immune functions: effector, recognition, and cell migration.

doi:10.1371/journal.pntd.0005398.g006

exact match, but 30 of the 69 transcripts displayed only a few differences, and could thus be clearly assigned according to the GenBank FREP nomenclature [37]. The 38 remaining sequences could not be assigned to the established nomenclature. Next, we aligned the 69 sequences with the *B. glabrata* genome draft (www.vectorbase.org/organisms/biomphalaria-glabrata; genome assembly version BglaB1) and assigned them to precise positions in the genome (see supplementary S3 Table). The 69 transcripts corresponded to 24 different genomic loci. Several *de novo* assembled transcripts were validated by traditional Sanger sequencing of PCR products to confirm that this FREPs were not the result of wrong assemblies (S1 Fig). For this study, we classified FREPs by their identity and assigned genomic locus. Any two FREPs that shared more than 85% identity were considered as homologous and were given the same letter (designating FREP class); however if they occupied different genomic loci, the letter is followed by a different number (e.g., C1 and C2). We identified seven loci that encode FREPs with one IgSF domain (grouped into classes A to F), and 16 loci corresponding to FREPs with two IgSF domains (grouped into classes H to M). Finally *B. glabrata* genome assembly did not allow us to establish the number of IgSF domains for a last FREP class named “O”.

After we characterized these 24 loci of FREPs, we used RNA-seq to analyze the representation of the corresponding transcripts in the four *B. glabrata* strains. We built a FREP transcriptome containing the 69 selected sequences, mapped the reads obtained from each snail strain to these sequences using the Bowtie2 software, and normalized the hit count values for each transcript using the upper quartile method. The normalized hit count values were then summed for transcripts belonging to the same class. The results (Fig 7A) showed that there was a high degree of heterogeneity in the representation of FREPs between classes and snail strains. For example, FREP A was 2000 fold more highly expressed than FREP E regardless of the snail strain. Between strains, the majority of the transcripts corresponding to FREPs containing one IgSF domain (e.g., FREPs A, D, C1, and C2) were more highly represented in the less-compatible BgBAR strain. Fig 7B shows the summed transcript levels of FREPs containing one or two IgSFs for the four snail strains. The one-IgSF FREPs comprised 80% of the FREP transcripts expressed by BgBAR, but only 55% to 70% of those expressed in the three other snail strains. Moreover, BgBAR was found to globally express more FREPs than the other snail strains (13%, 48% and 52% more than BgGUA, BgVEN, and BgBRE, respectively) (Fig 7B).

FREP polymorphism analysis

As most of the recovered FREP transcripts were only partial sequences, we could not align and compare the sequences of the FREP variants. Thus, to estimate their degree of polymorphism, we selected the longest transcript sequence for each of the 24 FREP loci and performed Blastn alignments with each of the *B. glabrata* transcriptomes (before CD-Hit EST treatment; Blastn cutoff, 95%). The number of hits was further normalized by the total number of transcripts of each transcriptome. The results (Fig 8A) showed that the variant numbers differed between

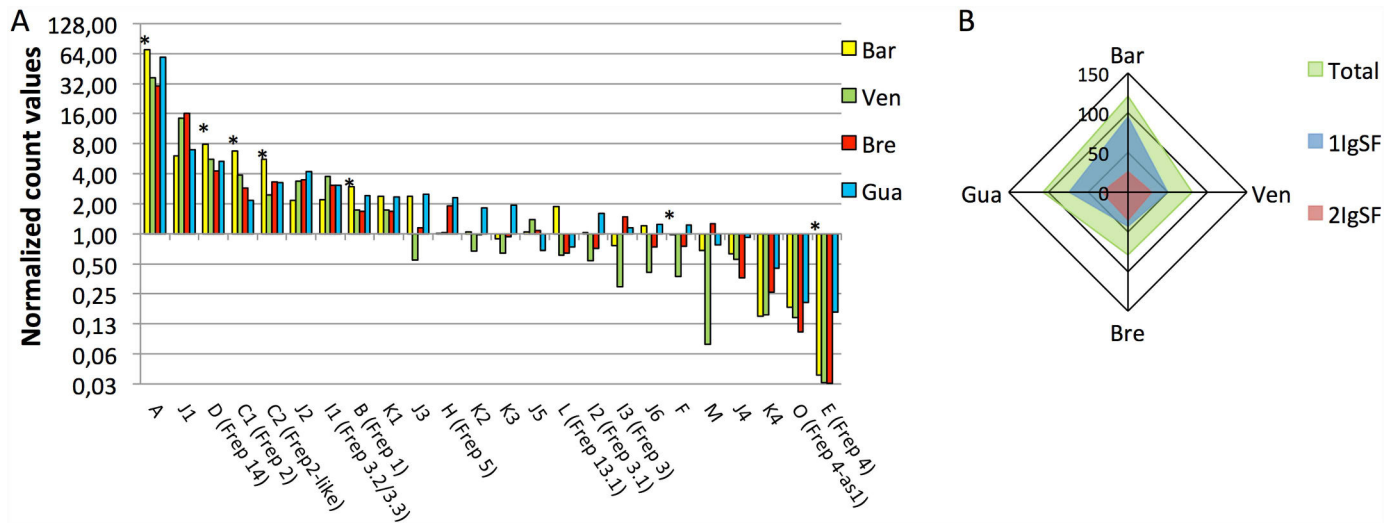


Fig 7. Abundances of transcripts for the different FREP classes in the four *B. glabrata* strains. (A) The histograms represent normalized count values (upper quartile method) obtained when Bowtie 2 was used to map the *BgBAR*, *BgVEN*, *BgBRE*, and *BgGUA* reads with respect to the sequences of the 24 classes of FREP transcripts. Asterisks indicate FREP classes that contain only one IgSF domain. (B) The diagram shows the sum of normalized count values for all the FREPs (total) or the FREPs containing one or two IgSF domains.

doi:10.1371/journal.pntd.0005398.g007

FREP loci at the intra-strain level. For example, FREPs B and I3 had 50 times more variants than FREP A in *BgBRE*, while FREP I2 had 30 times fewer variants than FREPs C1 and L in *BgBAR*. There were also many differences at the inter-strain level; *BgBRE* (Fig 8B) displayed the highest number of normalized variants (480) followed by *BgGUA* (409), *BgVEN* (313), and *BgBAR* (302). FREP A, E, and M always presented a low number of variants, while FREP F, H, and I3 all had many variants, regardless of the strain (Fig 8A). For the other FREP classes, the numbers of variants differed among the strains, with more variants seen for: FREPs C1 and L in *BgBAR*; FREPs B, F, I2, I3, and J2 in *BgBRE*; and FREPs K2 and K4 in *BgGUA*. For *BgVEN*, the FREP variants were distributed more or less homogeneously. Notably, although *BgBAR* had the fewest normalized FREP variants, it displayed the highest numbers of variants for the one-IgSF FREP A, C1 (corresponding to FREP 2 in GenBank), and D (corresponding to FREP 14 in GenBank).

Thus, our results show that *BgBAR*, which is the least susceptible to *S. mansoni* presents the highest level of FREP expression and the lowest number of total FREP variants among the studied strains. Moreover, it expresses a higher proportion of FREPs containing one IgSF domain, and has the highest ratio of one-IgSF variants/all FREP variants.

Discussion

FREPs and *SmPoMucs* are key molecular determinants of the compatibility process between *B. glabrata* and *S. mansoni*. Here, we examined the expression patterns and polymorphisms of these important factors in four host isolates and four parasite isolates that displayed different levels of compatibility with one another. Among the schistosomes, strain *SmLE* was clearly the most infective against the different snail strains, *SmGH2* was the least infective, and the other two strains displayed intermediate compatibility patterns (Fig 1). Among the snails, strain *BgBAR* was clearly the least permissive, whereas the compatibility phenotypes of the other three strains were much higher and quite similar.

In an effort to explain the compatibility differences among the different schistosome strains, we first analyzed the diversity and expression levels of *SmPoMucs* in the different strains. The

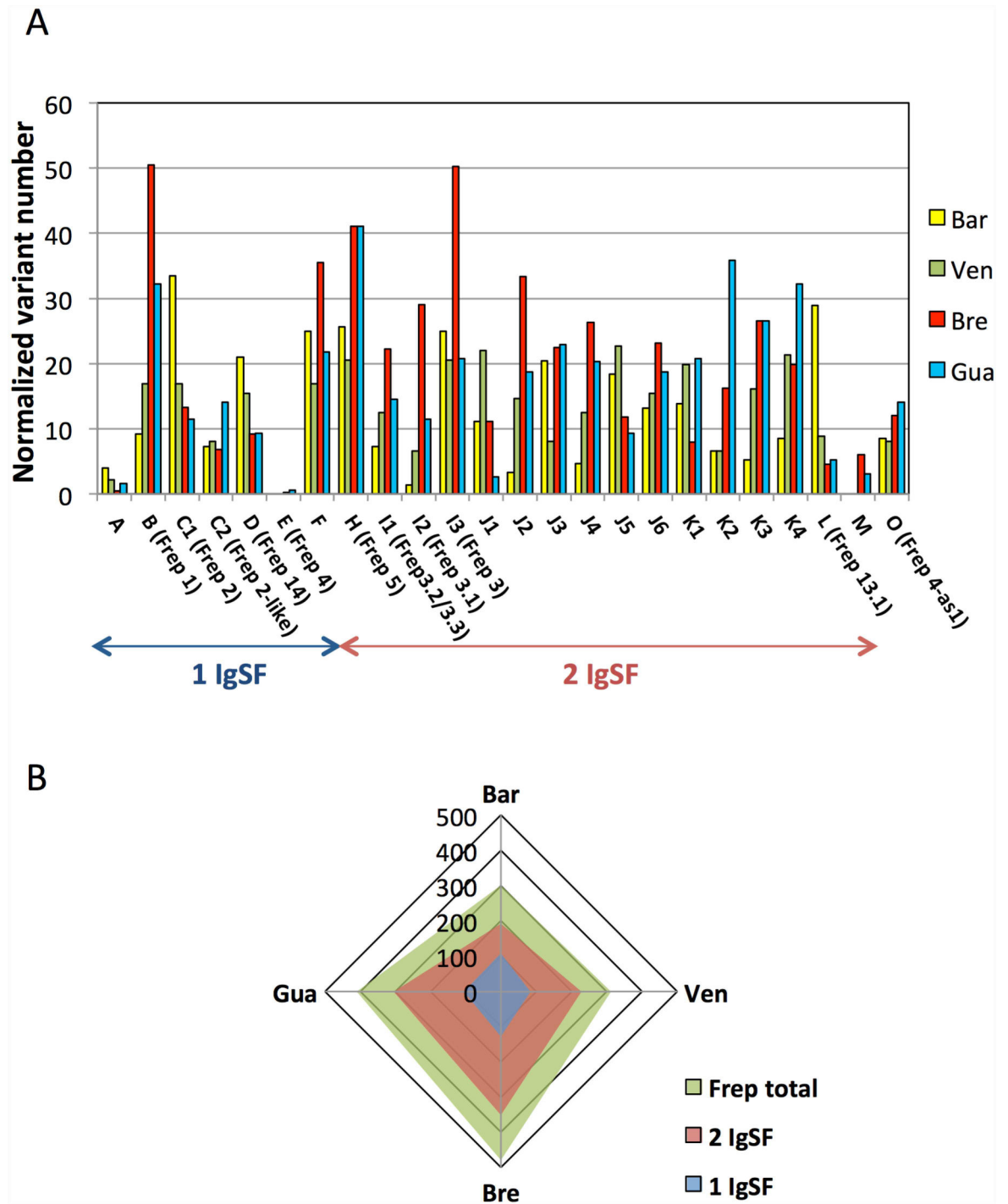


Fig 8. FREP variant polymorphism analysis. Diversity was assessed by aligning the longest transcript from each FREP class to the transcriptomes of *BgBAR*, *BgVEN*, *BgBRE*, and *BgGUA* using Blastn (similarity, at least 95%). (A) Histograms showing the number of variants normalized by the total number of transcripts for each FREP class in all *B. glabrata* strains. (B) For all *B. glabrata* strains, diagrams showing the sum of normalized FREP variant numbers based on whether they have one or two IgSF domains.

doi:10.1371/journal.pntd.0005398.g008

most compatible strains, *SmLE* and *SmVEN*, expressed similarly high numbers of *SmPoMuc* variants (around 270 and 250, respectively; Fig 3), while the two less compatible strains expressed fewer variants (45 and 75 for *SmBRE* and *SmGH2*, respectively; Fig 3). These results are clearly in agreement with the hypothesis of a matching phenotype [38]. Indeed, if a population of schistosomes is more diverse in their *SmPoMuc* patterns, they would be less prone to being recognized by a population of hosts that display a fixed number of FREP immune receptors. These results of *SmPoMuc* polymorphism analysis clearly agree with the theoretical results obtained from co-evolutionary models applied to plant and animal innate immunity [39]. In the multistep infectious interactions of parasites and hosts, a genetically explicit model revealed that polymorphism will be greater at recognition loci than at effector loci, and that host-genotype by parasite-genotype interactions are greater for the recognition phase than for the effector phase. Our results confirm these predictions, as we herein establish a link between *SmPoMuc* polymorphism and the compatibility phenotype. Additional results obtained by Nuismer and Dybdahl [39] also showed that, compared to the effector phase, the recognition phase contributes more to local adaptation. This also makes sense in the context of our observation that the compatibility level is generally higher for sympatric combinations. The expression level of *SmPoMucs* also appears to be relevant to the compatibility pattern. In terms of expression, *SmVEN* and *SmGH2* expressed around two fold more *SmPoMuc* genes than the other two strains. Conceivably, a schistosome expressing more *SmPoMucs* will generate more *SmPoMuc*/FREPs interactions at the parasite surface (even given a fixed number of possible combinations), which will favor the recognition, the immune response and the killing of the parasite. This could explain why strain *SmVEN* expressed a similar number of variants compared with strain *SmLE*, but displayed a lower compatibility phenotype. Similarly, the expression level of *SmPoMucs* could explain why *SmGH2* was less compatible than *SmBRE*, even though the former expressed more variants than the latter.

Concerning now the comparison of the proportion of variants belonging to the different groups of *SmPoMucs* in the studied schistosome strains, it revealed that: (i) the expression level and the number of variants of group 1 *SmPoMucs* is always low; (ii) the expression levels of groups 2 and 3 *SmPoMucs* are very similar within each strain; (iii) the proportions of group 2 and 3 variants can differ according to the strain (e.g., group 3 was more diversified in strain *SmLE*); and (iv) the group 3 of *SmPoMucs*, which containing intermingled repeats [19,20], are only expressed at significant levels in strain *SmGH2*. These differences in repeat types or numbers could also explain some of the compatibility differences between strains. However, our current understanding of the link between polymorphism, glycosylation status, and recognition by FREP lectins is too fragmentary to allow us to propose a more precise hypothesis. The *SmPoMuc* repeat number was shown to be related to the glycosylation status within a specific strain of schistosome [20], but the glycosylation of different variants and its influence on FREP recognition remain to be investigated.

Concerning the FREP contents of the studied snail strains, we first performed a global transcriptomic analysis; we then focused on immune genes, and finally on FREPs. As compatibility depends on molecules that are expressed constitutively by the host and the parasite [18], we conducted our transcriptomic analysis on naive snails. Our global comparative analysis revealed that, as expected, the transcriptome of *BgBAR* (which was much less permissive than the others (Fig 1) showed the greatest differences with respect to the others. Among the differentially expressed transcripts of this strain, we observed enrichment of numerous genes involved in the immune response and responses to biotic and abiotic stimuli. Accordingly, we performed a more targeted analysis of a *B. glabrata* immunome composed of 122 transcripts encoding proteins known to be involved in the immune recognition, immune signaling pathways, and effector functions of *B. glabrata* [17,40]. As expected, *BgBAR* had a higher

proportion of genes that were differentially represented in the majority of these molecular classes and the comparison of the transcription levels of these genes in the different strains showed again that *BgBAR* is the most important differences. This strain exhibited both over-representation (e.g., FREP1, FREP14, an LBP-BPI 1.1, an achacin, a biomphalysin-like, etc.) and under-representation (e.g., FREP 3.1 M, FREP J1, FREP K2, a biomphalysin-like, a macin, grctm 2, grctm 6, proteases, etc.) of transcripts. As some FREPs were differentially expressed between snail strains, we next analyzed the expression of all FREPs known to be present in the *B. glabrata* genome. Our results indicated that *BgBAR* expressed more FREPs than the three other strains, and that most of the FREPs containing one IgSF domain (5 of 7) were more highly expressed in *BgBAR* compared to the other strains. This suggests that this specific class of FREPs could play a key role in schistosome recognition and anti-schistosome defense. Indeed, this is consistent with a previous report showing that FREP 2 (containing one IgSF domain) interacts with *SmPoMuc* antigens [24]. Our analysis of FREP polymorphisms between and within strains showed that *BgBRE* and *BgGUA* had more variants than *BgVEN* and *BgBAR*, suggesting that the diversity of FREPs is not correlated with the compatibility level, as would be expected from an ongoing arms race between FREPs and *SmPoMucs*. We thus propose (very speculatively) that this absence of correlation could be a consequence of the recent introduction of the schistosome to the South American and Caribbean areas (from which the tested snail strains were sampled) through the Atlantic slave trade of the 16-19th centuries. This would have forced the schistosome to adapt to a new intermediate host of the same genus (*B. pfeifferi* in Africa versus *B. glabrata* in the New World). The parasite needed to develop an evolutionary strategy to increase its compatibility with its new host, and thus diversified its *SmPoMucs* to escape recognition by FREPs. In this context, the diversification of FREPs in some snail strain could be linked to co-evolution with other pathogens living in sympatry with snails of the New World.

Our present results clearly corroborate that FREPs and *SmPoMucs* seem to be molecular determinants of the compatibility between *B. glabrata* and *S. mansoni*. However, it is largely accepted that this compatibility process is a complex one that involves numerous genes engaged in an arms race (see [41] for a recent review). Compatibility can be viewed as a multi-step process through which the parasite escapes the recognition and effectors of the host. Although FREPs and *SmPoMucs* may play a crucial part in this recognition, other pattern recognition receptors (e.g., lectins) and antigens have been suggested to be involved [24]. In terms of the effectors of the host and the anti-effector systems of the parasite, it has been shown that highly reactive chemical compounds derived from molecular oxygen (ROS) are crucial to the snail's ability to defend itself against *S. mansoni* [42], and the parasite has developed ROS scavenger systems to counter these molecules [29]. Additional candidate genes have also been recently implicated in the compatibility process between *B. glabrata* and *S. mansoni*; for example, the biomphalysin from *B. glabrata* have been shown to exert high cytotoxicity against *S. mansoni* sporocysts [35], and a new family of putative immune receptors was identified using a RAD-seq approach [11]. The previous reports and present results therefore collectively suggest that the compatibility process is likely to be much more complex than previously thought. Indeed, our transcriptomic analysis of *B. glabrata* strains showed that the less permissive snail strain varied from the others in terms of immune-relevant transcripts that are involved not only in immune recognition, but also in signaling pathways and effector functions (Fig 6).

In conclusion, the arms race between *B. glabrata* and *S. mansoni* has selected for diversified molecular repertoires that allow the parasite to counter the immune recognition system of the host. The extraordinary diversification that enables *SmPoMucs* to avoid recognition by FREPs illustrates the outcome of these evolutionary dynamics. The present findings together with

other results obtained in recent decades show that the compatibility between *B. glabrata* and *S. mansoni* depends on multiple factors, including: (i) the genetics of the snail and the schistosome; (ii) the age of the snail; (iii) the previous interactions of the snail with schistosomes; and (iv) the ability of the environment to influence (through epigenetic mechanisms) the compatible/incompatible phenotypes of both partners (see [41] for a recent review). To solve this complicated puzzle, we need to develop novel integrative approaches that combine comparative genomic, epigenomic, and transcriptomic approaches performed under different environmental conditions. This should enable us to identify relevant candidate genes whose functions could then be validated using CRISPR/Cas or RNAi methodologies. Given the variability of the mechanisms involved in compatibility, such studies must be undertaken on different snail and schistosome populations and strains. These ambitious approaches are absolutely necessary if we hope to identify the molecular pathways that are most likely to be good targets for strategies aimed at blocking transmission through the snail intermediate host.

Materials and methods

Ethic statements

Our laboratory holds permit # A66040 for experiments on animals from both the French Ministry of Agriculture and Fisheries, and the French Ministry of National Education, Research, and Technology. The housing, breeding and animal care of the utilized animals followed the ethical requirements of our country. The experimenter also possesses an official certificate for animal experimentation from both French ministries (Decree # 87–848, October 19, 1987). Animal experimentation follows the guidelines of the French CNRS. The different protocols used in this study have been approved by the French veterinary agency from the DRAAF Languedoc-Roussillon (Direction Régionale de l'Alimentation, de l'Agriculture et de la Forêt), Montpellier, France (authorization # 007083).

Parasites, snails, and compatibility

The four studied strains of *S. mansoni* parasites and the four corresponding sympatric snail strains of *B. glabrata* originated from South America and had been maintained in the laboratory using Swiss OF1 mice (Charles River Laboratories, France) as the definitive host. The four sympatric host/parasite combinations were designated *BgVEN/SmVEN* (from Guaraca, Venezuela), *BgGUA/SmGH2* (le Lamentin, Guadeloupe), *BgBRE/SmBRE* (Recife, Brazil), and *BgBAR/SmLE* (Belo Horizonte, Brazil). Miracidia from the parasite strains (*SmBRE*, *SmLE*, *SmGH2* and *SmVEN*) were recovered from infected mouse livers and intestines and transformed into sporocysts in vitro as previously described [19]. Compatibility trials between the strains of parasites and snails were conducted as previously described [43]. For all sympatric and allopatric combinations, we experimentally infected snails with 20 miracidia per snail, which was previously shown to yield a maximum infection rate regardless of the utilized strain [43]. Two weeks later, we assessed the prevalence (percentage of infected snails) and intensity (number of developed parasites per infected snail) of infection. Three independent experiments were performed. The data presented in the present manuscript correspond to the mean values obtained in a previous published work [43] and the data obtained from two other experiments performed in 2010 and 2012.

Western blot analysis of SmPoMucs in *S. mansoni* sporocysts

Five thousand sporocysts were collected and counted for each *S. mansoni* strain (*SmBRE*, *SmLE*, *SmGH2*, and *SmVEN*). The samples were ground with a pestle and vortexed, and

proteins were extracted using UTC buffer (7 M urea, 2 M thiourea, 30 mM Tris, pH 8.5, and 4% CHAPS) for 2 h at room temperature. The samples were centrifuged at 10,000 g for 5 min, the supernatants were recovered, and the protein concentrations were estimated using a 2D Quant kit (GE Healthcare Life Sciences). For each sample, proteins (8 µg) were incubated with Laemmli buffer for 5 min at 99°C, resolved by 12% SDS-PAGE, and blotted on a nitrocellulose membrane (Trans-blot Turbo; Bio-Rad). The membrane was blocked with 5% skimmed dry milk in TBST (TBS containing 0.05% Tween 20) for 3 h at room temperature and incubated with the previously described anti-SmPoMuc [24] diluted 1/1000 in TBST overnight at 4°C. The membrane was washed three times in TBST (10 min each, room temperature), and then incubated with peroxidase-conjugated purified anti-rabbit IgG (Sigma Aldrich) diluted 1/5000 in TBST with 5% skimmed dry milk for 90 min at room temperature. The membrane was washed three times in TBST and once in TBS, and proteins were detected with a ChemiDoc MP Imaging system (Bio-Rad) using ECL reagents. As a loading control, we performed a parallel Western blot using anti-actin (Thermo Scientific) diluted 1/1000 and HRP-conjugated anti-mouse IgG (Sigma Aldrich) diluted 1/10,000.

SmPoMuc polymorphism analysis using individual sporocysts of *S. mansoni*

Eleven sporocysts were recovered individually from each *S. mansoni* strain. RNA was isolated from each individual using a Dynabeads mRNA Direct Micro kit (Ambion Life Technologies) as previously described [20]. The RNA was reverse transcribed by adding the enzyme mix (Superscript II, Invitrogen) directly to the paramagnetic Dynabeads. The generated cDNA was recovered using the magnetic system, washed twice in 10 mM Tris (pH 7.5) and SmPoMuc sequences were directly amplified using PCR and nested PCR, as previously described [20]. The obtained products were separated by 1% agarose gel electrophoresis. Each band was excised, purified, and cloned into pCR4-TOPO, and 100 clones were sequenced.

Quantitative PCR analysis of SmPoMuc expression on *S. mansoni* sporocysts

Five thousand sporocysts from each *S. mansoni* strain were recovered and stored at -80°C. RNA was extracted using a Dynabeads mRNA Direct Micro kit (Ambion Life Technologies) according to the manufacturer's instructions. Between the washing and elution steps of the RNA purification, an additional on-bead DNase treatment was performed using the TURBO DNA-free kit (Ambion Life Technologies). Reverse transcription was performed using the Maxima H Minus First Strand cDNA Synthesis kit (Thermo Scientific) with a 1:1 mixture of oligo dT and random primers. Quantitative PCR amplifications were performed with 2 µl of 20-fold diluted cDNA and 0.5 µM of each primer in a final volume of 10 µl, using a LightCycler 480 SYBR Green I Master kit and a LightCycler 480 II Real Time instrument (both from Roche). An initial denaturation at 95°C for 12 min was followed by: 45 cycles of 11 sec denaturation at 95°C, 11 sec annealing at 60°C, and 19 sec elongation at 72°C; a melting curve step from 65 to 97°C with a heating rate of 0.11°C/sec and continuous fluorescence measurement; and a cooling step to 40°C. For each reaction, the cycle threshold (Ct) was determined using the 2nd derivative method of the LightCycler 480 Software release 1.5 (Roche). PCR experiments were performed in triplicate (technical replicates) from three biological replicates. The mean value of Ct was calculated. Corrected melting curves were checked using the Tm-calling method of the LightCycler 480 Software release 1.5. Results were normalized with respect to the α -tubulin gene, as previously described [22], and Δ Ct values were calculated. The primers used to analyze the different SmPoMuc groups are indicated in Fig 1A.

B. glabrata cDNA library construction and sequencing

RNA extraction, cDNA library construction, and Illumina SOLEXA sequencing were performed as previously described [33,44]. Briefly, total RNA was extracted from whole snail body tissues from 10 juvenile, 10 adult, and 10 old snails for each *B. glabrata* strain (*BgVEN*, *BgGUA*, *BgBRE*, and *BgBAR*). Tissues were disrupted in liquid nitrogen and total RNA was extracted using the TRIzol reagent (Life Technologies) according to the manufacturer's instructions. Equimolar amounts of RNA from juvenile, adult and old *B. glabrata* were combined to yield two pools of 30 individuals for *BgBRE* (BRE1 and BRE2) and one pool of 30 individuals for each of the other strains (*BgBAR*, *BgVEN* and *BgGUA*).

Paired-end 72-bp cDNA libraries were generated using an mRNA-seq kit for transcriptome sequencing (Solexa, Illumina) on a Genome analyzer II platform (Illumina). Three samples were multiplexed per lane. Library construction and sequencing were performed by MGX (Montpellier Genomix, c/o Institut de Génomique Fonctionnelle, Montpellier, France). For library constructions cDNA fragments following RT-PCR amplification ranged from 220 to 500 bp (average, 300 bp). The numbers of 72-bp reads obtained from *BgBRE1*, *BgBRE2*, *BgBAR*, *BgGUA*, and *BgVEN* were 99,316,948, 73,000,210, 116,679,444, 100,640,190 and 111,178,786, respectively. The reads that passed the Illumina quality filter were further cleaned via a previously described workflow, using the Galaxy server [33,44]. The 13 first and three last low-quality bases were then trimmed; yielding paired-end reads of 56 nucleotides each. Reads without a pair (orphan reads) were removed. The final high-quality libraries used for transcriptome assemblies contained 90,331,578, 66,558 544, 105,718,404, 88,800,366, and 85,918,982 reads for *BgBRE1*, *BgBRE2*, *BgBAR*, *BgGUA*, and *BgVEN*, respectively.

Processing of read sequences obtained from RNA-seq of *B. glabrata*

De novo transcriptomic assemblies were performed using the Velvet version 1.2.02 software implemented by the python script provided by Oases version 0.2.06, as previously described [33,44]. The resulting contigs were merged into unigene clusters using CD-HIT-EST version 4.5.4 A multiple k-mer assembly approach was applied to optimize the assembly. Four high-quality reference transcriptomes were produced, comprising 117,269, 70,533, 82,500, and 79,664 transcripts for strains *BgBRE*, *BgBAR*, *BgGUA*, and *BgVEN*, respectively (for transcriptome details see S4 Table). The transcripts were automatically annotated using Blast2GO version 2.4.2.

Differential expression of transcripts in *B. glabrata*

Quality reads (Phred score >26) were aligned on the *BgBRE* transcriptome assembly using Bowtie2 (v2.0.2; mapping quality score 255) on the Galaxy server (<http://bioinfo.univ-perp.fr>) [45]. The count of the reads mapped to each transcript was assessed using Hitcount to BAM (SAM tools v0.1.18.0). The obtained values were normalized by the upper quartile method [46], which has been proposed to be more accurate than the RPKM method of normalization [47]. The DESeq2 software (v2.12; <http://www.bioconductor.org/packages/release/bioc/html/DESeq2.html>) [48] was used under default settings to identify genes that were differentially expressed in the two biological snail sample duplicates (BRE1 and BRE2) versus the other snail strain samples ($P < 0.05$). Hierarchical ascending clustering (HAC) with Pearson correlation, which was performed using the Cluster 3.0 [49] and JavaTreeView software packages, was used to generate a heatmap for analysis of the transcript expression patterns (log₂ fold change).

Gene Ontology (GO) enrichment analysis

To detect biological processes that were significantly over- or under-represented in the *BgBAR* strain, we examined the transcripts found to be differentially expressed between *BgBAR* and *BgBRE*. Functional enrichment was assessed using the GoStatsPlus package in R (<https://github.com/davfre/GOstatsPlus>). The enrichment was calculated for the 3 categories including of biological process (BP), molecular function (MF), and cellular component (CC), and. The results were imported into REVIGO [50] for further analysis and visualization.

Sequence analysis of *B. glabrata* FREPs

Transcripts corresponding to potential FREP sequences were selected from transcriptomes on the basis of Blast2Go annotations or the presence of immunoglobulin and/or fibrinogen domains (IPR013783 and IPR002181, respectively), as detected by Interproscan. As most of the obtained sequences were not full length, transcripts encoding complete sequences or partial sequences with contiguous Immunoglobulin superfamily (IgSF) and fibrinogen (FBG) domains were selected to be sure to have a transcript belonging to a real FREP family member and not to GREP, CREP, FREM or IgSF or FBG containing molecules. The retained transcripts were translated to predicted amino acid sequences and aligned with those of reference FREP proteins obtained from GenBank using BioEdit version 7.1.3.0. and were also aligned on the *B. glabrata* genome draft using the Blast tool available on the Vector Base website (<https://www.vectorbase.org/>). For quantitative analysis of FREPs, the reads from each *B. glabrata* strain were mapped to a reference FREP transcriptome (FREPs transcripts assembled from the transcriptomes of the four snail strains) using Bowtie2 (v2.0.2). The mapped reads were counted per transcript and the values were normalized by the upper quartile method, as described above. To estimate the FREP polymorphism in each strain, the longest transcript from each FREP class was aligned against the *B. glabrata* transcriptomes obtained before CD-Hit EST treatment, using Blastn (cutoff, 95% identity) on the Galaxy server. The number of mapped hits was further normalized to the total number of transcripts for each *B. glabrata* transcriptome, and the level of polymorphism was calculated as follows for each FREP class and snail strain: normalized number of FREP variants = (number of blast hits / total number of transcripts) x 10⁵.

Validation of *de novo* transcript assembly

Several *de novo* assembled transcripts were validated by traditional Sanger sequencing of PCR products. Briefly, depending of the strain, total RNA was reverse transcribed with random primers and RevertAid premium enzyme (Thermo scientific). Two µl of the RT reaction was then used for PCR (Advantage 2 PCR system, Invitrogen, Carlsbad, CA, USA) with primers that were designed to specially target and amplify novel predicted transcripts, and amplicons were sequenced (GATC Biotech, Konstanz, Germany). Sequences from Sanger sequencing and from computational assembly of *de novo* transcripts were aligned (Supplementary S1 Fig).

Accession numbers

The obtained *SmPoMuc* sequences were deposited in GenBank under the following accession numbers: KX645102-KX645377 for *SmLE*, KX645378-KX645624 for *SmVEN*, EU676447-EU676459/EU676503-EU676530/EU676572-EU676583/EU676595-EU676625 for *SmBRE*, and EU676460-EU676502/EU676531-EU676571/EU676584-EU676594/EU676556-EU676626 for *SmGH2*.

The obtained FREP sequences were deposited in GenBank under the following accession numbers: KY024239 to KY024307.

Supporting information

S1 Fig. Validation of FREP by alignment of sequences obtained from Sanger sequencing and from computational assembly of *de novo* transcripts. To confirm that *de novo* assembly will not generate miss assemblies for highly diverse FREP molecules, assembled FREP transcripts were validated by traditional Sanger sequencing of PCR products. Sanger sequences and computational assembled transcripts were aligned. Nucleotides underlined in black color are common to both sequences. Forward and reverse primers positions were indicated by asterisks.

(PDF)

S1 Table. Variants of cDNA obtained from 11 individual sporocysts of *S. mansoni* strains.

(XLS)

S2 Table. *B. glabrata* immunome. List of molecule categories previously shown to play roles in snail immunity, and the corresponding loci in the *BgBRE* transcriptome. Underlined lanes correspond to transcripts that are differentially expressed across the four *B. glabrata* strains. Molecules were selected from: *B. glabrata* transcriptomes and the literature [11,17,30]. FREP nomenclature is detailed in the chapter “Characterization and expression analysis of FREPs in the four *B. glabrata* strains”, see [Results](#) section.

(XLSX)

S3 Table. Annotation of the 24 classes of FREP molecules identified. The longest transcript number of each class is mentioned, as are: its length in nucleotides and in amino acids (after virtual translation); its new FREP class and standard classification when possible; its IgSF domain number; its position on the *B. glabrata* genome draft (genome assembly version *BglaB1*); the corresponding exon number; and, when determined, the predicted transcript in the Vectorbase database.

(XLSX)

S4 Table. Transcriptome statistics. The characteristics of *BgBAR*, *BgBRE1*, *BgBRE2*, *BgVEN* and *BgGUA* transcriptomes were detailed in terms of reads, contig length, contig number and nucleotide numbers. *BgBRE1* and *BgBRE2* transcriptomes came from 2 independent biological replicates.

(XLSX)

Acknowledgments

Authors acknowledge the use of the *B. glabrata* genome draft (genome assembly version *BglaB1*) in the Vectorbase database.

Author Contributions

Conceptualization: RG ER CGr AT BG GM.

Data curation: CC CGr.

Formal analysis: RG ER CGe ED CC BG.

Funding acquisition: BG GM.

Investigation: RG ER YM DD AP SP CC CGe ED AR NA BD.

Methodology: GR ER AR AT BG GM.

Project administration: RG ER BG GM.

Resources: AR NA BD.

Software: AP SP CC CGe ED CGr.

Supervision: AT BG GM.

Validation: RG ER YM BG GM.

Visualization: RG ER AP SP BG GM.

Writing – original draft: RG ER BG GM.

Writing – review & editing: RG ER YM DD AP SP CGr AT BG GM.

References

1. King CH (2010) Parasites and poverty: the case of schistosomiasis. *Acta Trop* 113: 95–104. doi: [10.1016/j.actatropica.2009.11.012](https://doi.org/10.1016/j.actatropica.2009.11.012) PMID: [19962954](https://pubmed.ncbi.nlm.nih.gov/19962954/)
2. King CH, Dickman K, Tisch DJ (2005) Reassessment of the cost of chronic helminthic infection: a meta-analysis of disability-related outcomes in endemic schistosomiasis. *Lancet* 365: 1561–1569. doi: [10.1016/S0140-6736\(05\)66457-4](https://doi.org/10.1016/S0140-6736(05)66457-4) PMID: [15866310](https://pubmed.ncbi.nlm.nih.gov/15866310/)
3. Doenhoff MJ, Hagan P, Cioli D, Southgate V, Pica-Mattoccia L, et al. (2009) Praziquantel: its use in control of schistosomiasis in sub-Saharan Africa and current research needs. *Parasitology* 136: 1825–1835. doi: [10.1017/S0031182009000493](https://doi.org/10.1017/S0031182009000493) PMID: [19281637](https://pubmed.ncbi.nlm.nih.gov/19281637/)
4. Fallon PG, Doenhoff MJ (1994) Drug-resistant schistosomiasis: resistance to praziquantel and oxamniquine induced in *Schistosoma mansoni* in mice is drug specific. *Am J Trop Med Hyg* 51: 83–88. PMID: [8059919](https://pubmed.ncbi.nlm.nih.gov/8059919/)
5. Melman SD, Steinauer ML, Cunningham C, Kubatko LS, Mwangi IN, et al. (2009) Reduced susceptibility to praziquantel among naturally occurring Kenyan isolates of *Schistosoma mansoni*. *PLoS Negl Trop Dis* 3: e504. doi: [10.1371/journal.pntd.0000504](https://doi.org/10.1371/journal.pntd.0000504) PMID: [19688043](https://pubmed.ncbi.nlm.nih.gov/19688043/)
6. Richards CS, Knight M, Lewis FA (1992) Genetics of *Biomphalaria glabrata* and its effect on the outcome of *Schistosoma mansoni* infection. *Parasitol Today* 8: 171–174. PMID: [15463608](https://pubmed.ncbi.nlm.nih.gov/15463608/)
7. Richards CS, Merritt JW Jr. (1972) Genetic factors in the susceptibility of juvenile *Biomphalaria glabrata* to *Schistosoma mansoni* infection. *Am J Trop Med Hyg* 21: 425–434. PMID: [5050093](https://pubmed.ncbi.nlm.nih.gov/5050093/)
8. Blouin MS, Bonner KM, Cooper B, Amarasinghe V, O'Donnell RP, et al. (2013) Three genes involved in the oxidative burst are closely linked in the genome of the snail, *Biomphalaria glabrata*. *Int J Parasitol* 43: 51–55. doi: [10.1016/j.ijpara.2012.10.020](https://doi.org/10.1016/j.ijpara.2012.10.020) PMID: [23207063](https://pubmed.ncbi.nlm.nih.gov/23207063/)
9. Bonner KM, Bayne CJ, Larson MK, Blouin MS (2012) Effects of Cu/Zn superoxide dismutase (*sod1*) genotype and genetic background on growth, reproduction and defense in *Biomphalaria glabrata*. *PLoS Negl Trop Dis* 6: e1701. doi: [10.1371/journal.pntd.0001701](https://doi.org/10.1371/journal.pntd.0001701) PMID: [22724037](https://pubmed.ncbi.nlm.nih.gov/22724037/)
10. Goodall CP, Bender RC, Brooks JK, Bayne CJ (2006) *Biomphalaria glabrata* cytosolic copper/zinc superoxide dismutase (*SOD1*) gene: association of *SOD1* alleles with resistance/susceptibility to *Schistosoma mansoni*. *Mol Biochem Parasitol* 147: 207–210. doi: [10.1016/j.molbiopara.2006.02.009](https://doi.org/10.1016/j.molbiopara.2006.02.009) PMID: [16564582](https://pubmed.ncbi.nlm.nih.gov/16564582/)
11. Tennesen JA, Theron A, Marine M, Yeh JY, Rognon A, et al. (2015) Hyperdiverse gene cluster in snail host conveys resistance to human schistosome parasites. *PLoS Genet* 11: e1005067. doi: [10.1371/journal.pgen.1005067](https://doi.org/10.1371/journal.pgen.1005067) PMID: [25775214](https://pubmed.ncbi.nlm.nih.gov/25775214/)
12. Ittiprasert W, Miller A, Myers J, Nene V, El-Sayed NM, et al. (2010) Identification of immediate response genes dominantly expressed in juvenile resistant and susceptible *Biomphalaria glabrata* snails upon exposure to *Schistosoma mansoni*. *Mol Biochem Parasitol* 169: 27–39. doi: [10.1016/j.molbiopara.2009.09.009](https://doi.org/10.1016/j.molbiopara.2009.09.009) PMID: [19815034](https://pubmed.ncbi.nlm.nih.gov/19815034/)
13. Lockyer AE, Noble LR, Rollinson D, Jones CS (2004) *Schistosoma mansoni*: resistant specific infection-induced gene expression in *Biomphalaria glabrata* identified by fluorescent-based differential display. *Exp Parasitol* 107: 97–104. doi: [10.1016/j.exppara.2004.04.004](https://doi.org/10.1016/j.exppara.2004.04.004) PMID: [15208043](https://pubmed.ncbi.nlm.nih.gov/15208043/)

14. Lockyer AE, Spinks J, Noble LR, Rollinson D, Jones CS (2007) Identification of genes involved in interactions between *Biomphalaria glabrata* and *Schistosoma mansoni* by suppression subtractive hybridization. *Mol Biochem Parasitol* 151: 18–27. doi: [10.1016/j.molbiopara.2006.09.009](https://doi.org/10.1016/j.molbiopara.2006.09.009) PMID: [17081633](https://pubmed.ncbi.nlm.nih.gov/17081633/)
15. Schneider O, Zelck UE (2001) Differential display analysis of hemocytes from schistosome-resistant and schistosome-susceptible intermediate hosts. *Parasitol Res* 87: 489–491. PMID: [11411951](https://pubmed.ncbi.nlm.nih.gov/11411951/)
16. Lockyer AE, Emery AM, Kane RA, Walker AJ, Mayer CD, et al. (2013) Early differential gene expression in haemocytes from resistant and susceptible *Biomphalaria glabrata* strains in response to *Schistosoma mansoni*. *PLoS One* 7: e51102.
17. Coustau C, Gourbal B, Duval D, Yoshino TP, Adema CM, et al. (2015) Advances in gastropod immunity from the study of the interaction between the snail *Biomphalaria glabrata* and its parasites: A review of research progress over the last decade. *Fish Shellfish Immunol* 46: 5–16. doi: [10.1016/j.fsi.2015.01.036](https://doi.org/10.1016/j.fsi.2015.01.036) PMID: [25662712](https://pubmed.ncbi.nlm.nih.gov/25662712/)
18. Roger E, Mitta G, Mone Y, Bouchut A, Rognon A, et al. (2008) Molecular determinants of compatibility polymorphism in the *Biomphalaria glabrata*/*Schistosoma mansoni* model: New candidates identified by a global comparative proteomics approach. *Mol Biochem Parasitol* 157: 205–216. doi: [10.1016/j.molbiopara.2007.11.003](https://doi.org/10.1016/j.molbiopara.2007.11.003) PMID: [18083248](https://pubmed.ncbi.nlm.nih.gov/18083248/)
19. Roger E, Gourbal B, Grunau C, Pierce RJ, Galinier R, et al. (2008) Expression analysis of highly polymorphic mucin proteins (Sm PoMuc) from the parasite *Schistosoma mansoni*. *Mol Biochem Parasitol* 157: 217–227. doi: [10.1016/j.molbiopara.2007.11.015](https://doi.org/10.1016/j.molbiopara.2007.11.015) PMID: [18187213](https://pubmed.ncbi.nlm.nih.gov/18187213/)
20. Roger E, Grunau C, Pierce RJ, Hirai H, Gourbal B, et al. (2008) Controlled Chaos of Polymorphic Mucins in a Metazoan Parasite (*Schistosoma mansoni*) Interacting with Its Invertebrate Host (*Biomphalaria glabrata*). *PLoS Negl Trop Dis* 2: e330. doi: [10.1371/journal.pntd.0000330](https://doi.org/10.1371/journal.pntd.0000330) PMID: [19002242](https://pubmed.ncbi.nlm.nih.gov/19002242/)
21. Fneich S, Theron A, Cosseau C, Rognon A, Aliaga B, et al. (2016) Epigenetic origin of adaptive phenotypic variants in the human blood fluke *Schistosoma mansoni*. *Epigenetics Chromatin* 9: 27. doi: [10.1186/s13072-016-0076-2](https://doi.org/10.1186/s13072-016-0076-2) PMID: [27379173](https://pubmed.ncbi.nlm.nih.gov/27379173/)
22. Perrin C, Lepesant JM, Roger E, Duval D, Fneich S, et al. (2013) *Schistosoma mansoni* mucin gene (SmPoMuc) expression: epigenetic control to shape adaptation to a new host. *PLoS Pathog* 9: e1003571. doi: [10.1371/journal.ppat.1003571](https://doi.org/10.1371/journal.ppat.1003571) PMID: [24009504](https://pubmed.ncbi.nlm.nih.gov/24009504/)
23. Roquis D, Rognon A, Chaparro C, Boissier J, Arancibia N, et al. (2016) Frequency and mitotic heritability of epimutations in *Schistosoma mansoni*. *Mol Ecol* 25: 1741–1758. doi: [10.1111/mec.13555](https://doi.org/10.1111/mec.13555) PMID: [26826554](https://pubmed.ncbi.nlm.nih.gov/26826554/)
24. Mone Y, Gourbal B, Duval D, Du Pasquier L, Kieffer-Jaquinod S, et al. (2010) A large repertoire of parasite epitopes matched by a large repertoire of host immune receptors in an invertebrate host/parasite model. *PLoS Negl Trop Dis* 4.
25. Adema CM, Hertel LA, Miller RD, Loker ES (1997) A family of fibrinogen-related proteins that precipitates parasite-derived molecules is produced by an invertebrate after infection. *Proc Natl Acad Sci U S A* 94: 8691–8696. PMID: [9238039](https://pubmed.ncbi.nlm.nih.gov/9238039/)
26. Zhang SM, Adema CM, Kepler TB, Loker ES (2004) Diversification of Ig superfamily genes in an invertebrate. *Science* 305: 251–254. doi: [10.1126/science.1088069](https://doi.org/10.1126/science.1088069) PMID: [15247481](https://pubmed.ncbi.nlm.nih.gov/15247481/)
27. Hanington PC, Fors MA, Loker ES (2012) A somatically diversified defense factor, FREP3, is a determinant of snail resistance to schistosome infection. *PLoS Negl Trop Dis* 6: e1591. doi: [10.1371/journal.pntd.0001591](https://doi.org/10.1371/journal.pntd.0001591) PMID: [22479663](https://pubmed.ncbi.nlm.nih.gov/22479663/)
28. Goodall CP, Bender RC, Brooks JK, Bayne CJ (2006) *Biomphalaria glabrata* cytosolic copper/zinc superoxide dismutase (SOD1) gene: association of SOD1 alleles with resistance/susceptibility to *Schistosoma mansoni*. *Molecular and Biochemical Parasitology* 147: 207–210. doi: [10.1016/j.molbiopara.2006.02.009](https://doi.org/10.1016/j.molbiopara.2006.02.009) PMID: [16564582](https://pubmed.ncbi.nlm.nih.gov/16564582/)
29. Mone Y, Ribou AC, Cosseau C, Duval D, Theron A, et al. (2011) An example of molecular co-evolution: reactive oxygen species (ROS) and ROS scavenger levels in *Schistosoma mansoni*/*Biomphalaria glabrata* interactions. *Int J Parasitol* 41: 721–730. doi: [10.1016/j.ijpara.2011.01.007](https://doi.org/10.1016/j.ijpara.2011.01.007) PMID: [21329695](https://pubmed.ncbi.nlm.nih.gov/21329695/)
30. Hahn UK, Bender RC, Bayne CJ (2001) Involvement of nitric oxide in killing of *Schistosoma mansoni* sporocysts by hemocytes from resistant *Biomphalaria glabrata*. *Journal of Parasitology* 87: 778–785. doi: [10.1645/0022-3395\(2001\)087\[0778:IONOIK\]2.0.CO;2](https://doi.org/10.1645/0022-3395(2001)087[0778:IONOIK]2.0.CO;2) PMID: [11534641](https://pubmed.ncbi.nlm.nih.gov/11534641/)
31. Mourao MdM, Dinguirard N, Franco GR, Yoshino TP (2009) Role of the Endogenous Antioxidant System in the Protection of *Schistosoma mansoni* Primary Sporocysts against Exogenous Oxidative Stress. *PLoS Negl Trop Dis* 3: e550. doi: [10.1371/journal.pntd.0000550](https://doi.org/10.1371/journal.pntd.0000550) PMID: [19924224](https://pubmed.ncbi.nlm.nih.gov/19924224/)
32. Bender RC, Goodall CP, Blouin MS, Bayne CJ (2007) Variation in expression of *Biomphalaria glabrata* SOD1: a potential controlling factor in susceptibility/resistance to *Schistosoma mansoni*. *Dev Comp Immunol* 31: 874–878. doi: [10.1016/j.dci.2006.12.005](https://doi.org/10.1016/j.dci.2006.12.005) PMID: [17292470](https://pubmed.ncbi.nlm.nih.gov/17292470/)

33. Dheilly NM, Duval D, Mouahid G, Emans R, Allienne JF, et al. (2015) A family of variable immunoglobulin and lectin domain containing molecules in the snail *Biomphalaria glabrata*. *Dev Comp Immunol* 48: 234–243. doi: [10.1016/j.dci.2014.10.009](https://doi.org/10.1016/j.dci.2014.10.009) PMID: [25451302](https://pubmed.ncbi.nlm.nih.gov/25451302/)
34. Hathaway JJ, Adema CM, Stout BA, Mobarak CD, Loker ES (2010) Identification of protein components of egg masses indicates parental investment in immunoprotection of offspring by *Biomphalaria glabrata* (gastropoda, mollusca). *Dev Comp Immunol* 34: 425–435. doi: [10.1016/j.dci.2009.12.001](https://doi.org/10.1016/j.dci.2009.12.001) PMID: [19995576](https://pubmed.ncbi.nlm.nih.gov/19995576/)
35. Galinier R, Portela J, Mone Y, Allienne JF, Henri H, et al. (2013) Biomphalysin, a new beta pore-forming toxin involved in *Biomphalaria glabrata* immune defense against *Schistosoma mansoni*. *PLoS Pathog* 9: e1003216. doi: [10.1371/journal.ppat.1003216](https://doi.org/10.1371/journal.ppat.1003216) PMID: [23555242](https://pubmed.ncbi.nlm.nih.gov/23555242/)
36. Baron OL, van West P, Industri B, Ponchet M, Dubreuil G, et al. (2013) Parental transfer of the antimicrobial protein LBP/BPI protects *Biomphalaria glabrata* eggs against oomycete infections. *PLoS Pathog* 9: e1003792. doi: [10.1371/journal.ppat.1003792](https://doi.org/10.1371/journal.ppat.1003792) PMID: [24367257](https://pubmed.ncbi.nlm.nih.gov/24367257/)
37. Adema CM (2015) Fibrinogen-Related Proteins (FREPs) in Mollusks. *Results Probl Cell Differ* 57: 111–129. doi: [10.1007/978-3-319-20819-0_5](https://doi.org/10.1007/978-3-319-20819-0_5) PMID: [26537379](https://pubmed.ncbi.nlm.nih.gov/26537379/)
38. Mitta G, Adema CM, Gourbal B, Loker ES, Theron A (2012) Compatibility polymorphism in snail/schistosome interactions: From field to theory to molecular mechanisms. *Dev Comp Immunol* In Press.
39. Nuismer SL, Dybdahl MF (2016) Quantifying the coevolutionary potential of multistep immune defenses. *Evolution* 70: 282–295. doi: [10.1111/evo.12863](https://doi.org/10.1111/evo.12863) PMID: [26792644](https://pubmed.ncbi.nlm.nih.gov/26792644/)
40. Mitta G, Galinier R, Tisseyre P, Allienne JF, Girerd-Chambaz Y, et al. (2005) Gene discovery and expression analysis of immune-relevant genes from *Biomphalaria glabrata* hemocytes. *Dev Comp Immunol* 29: 393–407. doi: [10.1016/j.dci.2004.10.002](https://doi.org/10.1016/j.dci.2004.10.002) PMID: [15707661](https://pubmed.ncbi.nlm.nih.gov/15707661/)
41. Mitta G, Gourbal B, Grunau C, Knight M, Bridger JM, et al. (2016) The compatibility between *Biomphalaria* snails and *Schistosoma mansoni*: an increasingly complex puzzle. *Advances in parasitology* In press.
42. Bayne CJ, Hahn UK, Bender RC (2001) Mechanisms of molluscan host resistance and of parasite strategies for survival. *Parasitology* 123 Suppl: S159–167.
43. Theron A, Rognon A, Gourbal B, Mitta G (2014) Multi-parasite host susceptibility and multi-host parasite infectivity: a new approach of the *Biomphalaria glabrata*/*Schistosoma mansoni* compatibility polymorphism. *Infect Genet Evol* 26: 80–88. doi: [10.1016/j.meegid.2014.04.025](https://doi.org/10.1016/j.meegid.2014.04.025) PMID: [24837670](https://pubmed.ncbi.nlm.nih.gov/24837670/)
44. Pinaud S, Portela J, Duval D, Nowacki FC, Olive MA, et al. (2016) A Shift from Cellular to Humoral Responses Contributes to Innate Immune Memory in the Vector Snail *Biomphalaria glabrata*. *PLoS Pathog* 12: e1005361. doi: [10.1371/journal.ppat.1005361](https://doi.org/10.1371/journal.ppat.1005361) PMID: [26735307](https://pubmed.ncbi.nlm.nih.gov/26735307/)
45. Giardine B, Riemer C, Hardison RC, Burhans R, Elnitski L, et al. (2005) Galaxy: a platform for interactive large-scale genome analysis. *Genome Res* 15: 1451–1455. doi: [10.1101/gr.4086505](https://doi.org/10.1101/gr.4086505) PMID: [16169926](https://pubmed.ncbi.nlm.nih.gov/16169926/)
46. Bullard JH, Purdom E, Hansen KD, Dudoit S (2010) Evaluation of statistical methods for normalization and differential expression in mRNA-Seq experiments. *BMC Bioinformatics* 11: 94. doi: [10.1186/1471-2105-11-94](https://doi.org/10.1186/1471-2105-11-94) PMID: [20167110](https://pubmed.ncbi.nlm.nih.gov/20167110/)
47. Dillies MA, Rau A, Aubert J, Hennequet-Antier C, Jeanmougin M, et al. (2013) A comprehensive evaluation of normalization methods for Illumina high-throughput RNA sequencing data analysis. *Briefings in Bioinformatics* 14: 671–683. doi: [10.1093/bib/bbs046](https://doi.org/10.1093/bib/bbs046) PMID: [22988256](https://pubmed.ncbi.nlm.nih.gov/22988256/)
48. Love MI, Huber W, Anders S (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 15: 550. doi: [10.1186/s13059-014-0550-8](https://doi.org/10.1186/s13059-014-0550-8) PMID: [25516281](https://pubmed.ncbi.nlm.nih.gov/25516281/)
49. Eisen MB, Spellman PT, Brown PO, Botstein D (1998) Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci U S A* 95: 14863–14868. PMID: [9843981](https://pubmed.ncbi.nlm.nih.gov/9843981/)
50. Supek F, Bosnjak M, Skunca N, Smuc T (2011) REVIGO summarizes and visualizes long lists of gene ontology terms. *PLoS One* 6: e21800. doi: [10.1371/journal.pone.0021800](https://doi.org/10.1371/journal.pone.0021800) PMID: [21789182](https://pubmed.ncbi.nlm.nih.gov/21789182/)

3. L'impact de l'adaptation locale sur l'interaction immunologique entre *Biomphalaria glabrata* et *Schistosoma*

Publication : Towards an integrative view of local adaptation theory in host/parasite systems.

PORTET Anaïs, PINAUD Silvain, CHAPARRO Cristian, GALINIER Richard, CHARRIÈRE Guillaume M., ALLIENNE Jean-François, DUVAL David and GOURBAL Benjamin

*Article re-soumis dans Molecular Biology and Evolution après premier retour des reviewers

Towards an integrative view of local adaptation in host/parasite systems

Journal:	<i>Molecular Biology and Evolution</i>
Manuscript ID	Draft
Manuscript Type:	Article
Date Submitted by the Author:	n/a
Complete List of Authors:	<p>Portet, Anaïs; Interaction Hôtes Pathogènes Environnements UMR 5244, Univ. Montpellier, Univ. Perpignan Via Domitia, CNRS, IFREMER</p> <p>Pinaud, Silvain; Interaction Hôtes Pathogènes Environnements UMR 5244, Univ. Montpellier, Univ. Perpignan Via Domitia, CNRS, IFREMER</p> <p>Chaparro, Cristian; Université de Perpignan via Domitia, Interaction Hôtes Pathogènes Environnements UMR 5244, Univ. Montpellier, Univ. Perpignan Via Domitia, CNRS, IFREMER</p> <p>Galinier, Richard; Interaction Hôtes Pathogènes Environnements UMR 5244, Univ. Montpellier, Univ. Perpignan Via Domitia, CNRS, IFREMER</p> <p>Charrière, Guillaume; Université de Montpellier, Interaction Hôtes Pathogènes Environnements UMR 5244, Univ. Montpellier, Univ. Perpignan Via Domitia, CNRS, IFREMER</p> <p>Allienne, Jean-François; Université Perpignan, Interaction Hôtes Pathogènes Environnements UMR 5244, Univ. Montpellier, Univ. Perpignan Via Domitia, CNRS, IFREMER</p> <p>Duval, David; BETM, Interaction Hôtes Pathogènes Environnements UMR 5244, Univ. Montpellier, Univ. Perpignan Via Domitia, CNRS, IFREMER</p> <p>Gourbal, benjamin; IHPE UMR 5244 CNRS UPVD,</p>
Key Words:	local adaptation, host-parasite interaction, transcriptomic, immunity

ARTICLE

Towards an integrative view of local adaptation in host/parasite systems

Anaïs PORTET, Silvain PINAUD, Cristian CHAPARRO, Richard GALINIER, Guillaume M. CHARRIERE, Jean-François ALLIENNE, David DUVAL, Benjamin GOURBAL[§]

Univ. Perpignan Via Domitia, Interactions Hôtes Pathogènes Environnements UMR 5244, CNRS, IFREMER, Univ. Montpellier, F-66860 Perpignan, France

[§] corresponding author:

Benjamin Gourbal

UMR5244 IHPE, Université de Perpignan

58 avenue Paul Alduy, 66860 Perpignan

benjamin.gourbal@univ-perp.fr

1
2
3 **Abstract:**

4 In the interaction between schistosomes and their vector snails, *Biomphalaria sp.*, co-evolution is
5 illustrated by a polymorphism of compatibility. Most of the time, infection phenotype varies from
6 compatible in sympatry to incompatible in allopatry, as expected by the “host-parasite local adaptation
7 hypothesis” that predicts sympatric interactions to perform better than allopatric ones. However,
8 similar prevalence/intensity phenotypes could be observed between sympatric and allopatric
9 combinations, in these cases would it be concluded to the absence of local adaptation?
10

11 To answer this intriguing question, we used an integrative approach from the molecular mechanisms
12 to population phenotypes. Dual-comparative approaches were used to investigate the molecular
13 mechanisms of sympatric and allopatric interactions, all displaying similar compatible phenotypes.
14 Snail-RNAseq analyses revealed that infection triggered an immuno-suppression in sympatry, and the
15 activation of immunity in allopatry. This difference in immune cellular response activation between
16 sympatric and allopatric combinations was confirmed by hemocyte microscopy and flow cytometry. In
17 addition, parasite-RNAseq analyses revealed that the sympatric parasite mounts an efficient infection
18 program resulting in such host immuno-suppression. We demonstrate the ability for the sympatric
19 parasite to hijack host immune response using miRNAs. Therefore, the effect of host immune
20 response on parasites was thus reduced in sympatric interaction resulting in the early and efficient
21 initiation of developmental program for sympatric parasites.
22

23 Using sympatric and allopatric interactions, we were able to demonstrate that the “host-parasite local
24 adaptation hypothesis”, which has often been questioned for prevalence or infectivity phenotypes, can
25 hold true at the molecular level in snail/schistosome interactions.
26
27

28
29
30 **Key Words:** local adaptation, host-parasite interaction, transcriptomic, immunity
31
32

33
34
35
36
37
38
39 **Introduction:**

40 High selective pressures occurred in host-parasite systems driving the evolution or adaptation of their
41 life history traits (Minchella 1985; Howard 1991). Thus, host/parasite interactions represent good
42 models for investigating evolutionary concepts or theories.
43

44 Schistosomiasis is the second most widespread human parasitic disease after malaria and affects over
45 200 million people worldwide (WHO 2010). *Schistosoma mansoni* (Platyhelminthes, Lophotrochozoa)
46 causes intestinal schistosomiasis. As is true for all trematode species, a schistosome needs a mollusc to
47 act as its first intermediate host, where it undergoes part of its life cycle before infecting humans.
48 Patently infected snails support the continuous production of hundreds of generations of cercariae, the
49 free-living human-infective stage of the parasite. Snail-parasite immunobiological interactions and the
50 molecular mechanisms by which snails and schistosomes interact represent major driving forces of
51 their reciprocal evolution. *Biomphalaria / Schistosoma* compatibility is based on a complex
52 phenotype-to-phenotype or matching-phenotype model (Basch 1976; Theron, et al. 1997; Mitta, et al.
53
54
55
56
57
58
59
60

1
2
3 74 2012; Theron, et al. 2014). The success or failure of the infection of *B. glabrata* by *S. mansoni* reflects
4 75 a complex interplay between the host's defense mechanisms and the parasite's infective strategies. In
5 76 the past 15 years, the molecular basis of this compatibility polymorphism has been investigated at the
6 77 genomic (Goodall, et al. 2006; Bonner, et al. 2012; Tennessen, et al. 2015), transcriptomic (Lockyer,
7 78 et al. 2004; Lockyer, et al. 2007; Roger, Grunau, et al. 2008; Ittiprasert, et al. 2010; Lockyer, et al.
8 79 2012; Galinier, et al. 2017), proteomic (Hahn, et al. 2001; Bender, et al. 2007; Myers, et al. 2008;
9 80 Roger, Mitta, et al. 2008; Mone, et al. 2010; Galinier, et al. 2013; Larson, et al. 2014; Pila, et al. 2016)
10 81 and epigenomic levels (Ittiprasert, et al. 2009; Ittiprasert and Knight 2012; Perrin, et al. 2012; Bridger,
11 82 et al. 2014; Knight, et al. 2015; Nelson, et al. 2016). These studies have revealed that various
12 83 molecules and pathways involved in immune recognition (snail immune receptors versus parasite
13 84 antigens), immune effector/anti-effector systems, and immune regulation/activation participate in a
14 85 complex interplay that governs the (mis)match of host and parasite phenotypes (Portet, et al. 2017).
15 86 This complex phenotype-by-phenotype interaction (compatibility polymorphism) varies between
16 87 populations and individuals resulting in a "multi-parasite susceptibility" or "multi-host infectivity"
17 88 phenotypes (Theron, et al. 2014) that reflect between-population variations in parasite
18 89 infectivity/virulence and host defense/resistance (Tack, et al. 2012; Osnas, et al. 2015). This variation
19 90 may be adaptive, suggesting that there may be local adaptation between host and parasite (Thrall, et al.
20 91 2002; Laine 2005; Greischar and Koskella 2007; Koskella 2014; Penczykowski, et al. 2015). In *B.*
21 92 *glabrata/Schistosoma sp.* models numerous sympatric and allopatric combinations have been tested;
22 93 and the results have shown that: (i) different *B. glabrata* laboratory strains (or isolates) show various
23 94 degrees of susceptibility to *S. mansoni* infection and (ii) different strains of *S. mansoni* display
24 95 different levels of infectivity towards a particular strain of snail host (Richards 1975; Basch 1976;
25 96 Theron, et al. 1997; Prugnolle, et al. 2006; Theron, et al. 2014). The general assumption is that the
26 97 parasites, which have a shorter generation time, larger population sizes and higher reproductive
27 98 outputs, are ahead in this co-evolutionary conflict and are therefore more likely to locally adapt to
28 99 their hosts (Frank 1996; Kawecki and Ebert 2004). This is exactly what is stated by the "host-parasite
29 100 local adaptation hypothesis", in which the sympatric parasite is supposed to perform better than the
30 101 allopatric one (Lively 1989; Ebert 1994; Kawecki and Ebert 2004; Kalbe, et al. 2016). Local
31 102 adaptation in *B. glabrata/Schistosoma* models has been investigated by comparing mostly two cases:
32 103 the sympatric/compatible and the allopatric/incompatible host-parasite association. However sometime
33 104 infectivity phenotypes were the same comparing sympatric and allopatric combinations, and thus they
34 105 did not fulfill the "local versus foreign" criterion of the "host-parasite local adaptation hypothesis"
35 106 (Morand, et al. 1996; Osnas and Lively 2004; Prugnolle, et al. 2006). Such discrepancy could be due
36 107 to the scale of analyses and/or the proxies used to evaluate the compatibility phenotypes. Indeed,
37 108 compatibility phenotypes have been investigated mostly in terms of parasite infectivity or
38 109 pathogenicity toward snail hosts, but to our knowledge very few studies have investigated the
39 110 molecular basis of local adaptation in host/parasite interactions (Mone, et al. 2010; Mone, et al. 2011;
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 111 Robertson, et al. 2017). Moreover, the outcome of a host-parasite encounter depends on the host and
4 112 parasite's genotype/phenotype matching; such interactions have mainly been examined using
5 113 theoretical models (Kamiya, et al. 2016; Nuismer and Dybdahl 2016), and few studies have tested
6 114 both sides of the interaction at the molecular level (Mone, et al. 2011; Penczykowski, et al. 2015).
7
8 115 Hence, in order to bridge these gaps, we herein conducted an integrative approach for studying
9 116 sympatric/allopatric-local adaptation interactions using dual-comparative approaches at the molecular
10 117 level on both host snails and parasite schistosomes.

11 118 In the present study we set out to analyze the compatibility between South American snail strains
12 119 originating from Recife Brazil or from Guacara Venezuela in combination with three different parasite
13 120 isolates that displayed nearly the same infectivity status (almost same prevalence and intensity of
14 121 infection): (i) *S. mansoni* Recife Brazil, (ii) *S. mansoni* Guacara Venezuela (both sympatric and
15 122 allopatric, narrow geographic scale), and (iii) *S. rodhaini* from Burundi Africa (allopatric, large
16 123 geographic and phylogenetic scales). Thus even if compatibility phenotypes (prevalence/intensity) are
17 124 similar, the characterization of the underlying molecular mechanisms of local adaptation demonstrates
18 125 a totally different immunobiological dialogue between *B. glabrata* vector snails and their sympatric or
19 126 allopatric schistosome parasites at the cellular and molecular scales.
20
21
22
23
24
25
26
27

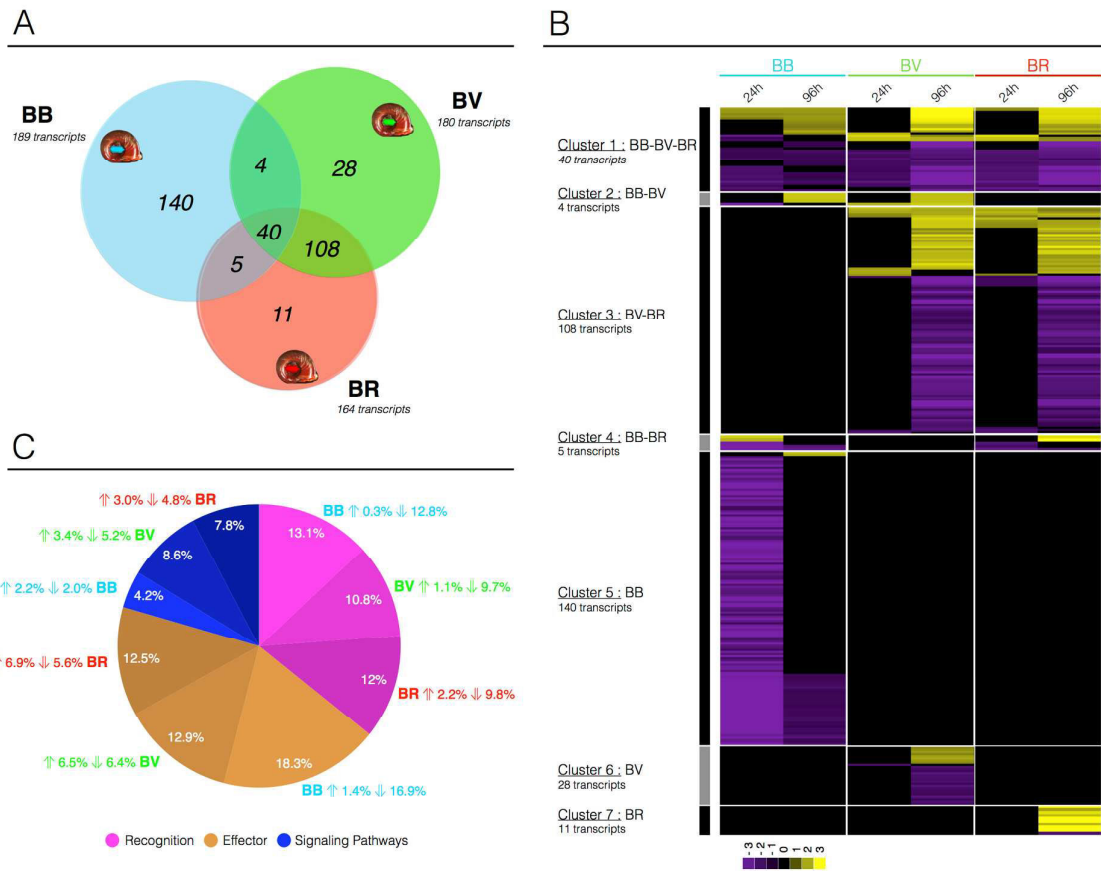
28 29 **Results**

30 **A whole-snail transcriptomic approach for investigating the molecular basis of the innate** 31 **immune response in the sympatric and allopatric contexts** 32

33 131 To the best of our knowledge very few studies have investigated the molecular basis of local
34 132 adaptation in host/parasite interactions (Robertson, et al. 2017). Moreover, the previous studies were
35 133 conducted on a limited set of candidate genes using targeted approaches (i.e. quantitative PCR). Here,
36 134 we used a powerful global approach to identify the differentially regulated transcripts in snails of a
37 135 single *B. glabrata* strain under infection by three sympatric or allopatric compatible strains or species
38 136 of the parasite, *Schistosoma* sp. The global transcript representation was analyzed using the previously
39 137 described RNAseq pipeline developed in our laboratory (Dheilly, et al. 2015; Pinaud, et al. 2016;
40 138 Galinier, et al. 2017). The *BgBRE* snail host had the same prevalence and intensity phenotypes when
41 139 exposed to the *SmBRE*, *SmVEN* or *Srod* parasites (see Materials and Methods section). To compare
42 140 the host responses during the sympatric or allopatric interactions, we used the DEseq2 software to
43 141 analyse the response of duplicate *BgBRE* control strains (uninfected *BgBRE1* and *BgBRE2*) to the
44 142 sympatric and allopatric conditions. Of the 159,711 transcripts of the *BgBRE* transcriptome, 1,895
45 143 (1.2%) were differentially represented in all sympatric and allopatric conditions compare to naive
46 144 snails (table 1, supplementary fig. S1). We performed automatic Blast2GO annotation and discard the
47 145 non-annotated transcripts, and retained 1,017 annotated transcripts (53.7% of the differentially
48 146 represented transcripts). Finally, as we were interested in the innate immune response of the snail to
49 147 the sympatric and allopatric parasites, we focused on transcripts known to have immune-related
50
51
52
53
54
55
56
57
58
59
60

148 functions. Of the annotated differentially expressed transcripts, 336 were identified as being immune-
 149 related; this subset corresponded to 17.7% of all differentially represented transcripts. The Heatmap
 150 generated for all differentially represented transcripts was presented in supplementary fig. S1.

151



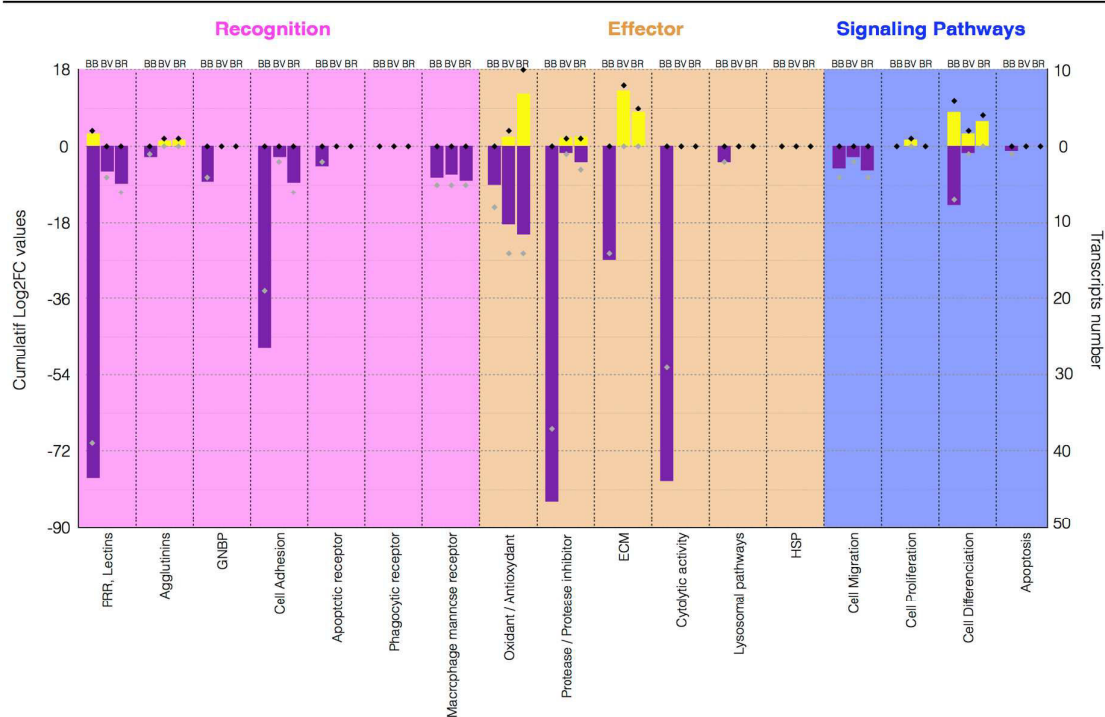
152
 153 *Fig. 1: Dual-RNAseq of Biomphalaria immune-related transcripts*

154
 155 Venn diagrams were used to highlight the relationships of the transcripts that were differentially
 156 represented in the *B. glabrata* and *Schistosoma sp.* interactions. Overall, 189, 180, and 164 transcripts
 157 were differentially represented in the BB (BgBRE/SmBRE, sympatric), BV (BgBRE/SmVEN,
 158 allopatric), and BR (BgBRE/Srod, allopatric) interactions, respectively (fig. 1A). Of the selected
 159 transcripts, 40 were shared by the BB, BV and BR interactions (fig. 1A), and displayed globally the
 160 same differentially expressed pattern of expression (fig. 1B, cluster 1). These transcripts may
 161 correspond to the snail's basal immune response against *Schistosoma* parasites.

162 Of the 140 transcripts exclusive to the BB interaction, which represented 74.1% of the immune
 163 transcripts found to be differentially represented in this interaction (fig. 1A), 100% were under-
 164 represented at 24 h post-infection, and 74.6% of these transcripts were observed exclusively at this
 165 time point (fig. 1B, cluster 5).

1
2
3 166 Very similar transcript expression patterns were observed for the two allopatric interactions (BV and
4 167 BR). Clusters 6 and 7, comprising only 28 and 11 transcripts, were exclusive to BV and BR,
5
6 168 respectively. In BV interaction (fig. 1B, cluster 6), at 96 h after infection 96.5% of the transcripts were
7
8 169 expressed: 22% over-represented. In BR interaction (fig. 1B, cluster 7), 100% of the transcripts were
9
10 170 expressed at 96 h after infection: 82% over-represented. Of the 108 transcripts shared by BV and BR
11
12 171 (cluster 3, representing 60% and 65.9 % of the transcripts, respectively) (fig. 1A; fig. 1B, cluster 3),
13
14 172 98.1% were expressed at 96 h post-infection: 28.2% over-represented (fig. 1B, cluster 3).
15
16 173 Altogether, these results show that the host immune-related transcriptomic response differed widely
17
18 174 between the sympatric and allopatric interactions. While most of the selected transcripts were
19
20 175 differentially represented (all under-expressed) at 24 h after infection in sympatry, the majority of
21
22 176 differentially represented transcripts (one third over-expressed) were seen at 96 h in allopatry.
23
24 177 Moreover, the sympatric interaction was associated with a strong immunosuppression (fig. 1B, cluster
25
26 178 5), whereas allopatry yielded an activation of the immune response (fig. 1B, cluster 3).
27
28 179 We next questioned whether the transcripts that exhibited different regulation between the sympatric
29
30 180 and allopatric interactions might represent different functional categories. To test this, we realized an
31
32 181 automatic annotation with Blast2GO (supplementary table S2) and we distributed the relevant
33
34 182 differentially expressed immune transcripts into the groups of immune recognition molecules, immune
35
36 183 effectors, and immune signaling molecules (fig. 1C), and then subdivided these groups into functional
37
38 184 categories (fig. 2). In the BB sympatric interaction, immune recognition molecules, immune effectors
39
40 185 and immune signalling pathways correspond to 13.1%, 18.3% and 4.2% respectively (sums of all
41
42 186 relevant transcripts at all infection time points). Among the transcripts distributed into these groups,
43
44 187 89.0% were under-represented. In the BV allopatric interaction, the three groups corresponded
45
46 188 respectively to 10.8%, 12.9% and 8.6% and among them 65.9% were under-represented. Finally, in
47
48 189 the BR allopatric interaction, they correspond to 12%, 12.5% and 7.8% and 62.5% of them were
49
50 190 under-represented. When we compared the percentage of each immunological group in the sympatric
51
52 191 and allopatric interactions, no specific subset was particularly repressed in the BB sympatric
53
54 192 interaction (fig. 1C; fig. 2). In fact, different immune transcripts (grey and black diamond in fig. 2)
55
56 193 belonging to the same immune functions showed similar differentially regulations following sympatric
57
58 194 and allopatric infections (fig. 2).
59
60 195
196

A



B

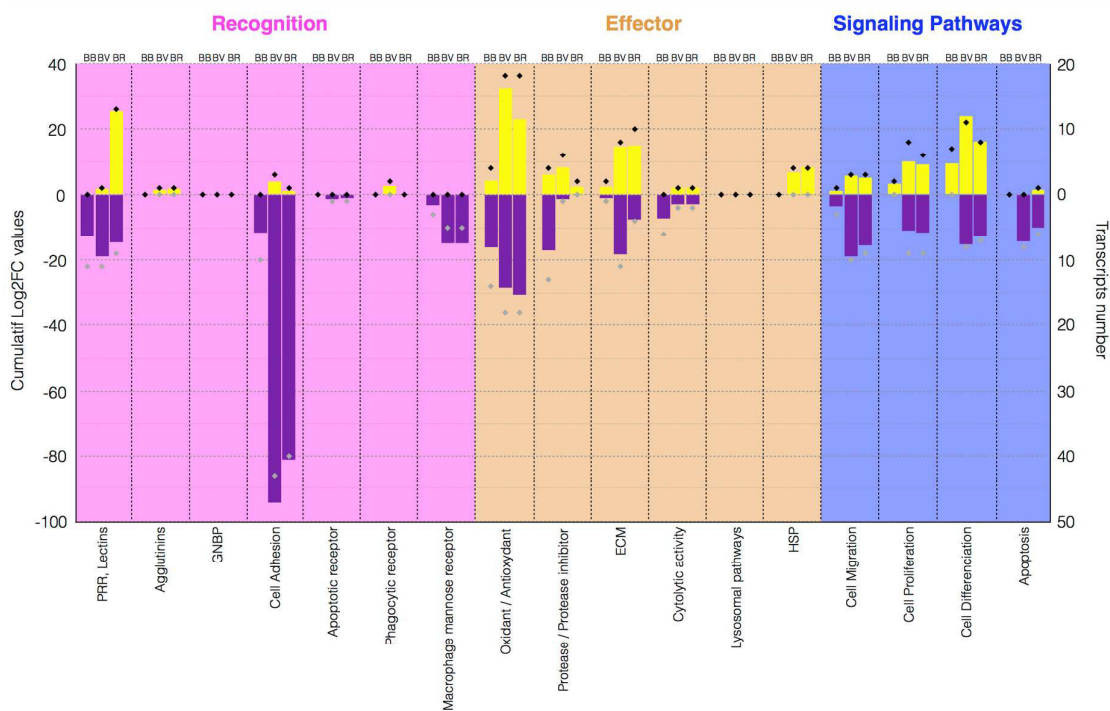


Fig. 2: Differentially represented immune-related transcripts in sympatric and allopatric interactions.

The differentially regulated transcripts belonging to the three immunological groups (fig. 2) were largely involved in immune cellular responses, cell adhesion, extra cellular matrix component, cell

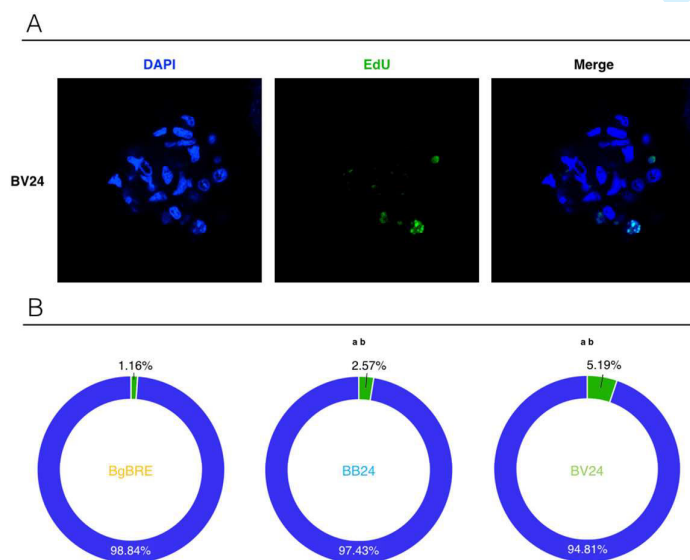
202 migration, cell differentiation and cell proliferation. All of these functions were under-represented in
 203 sympatric interaction (76%), whereas many were over-represented in allopatric ones (39%) (fig. 2).

204

205 **Immune cellular responses in the sympatric and allopatric contexts**

206 As the transcriptomic approach indicated that multiple immunobiological functions seems to be
 207 altered in the host response during the interaction with the parasites, we next investigated whether
 208 some differences in immune responses in sympatric versus allopatric infections could be seen at the
 209 cellular level. Hemocytes are the snail immune cells that participated directly in the immune response
 210 against the parasites, and immune cells activation under an immunological challenge can translate into
 211 cell proliferation and/or cell morphology modifications.

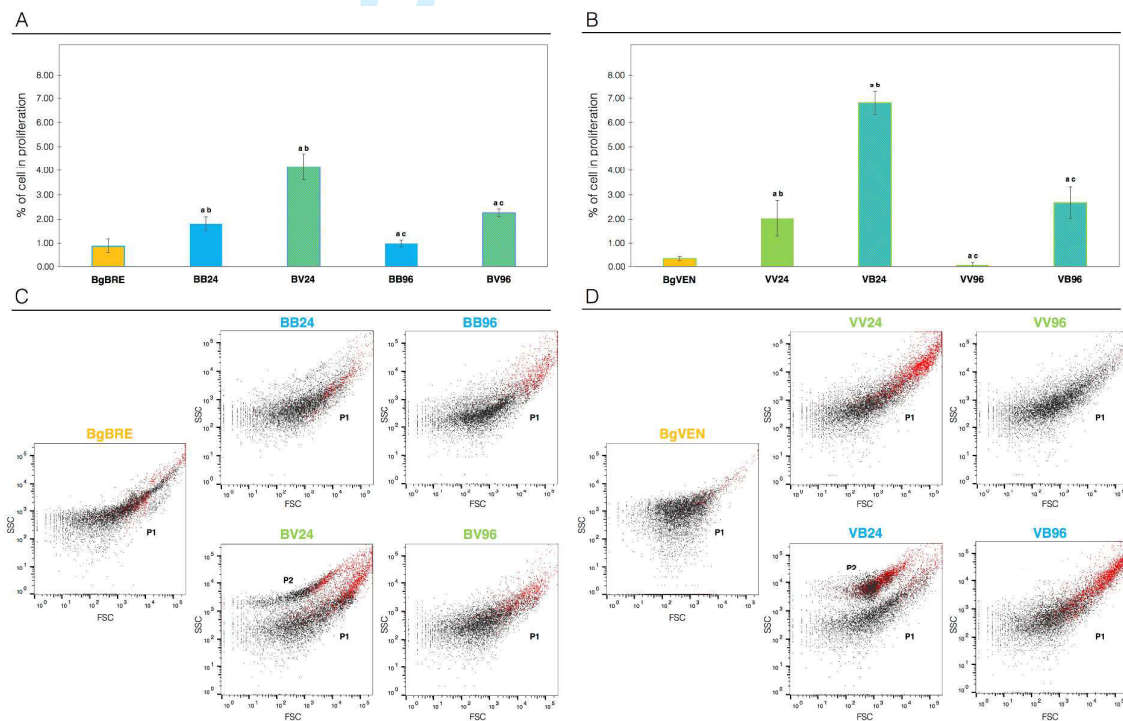
212 Thus to investigate such immune cell activation, cell proliferation was quantified using EdU nuclear
 213 labelling (fig. 3). The EdU is a nucleoside analogue of thymine incorporated into DNA during DNA
 214 synthesis. This incorporation reflects the mitotic activity of hemocytes. As the two tested allopatric
 215 interactions appeared extremely similar at the transcriptomic level, we compared only the BV
 216 (allopatric) interaction and BB (sympatric) interaction for further analyses. Circulating hemocytes
 217 recovered in snail hemolymph were EdU-labelled *in-vitro* to identify mitotic circulating hemocytes
 218 (fig. 3A). Edu-positive hemocytes were quantified using confocal microscopy examination and
 219 showed that hemocyte proliferation was induced by 2 fold following *Schistosoma* infection in BV
 220 compared to BB (fig. 3B). Twenty-four hours after infection, proliferative circulating cells increase
 221 significantly in BV, with 5.2% of proliferative cells compared to BB with 2.6% (Fisher exact test two-
 222 tailed $p = 7.6 \times 10^{-6}$) (fig. 3B). Moreover, this result demonstrates for the first time that “circulating”
 223 hemocytes are able to proliferate following *Schistosoma* infections.



224 Fig. 3: Microscopic investigation of snail hemocyte proliferations

225 Hemocyte proliferation was assessed using flow cytometry after *in-vivo* EdU-labelling (fig. 4A). At
 226 24h after infection, the rate of proliferating cells was increased significantly (Mann Whitney U test:
 227

228 U=36; $z = -2.8$; $p = 0.0022$) by more than two fold between the allopatric and sympatric interactions,
 229 4.2% compared to 1.8% respectively. At 96 h after infection, there were fewer proliferating cells: the
 230 percentage of proliferating hemocytes was similar in sympatric BB interaction compared to the control
 231 (1%, Mann Whitney U test: $U=17$; $z=-0.27$; $p = 0.3936$), while remaining somewhat higher in
 232 allopatric BV interaction (2.3%, Mann Whitney U test: $U=0$; $z=2.65$; $p = 0.009$).
 233 To confirm that our observations were related to the type of interaction rather than to a strain effect,
 234 we performed the same experiments using BgVEN as the host (fig. 4B). The rate of proliferating
 235 hemocytes increased significantly (Mann Whitney U test: $U=36$; $z=-2.8$; $p = 0.0022$) by more than 3
 236 fold between the allopatric (VB: BgVEN/SmBRE) and the sympatric (VV: BgVEN/SmVEN)
 237 interactions at 24 h after infection (6.8% compared to 2.0% respectively). At 96 h after infection, both
 238 rates were decreased, albeit more for VV (0.1%, Mann Whitney U test: $U=2$; $z=2.48$; $p = 0.013$) than
 239 for VB (2.7%, Mann Whitney U test: $U=36$; $z=2.8$; $p = 0.0022$). These results similar to the one
 240 observed for BgBRE interactions confirmed that the effects observed at the cellular level were related
 241 to the type of interactions (sympatric versus allopatric) but not to the host or parasite strains used.



242 *Fig. 4: Flow cytometric investigation of the hemocyte response in sympatric and allopatric interactions*

245 In addition to the mitotic activity, hemocyte morphology was estimated using flow cytometry by
 246 measuring their size and granularity. We analysed the circulating hemocyte populations in non-
 247 infected versus infected snails in two *B. glabrata* snail strains (BgBRE and BgVEN) in sympatric and
 248 allopatric interactions with parasites (fig. 4C and 4D). Morphology and heterogeneity of circulating
 249 hemocytes varied in a similar manner between BgBRE and BgVEN snails subjected to infection (fig.
 250 4C and 4D) and we observed infection-related differences in cell morphology. In non-infected snails,

the content of circulating hemocytes was very heterogeneous, but appeared to represent a single population that exhibited the continuous gradient of size and granularity (fig. 4C and 4D), which is typical of *B. glabrata* hemocytes (Baeza Garcia, et al. 2010). However, hemocyte population's heterogeneity changed quickly after infection. In allopatric interactions, 24 h after infection (fig. 4C, BV24, and 4D, VB24) two populations could be distinguished: a population P1 (corresponding to that seen in non-infected snails) and a population P2 (a new population). P2 cells exhibited increased granularity, retained a high degree of size variability, and showed a mitotic activity, as indicated by EdU labeling (fig. 4C and 4D, red dots). This profile was transitory, with the P2 population disappearing by 96 h after infection (fig. 4C, BV96, and 4D, VB96). Altogether, these results show that, upon infection, the snail circulating immune cells exhibit a particular population dynamics with transient increased of the mitotic activity associated with morphology modifications that are likely to be involved in the host defense response against the parasite during interactions. Moreover, this response of the immune cells appears to be exacerbated in allopatric interactions when compared with sympatric interaction.

Schistosoma growth and development in *Biomphalaria* tissues

Parasite development

To investigate the development of *S. mansoni* in *B. glabrata* tissues, we examined the fate of sporocyst into sympatric and allopatric compatible interactions using a histological approach. For both interactions, miracidia were able to penetrate, transform into primary sporocysts (SpI), and develop. At 24 h after infection, we observed a significant difference (Mann Whitney U test: $U=40$; $z=4.33$; $p = 1.42 \times 10^{-6}$) in the size of sporocyst from sympatric parasites ($11,838 \mu\text{m}^2$ average size) versus allopatric parasites ($7,402 \mu\text{m}^2$ average size) (fig. 5). This difference was no longer seen at 96 h after infection ($41,413 \mu\text{m}^2$ and $36,920 \mu\text{m}^2$, respectively, Mann Whitney U test: $U=280$; $z=-1.31$; $p = 0.1917$) (fig. 5). These results show that during the early events following infection, the parasites develop more slowly in allopatry compared to sympatry; thereafter, however, the allopatric parasites catch up quickly, such that no difference is seen at 96 h post-infection (fig. 5).

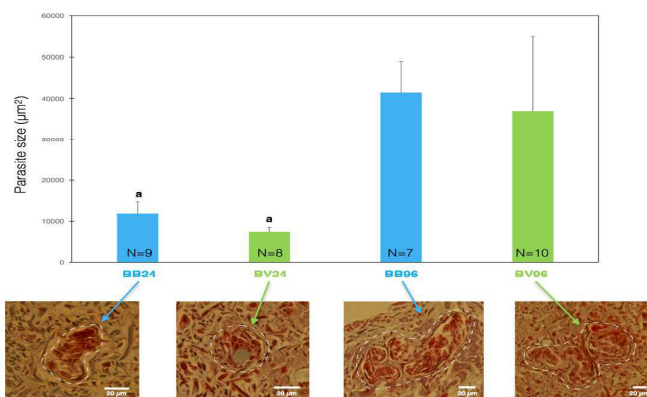
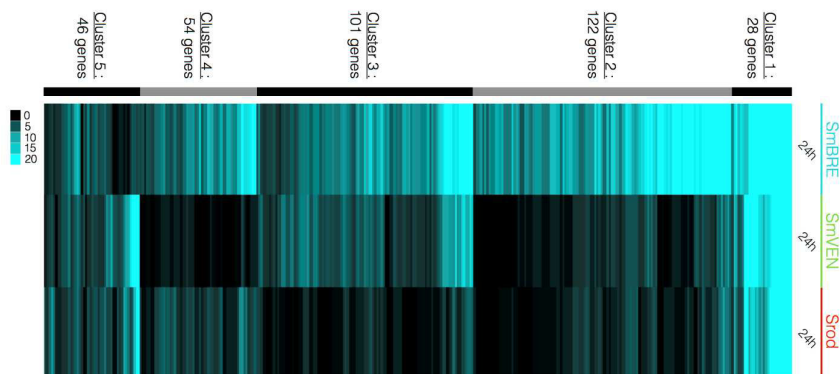


Fig. 5: Development of parasites into snail tissues

280 *Parasite transcript expression analysis*

281 In order to investigate the biological responses of the parasites within their host, dual RNAseq
 282 analyses allowed us to identify regulated transcripts from *Schistosoma* parasites during their intra-
 283 molluscal development. The parasite-RNAseq data at 24 h after infection, revealed five clusters of
 284 differentially expressed genes from the sympatric (SmbRE) and the allopatric (SmVEN, Srod)
 285 parasite responses (fig. 6). The cluster 1 corresponds to genes highly expressed and cluster 5 weakly
 286 expressed for all parasites strains. The cluster 2 represents genes over-expressed in SmbRE versus
 287 SmVEN and Srod. Clusters 3 contained genes over-expressed in SmbRE and SmVEN versus Srod
 288 and cluster 4 SmBRE and Srod versus SmVEN. In all clusters, the gene expression levels in SmbRE
 289 sympatric parasite are always greater than in the other allopatric parasites. We used automatic
 290 Blast2GO annotation to characterize 70% of the 351 genes present in the five clusters (supplementary
 291 table S3). According to the global Gene Ontology (GO): 70% of the annotated genes were involved in
 292 the general metabolism and growth, the translation processes, regulation of cellular processes and
 293 RNA biosynthesis; 25% were involved in molecular transports or cell organization; and 5% were
 294 involved in organism defense or response to stimuli. In all these clusters, we identified 6 parasite gene
 295 products that could be involved in parasite modulation or suppression of snail immunity;
 296 corresponding to heat shock proteins (fig. 6, clusters 1 and 2) (Knight, et al. 2015); glutathione-S-
 297 transferase, NADH dehydrogenase subunit, and calreticulin (fig. 6, cluster 2) (Guillou, et al. 2007;
 298 Mone, et al. 2010; Raghavan, et al. 2013); Alpha-2-macroglobulin (fig. 6, cluster 4) (Ryu and Park
 299 2009); von willebrand factor type EGF with pentraxin domain (fig. 6, cluster 5) (Gilges, et al. 2000).
 300 These particular genes could be involved in the strategy used by the parasite to ensure its development
 301 and growth within host tissues.

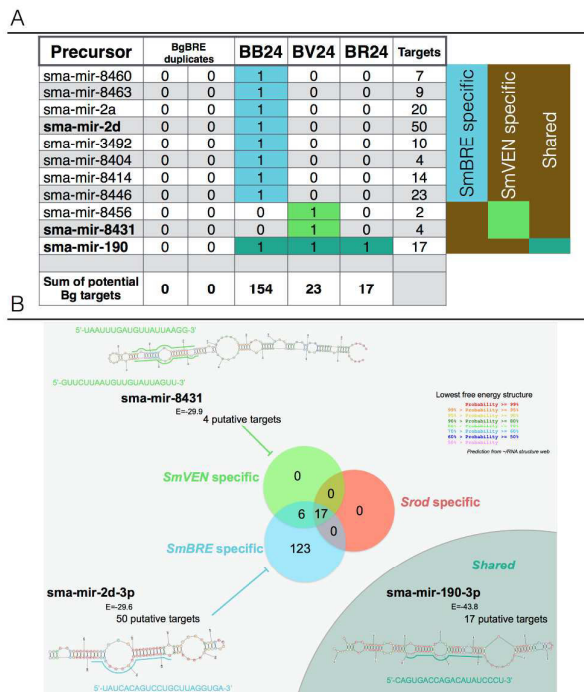


310 Fig. 6: Clustering of intra-molluscal *Schistosoma* expression patterns

312 *Parasite microRNAs analysis*

313 The microRNAs (miRNAs) are known as non-coding small RNA (<24nt) able to regulate gene
 314 expressions. As we identified herein deep modification of transcriptional or post-transcriptional
 315 activities between sympatric and allopatric interactions, we investigated, *in-silico*, the potential
 316 presence of *Schistosoma mansoni* miRNAs in our parasite-RNAseq data. At 24 h post-infections, 54

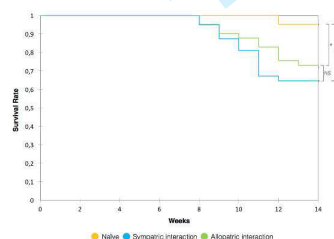
miRNA precursors from miRBase were identified by high quality alignment scores with the different RNAseq read libraries (naïve BgBRE, BB24, BV24, BR24). To avoid cross-species misidentification, we selected precursors that were exclusively identified in infected samples and never identified in uninfected snails (naive BgBRE). Eleven miRNA precursors corresponding to *Schistosoma mansoni* were identified (fig. 7A). Eight of the parasite miRNA precursors were specific to the Brazil-infected libraries (BB24); two were specific of the Venezuela-infected libraries (BV24); and one was shared across the three infected conditions (BB24, BV24 and BR24). Although, we identify 49 miRNA precursor sequences specific to *S. mansoni* (fig. 7B), unfortunately, we obtained solely 11 read alignments (100% nucleotidic similarity) that allowed to predict the mature miRNA form (eg. sma-mir-2d-3p, sma-mir-190-3p) or only the precursor forms (sma-mir-8431). Then, in order to identify candidate sequences that could represent putative miRNA targets, we used the Miranda tool (supplementary table S4). Only RNA-RNA interactions that showed good scores for pairing (>140) and enthalpy (<-15 Kcal) were considered. The identified miRNAs were found to target anywhere from 2 targets for sma-mir-8456, to 50 targets for sma-mir-2d of the differentially expressed immune-related transcripts identified in Fig. 1. The miRNAs identified under the sympatric conditions (SmBRE) were predicted to specifically target 43.5% of the differentially represented immune-related transcripts (fig. 1B, fig. 7) whereas 6.8% and 5.1% were targeted in allopatric conditions, SmVEN and Srod, respectively (figure 1B, fig. 7).). Altogether, these data suggested that only during sympatric interaction, the parasite may hijacks the host immune response at gene regulation level (from transcriptional, post-transcriptional, translational and protein stability) by producing dedicated miRNAs.



340

341 **Survival of snail following infection**

342 To examine the potential impact of allopatric or sympatric parasites on snail survival, we investigated
 343 the mortality rates of infected snails over 4 months. The survival rate was significantly higher for non-
 344 infected snails compared to infected snails (sympatric interaction Kaplan-Meier Log Rank test $p =$
 345 1.39×10^{-5} and allopatric interaction $p = 0.0005$). However, there was no significant difference in the
 346 mortality rates of snails subjected to sympatric versus allopatric interactions: at the end of the
 347 experiment, the survival rates were 72% and 65% for the allopatric and sympatric interactions,
 348 respectively (Kaplan-Meier Log Rank test $p = 0.243$) (supplementary fig. S2).



349

350 *Supplementary fig. S2: Mortalities of B. glabrata snails after infections*

351

352 **DISCUSSION:**

353 In the natural environment, it is assumed that the parasitic genes responsible for infectivity will evolve
 354 alongside the host defense genes, resulting in adaptation of the interactions between local host and
 355 parasite populations (Dybdahl and Storfer 2003; Osnas and Lively 2005). In this context,
 356 local/sympatric parasites were expected to display greater infectiveness, reproductive success, and
 357 virulence in host populations compared to foreign/allopatric parasites (Minchella 1985; Lively 1989;
 358 Ebert 1994, 1998). However, in some cases this rule may be contradicted, as certain allopatric
 359 parasite-host interactions have been reported to be significantly more compatibles than the
 360 corresponding sympatric combinations (Morand, et al. 1996; Greischar and Koskella 2007).

361 To our knowledge, most of the previous studies on local adaptation in host/parasite systems focused
 362 on parasite infectivity (prevalence, intensity) and/or pathogenicity (severity of infection, host
 363 survival/mortality, host reproductive success) (Ebert 1994, 1998). We propose that it would be
 364 important to develop integrative analysis of local adaptation by paying a particular attention to the
 365 underlying molecular mechanisms (immuno-biological interactions) as well as investigating the
 366 infectivity or pathogenicity phenotypes to depict a unified theory of local adaptation. Local adaptation
 367 has often been investigated using either the “home vs. away host strategy”, wherein researchers
 368 compared the performance of a sympatric or allopatric host confronted with a single parasite
 369 population, or the “local vs. foreign parasite strategy”, wherein researchers compare the performance
 370 of a sympatric or allopatric parasite against a single host population (Greischar and Koskella 2007).

1
2
3 371 However, global and integrative approaches to distinguish the main host- and parasite related effects
4 372 remain scarce.

5
6 373 Using field data, Morand et al. (1996) and Prugnolle et al. (2006) showed that although sympatric
7 374 parasite-host combinations of schistosomes and snails do tend to be more compatible, as expected by
8
9 375 the “host-parasite local adaptation hypothesis”(Minchella 1985; Lively 1989; Ebert 1994, 1998), there
10 376 are exceptions wherein particular allopatric combinations are equally or significantly more
11 377 compatibles. Similar results were obtained for the interactions of Brazilian and Guadeloupien snails
12 378 versus schistosomes (Mone, et al. 2011). The authors found that allopatric Guadeloupien parasites
13 379 were not able to infect Brazilian snails; but Brazilian parasites were able to infect the allopatric
14 380 Guadeloupien snails. Furthermore, this work demonstrated the presence of local adaptation between
15 381 reactive oxygen species (ROS) and ROS scavengers in this system (Mone, et al. 2011). As some
16 382 allopatric parasite-host populations may be equally or more compatibles than the corresponding
17 383 sympatric combinations, it appears that certain *Biomphalaria* /*Schistosoma* interactions do not fulfill
18 384 the “host-parasite local adaptation hypothesis”, in which the sympatric parasite is expected to perform
19 385 better than the allopatric one.

20 386 Those data highlight the necessity to adopt a broader view of local adaptation, using integrative
21 387 approaches from the molecule to population phenotypes. Increasing the number of proxies used to
22 388 characterize local adaptation processes would be particularly relevant. One of these proxies would be
23 389 the molecular cross talk or immunological dialogue between the host and the parasite. Thus dual-
24 390 comparative approaches were used herein to simultaneously analyse the responses of *Biomphalaria*
25 391 snails toward sympatric or allopatric strains of *Schistosoma* displaying similar compatibilities.

26 392 Our RNAseq analysis demonstrated that the sympatric interaction (BB) triggered a huge
27 393 immunosuppression reaction, twenty-four hours after the infection, that concerned three
28 394 immunological processes: (i) immune recognition, (ii) effector and (iii) signalling pathways (fig. 1 and
29 395 2). Conversely, for the allopatric interactions (BV and BR), the same immunological processes were
30 396 mostly over-represented indicating that the host immune response was activated at 96 h post-infection
31 397 (fig. 1 and 2). Most of the differentially regulated transcripts were associated with immune cellular
32 398 responses, particularly cell activation, migration, proliferation, or differentiation (fig. 2). A EdU
33 399 labelling was used to detect proliferation and to confirm that more hemocyte proliferation was
34 400 observed in allopatry compared to sympatry (fig. 3, 4A and 4B). The flow cytometry size scatter and
35 401 granularity scatter analyses demonstrated that a new cell subpopulation (named P2) was observed
36 402 exclusively following allopatric infection at 24 h (fig. 4C and 4D); P2 was characterized by an
37 403 increased granularity and EdU-positive cells, indicating that the new P2 cell subtype could proliferate.
38 404 Thereafter, global hemocyte proliferation decreased more rapidly in sympatric rather than in allopatric
39 405 interactions (fig. 3 and 4).

40 406 To confirm that the observed sympatric versus allopatric differences were not associated with a
41 407 specific host or parasite strains, we conducted the same experiments using BgVEN snails as the host.

1
2
3 408 The results obtained from infecting BgVEN with its sympatric (SmVEN, VV) and allopatric (SmBRE,
4 409 VB) parasites were consistent with those described above (fig. 4B and 4D): hemocyte proliferation
5 410 was higher in allopatry and the P2 population was observed exclusively in the VB interaction at 24 h
6 411 after infection. These results confirmed that the observed sympatric and allopatric phenotypes
7 412 reflected an overall process that did not depend on the strain of snail host or parasite used.

8
9 413 The strong immunosuppression observed during the sympatric interaction versus the immune cell
10 414 activation of allopatry would certainly results in differences in the capabilities of sympatric and
11 415 allopatric parasites to infect and grow in such hosts. This hypothesis was confirmed by our
12 416 histological analysis of sporocysts development in snail tissues. We observed a significant difference
13 417 in sporocyst size 24 h after infection (fig. 5), when sympatric sporocysts were one-third bigger than
14 418 allopatric sporocysts. At 96 h after infection, in contrast, there was no size difference between
15 419 sympatric and allopatric parasites (fig. 5), indicating that the allopatric parasites seem apparently to
16 420 overcome this early delay in development. However, the consequences of this delay for duration of
17 421 cercariae development, number of cercariae produced, cercariae infectivity and pathogenicity in
18 422 vertebrate host, will deserve further investigations to conclude about a potential fitness cost between
19 423 sympatric and allopatric parasites.

20
21 424 We next used a dual-RNAseq approach to investigate transcript expression of the parasite by its intra-
22 425 molluscal stage (fig. 6). As the histological differences were solely observed at 24h after infection, we
23 426 used dual-RNAseq to investigate the parasite expression patterns at the same time point of infection.
24 427 Most of the identified infection-related parasite transcripts belonged to the processes of nucleotide
25 428 metabolism, transcription and translation and cell differentiation, development, and growth. We also
26 429 identified some transcripts with GO terms or functions related to immuno-modulation or immuno-
27 430 suppression (fig. 6 and supplementary table S4). Nearly all of the total identified transcripts were over-
28 431 represented in the sympatric interaction compared to the allopatric ones. Our results therefore suggest
29 432 that the installation, development and growth of the parasite occurred much more rapidly in the
30 433 sympatric combination perhaps because the parasite interfered efficiently with the host immune
31 434 system.

32
33 435 We next examined the generated dual-RNAseq libraries in an effort to identify whether sympatric
34 436 and/or allopatric Schistosomes could hijack the host immune system. We confronted the dual-RNAseq
35 437 data to the *Schistosoma mansoni* subset of miRBase to identify the presence of microRNAs (miRNAs)
36 438 in our datasets. At 24 h after infection, several parasite miRNAs were identified and differentially
37 439 expressed. As sRNAs and miRNAs are known to regulate numerous biological processes, including
38 440 key immune response genes (Li, et al. 2017; Zawada, et al. 2017), we investigated the role played by
39 441 these miRNAs on host immunobiological response to intruders. We identified some predicted targets
40 442 of such schistosome miRNAs in the *Biomphalaria* immune reference transcriptome. We found that
41 443 schistosomes miRNAs identified in the snails 24 h after sympatric infection may target 43.5% of the
42 444 differentially regulated immune transcripts (fig. 7). In contrast, far fewer correspondences were

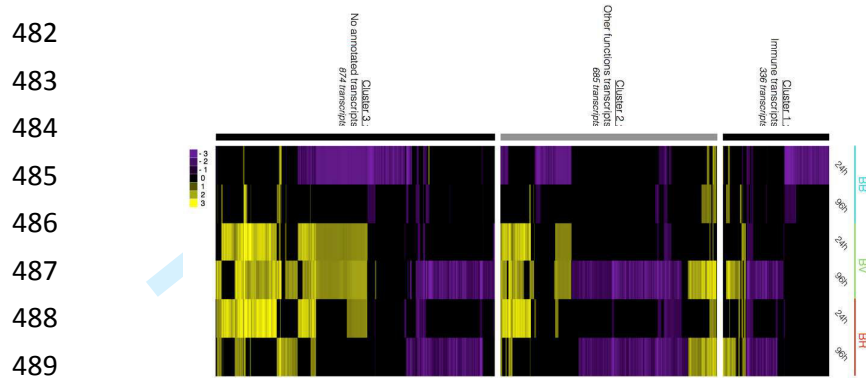
1
2
3 445 identified for the allopatric interaction (fig. 7). Recent work has demonstrated that circulating small
4 446 non-coding RNAs from parasites have hijack roles against host metabolism, especially in the
5
6 447 interaction of schistosomes with their vertebrate hosts (Nowacki, et al. 2015; Meninger, et al. 2017;
7
8 448 Walzer and Chi 2017). Such non-coding RNAs could act as exogenous miRNAs to interfere with or
9
10 449 circumvent the host immune system. These results and our findings show that parasite miRNAs might
11
12 450 help subvert the snail's immune defenses by targeting immune weapons or directly targeting genes
13
14 451 that regulate innate cellular response.. Furthermore, our observation that more host immune genes
15
16 452 appear to be more targeted in the sympatric combination (fig. 7) demonstrates that miRNAs may act as
17
18 453 potential support of local adaptation in the transcriptional regulation of a sympatric interaction
19
20 454 between host and parasite. This hypothesis deserves further investigation; small RNA (<50pb)
21
22 455 sequencing approach is actually on going to confirm the role of non-coding RNAs in host-parasite
23
24 456 local adaptation processes.

25
26 457 Compatibility reflects the outcome of complex immunobiological interactions and depends on:
27
28 458 (i) the ability of the snail immune system to recognize and kill the parasite; and (ii) the ability of the
29
30 459 parasite to circumvent or evade the host immune response (Van Der Knaap and Loker 1990; Guillou,
31
32 460 et al. 2007; Mone, et al. 2010).

33
34 461 Based on our present observations, we propose that sympatric and allopatric interactions trigger totally
35
36 462 different responses. In the sympatric interaction, the parasite is able to induce a host
37
38 463 immunosuppression within the first day of infection enabling it to quickly infect the host and readily
39
40 464 begins to develop. This very efficient strategy reflects a close match between the host and parasite,
41
42 465 originating from fine and complex local adaptation processes. In the allopatric interaction, the host's
43
44 466 immune system immediately recognizes the parasite and mounts a potent immune response. Although
45
46 467 the need to resist the immune system seems to disrupt the activation of the parasite's developmental
47
48 468 program during the first day of infection, however the allopatric parasite seems able to circumvent the
49
50 469 host immune response and develops in the snail tissues, such that no significant between-interaction
51
52 470 difference is seen at 96 h post-infection. Indeed, one major objective of the parasite is to block and/or
53
54 471 resist to the innate immune response of the host organism, however, this is not exclusive. Parasites
55
56 472 may also hijack more globally the host to their own benefit. Herein we demonstrate that the global
57
58 473 transcriptomic activity in sympatry was repressed whereas is not the case in allopatry (supplementary
59
60 474 fig. S1). The down-regulation of immunity in sympatry would thus appear as a collateral effect of the
475 overall parasite control of host's transcriptional and post-transcriptional programs.

476 Host tolerance would be also a possibility to explain the limited sympatric host immune response.
477 Tolerate the parasite would be an interesting host strategy to avoid host damages and costs induced by
478 the deleterious activity of the immune system. However, parasite immune-modulatory molecules and
479 miRNAs were identified in the parasite RNAseq data (fig.6 and 7, table S4), arguing for a direct
480 strategy of locally adapted parasites in this case.

481



Supplementary fig. S1: Clustering of all differential represented transcripts

Our results collectively suggest that the sympatric interaction exhibits a fine scale local adaptation within the first hours of interaction whereas the less well-adapted allopatric parasites seems to activate an early immune response that must be circumvented before they can develop in the snail host's tissues. Thereafter, in the medium- or long-term, there are no observable differences in the prevalence, intensity, or snail survival of sympatric and allopatric interactions (supplementary table S1, supplementary fig. S2). Our present work shows that analyses of specific differences in parasite infectivity and/or host susceptibility would benefit from being associated with molecular approaches to reveal local adaptation in snail/parasite interactions. If we hope to draw conclusions regarding the existence of local adaptation, we need to develop integrative approaches to explore fine-scale patterns of host-parasite interactions, studying the relevant factors at the relevant timing. We must consider the spatial scale at which comparisons are made, the patterns of disease occurrence, the population genetics, the physical barriers believed to reduce gene flow across the landscape, (Penczykowski, et al. 2015), and the involvements of physiological, immunological, and molecular processes.

When possible, researchers should also analyse the evolutionary history of the host/parasite association. Schistosomes originated in Asia, reached Africa 12 to 19 million years ago (MYA), and gave rise to all *Schistosoma* species known in Africa (Lockyer, et al. 2003). *S. mansoni* diverged from *S. rodhaini* around 2.8 MYA (Morgan, et al. 2005; Crellen, et al. 2016), and thereafter, 400 to 500 years ago, colonized South America since the slave trade of the 16th-19th Centuries (Lockyer, et al. 2003; Crellen, et al. 2016). This colonization of South America by *S. mansoni* was rendered possible by the presence of the snail host: *Biomphalaria glabrata*. All African species of *Biomphalaria* are monophyletic and seem to have originated from paraphyletic South American clades (Campbell, et al. 2000; DeJong, et al. 2001; Theron 2005). The ancestor of *B. glabrata* appears to have colonized Africa 1 to 5 MYA, giving rise to all to all 12 species of *Biomphalaria* known today in Africa (Morgan, et al. 2001). In South America and Caribbean Island, *S. mansoni* infects *B. glabrata*; in Africa, it infects mostly *B. pfeifferi* and *B. alexandrina*.

We found that South American *S. mansoni* parasites are highly compatibles with their sympatric South American snail hosts, whereas African *S. mansoni* parasites are incompatible (supplementary table

1
2
3 519 S1). This reflects that local adaptation has occurred in South America over the 400 to 500 years of
4 520 sympatric evolution. Interestingly, the South American parasites did not lose their compatibility for
5 521 African snail hosts (i.e., they performed equally as well as African parasites in African snails)
6 522 (supplementary table S1). This could be interpreted as an absence of local adaptation if we failed to
7 523 consider the recent African origin of the parasite, which have not yet diverged sufficiently in South
8 524 America to lose their compatibility for their original African snail hosts. These observations
9 525 highlighted that local adaptation is an evolutionary process that occurs over time. 500 years of co
10 526 evolution drive a very fine adaptation process at the molecular level in sympatry, but seems not
11 527 sufficient for parasites for having lost their compatibility with ancestral hosts.

12 528 Our work shows that it can be difficult to detect local adaptation in host-parasite interactions.
13 529 Such studies need to be integrative and carefully designed, and should yield a highly detailed
14 530 overview of the interaction, from the levels of population phenotypes to molecular processes. How
15 531 infectious agents divert or modify host metabolism and signalling/trafficking pathways to gain
16 532 selective advantages is the good question. This should allow us to conclude about the presence or
17 533 absence of local adaptation in host-parasite biological systems.

18 534

19 535 **Materials and Methods**

20 536 ***Ethical statements***

21 537 Our laboratory holds permit # A66040 for experiments on animals, which was obtained from the
22 538 French Ministry of Agriculture and Fisheries and the French Ministry of National Education,
23 539 Research, and Technology. The housing, breeding, and care of the utilized animals followed the
24 540 ethical requirements of our country. The experimenter possesses an official certificate for animal
25 541 experimentation from both of the above-listed French ministries (Decree # 87–848, October 19, 1987).
26 542 The various protocols used in this study have been approved by the French veterinary agency of the
27 543 DRAAF Languedoc-Roussillon (Direction Régionale de l'Alimentation, de l'Agriculture et de la
28 544 Forêt), Montpellier, France (authorization # 007083).

29 545

30 546 ***Biological materials***

31 547 Two snail strains of *Biomphalaria glabrata* were used in this study: the albino Brazilian strain,
32 548 (BgBRE) and the Venezuelan strain, (BgVEN). They were exposed to infection by a Brazilian
33 549 (SmBRE) or Venezuelan (SmVEN) strains of *Schistosoma mansoni*, or by another *Schistosoma*
34 550 species, *S. rodhaini* (Srod). All host and parasite strains of each different geographical origin were
35 551 recovered in the same locality and maintain in the laboratory always on their sympatric snail hosts.
36 552 The Brazilian strain originates from the locality of Recife, the Venezuelan strains of snail and parasite
37 553 were recovered from the locality of Guacara and African species, *Schistosoma rodhaini* originates
38 554 from Burundi. These *Schistosoma* isolates/species were selected because they exhibited similar
39 555 infectivities toward BgBRE or BgVEN strains (see prevalence and intensity in supplementary table

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

556 S1). Prevalence of SmBRE and SmVEN for the African vector snail *Biomphalaria pfeifferi* from
557 Senegal (BpSEN), and prevalence of the corresponding parasite SmSEN on South American snails
558 were also tested (supplementary table S1).

559

560 *RNAseq experimental protocol*

561 One hundred and twenty BgBRE snails were infected with SmBRE, SmVEN or Srod. Each snail was
562 individually exposed for 12 h to 10 miracidia in 5mL of pond water. For each experimental infection,
563 20 snails were recovered at 24 and 96 h after infection. The samples were named as follows: BB24,
564 BB96 for BgBRE infected with SmBRE; BV24, BV96 for BgBRE infected with SmVEN; and BR24,
565 BR96 for BgBRE infected with Srod. Two pools of 30 uninfected BgBRE snails (Bre1 and Bre2) were
566 used as controls.

567 - *Whole-snail RNA extraction and sequencing*

568 Total RNA was extracted using TRIZOL[®] (Sigma Life Science, USA) according to the
569 manufacturer's instructions. Sequencing was realized in paired-end 72-bp read lengths, using an
570 Illumina Genome Analyzer II (MGX-Montpellier GenomiX, Montpellier, France).

571 - *De novo transcriptome assembly*

572 *De novo* transcriptome assembly, using all time points, was performed using an in-house pipeline
573 created with the Velvet (1.2.02), Oases (v0.2.04), and CD-HIT-EST (v4.5.4) programs. The assembly
574 of consensus reference transcriptome was optimized using various parameters, including k-mer length,
575 insert length and expected coverage, as previously described (Dheilly, et al. 2015; Pinaud, et al. 2016).
576 A *de novo* transcriptome was created and contained 159,711 transcripts.

577 - *Differential expression analysis*

578 High-quality reads (Phred score >29) were aligned to the *de novo* transcriptome using Bowtie2
579 (v2.0.2), which was run locally on a Galaxy server. The DESeq2 (v2.12) software was used to identify
580 differential transcript representation between uninfected (Bre1 and Bre2) and infected conditions (p
581 value < 0.1) (Pinaud, et al. 2016). A Venn diagram was generated using the Venny 2.1 software to
582 highlight which differentially expressed transcripts were specific to a certain interaction versus shared
583 between them. A heatmap was then created to analyze transcript expression patterns, using log₂ Fold
584 Change with Hierarchical Ascending Clustering (HAC) and Pearson correlation (uncentered) as
585 applied by the Cluster (v3.0) and Java TreeView (v1.1.6r4) softwares packages. The differentially
586 represented transcripts were functionally classified using a BlastX analysis with the cutoff set to e-
587 value < 1e⁻³ (NCBI dataset; thanks to the Roscoff Datacenter Cluster, UPMC) and gene ontology was
588 assigned using Blast2GO (v3.0.8) (supplementary table S2).

589 *Innate immune cellular response analysis: microscopy and flow cytometry*

590 A total of 105 BgBRE and 90 BgVEN were used. Snails were infected as described above, using
591 either SmBRE or SmVEN parasites. For each experimental infection, snails were recovered at 24 and
592 96 h after infection, and designated as follows: BB24 and BB96 for BgBRE infected with SmBRE;

1
2
3 593 BV24 and BV96 for BgBRE infected with SmVEN; VV24 and VV96 for BgVEN infected with
4 594 SmVEN; and VB24 and VB96 for BgVEN infected with SmBRE. Snails of each strain (BgBRE and
5 595 BgVEN) were recovered and used as controls.

6 596 *- Hemocyte proliferation analysis: microscopy*

7 597 For our microscopic analysis, 12 infected BgBRE (6 BgBRExSmBRE and 6 BgBRExSmVEN) and 3
8 598 naive BgBRE are used. The hemocytes of 3 snails were counted by condition at 24h and 96h after
9 599 infection. The proliferation of circulating hemocytes was studied by using a Click-iT EdU Alexa Fluor
10 600 488 Flow Imaging Kit (Molecular Probes). At each time point, circulating hemocytes were recovered
11 601 by direct puncture after foot retraction and 1mM of EdU solution was added to the hemolymph. Three
12 602 hours later, the amount of EdU incorporated by the circulating hemocytes was visualized *in-vitro* after
13 603 fixation of the cells and performing a covalent coupling of Alexa Fluor 488 to the EdU residues through
14 604 a click chemistry reaction following manufacturer indications, then nuclei of hemocytes were
15 605 counterstained with DAPI (Biotum) staining, and the sample was subjected to confocal microscopy
16 606 using a Zeiss LSM 700, with 4 lasers (405, 488, 555 and 633 nm). Positive cells were counted and
17 607 between-sample differences in the percentage of proliferation were tested using a Fisher exact test,
18 608 with significance accepted at p value<0.05.

19 609 *- Hemocyte proliferations and population profiles analysis: flow cytometry*

20 610 For flow cytometry analysis, 72 infected BgBRE or BgVEN (36 infected by SmBRE and 36 infected
21 611 by SmVEN) and 18 naïve BgBRE or BgVEN are used. Six biological replicates (pools of 3 snails per
22 612 replicate) were realised for each conditions. Flow cytometry was used to profile and assess the
23 613 proliferation of circulating hemocytes using Click-iT EdU Alexa Fluor 647 labelling (Molecular
24 614 Probes). At each time point, 1mM of EdU solution was injected into pericardial cavity of each snail.
25 615 Three hours later six replicates of 3 snails were collected, and the hemolymph was extracted from the
26 616 head-foot according to standard procedures (Sminia and Barendsen 1980). The hemolymph was
27 617 pooled from the three snails, and 100 µl were subjected to analysis with the above-listed kit, according
28 618 to the manufacturer's instructions, the percentage of proliferative cells was calculated by flow
29 619 cytometry.

30 620 The hemocytes were profiled along the course of infection using Side Scatter Channel (SSC) to
31 621 estimate cell granularity and Forward Scatter Channel (FSC) to estimate cell size. The cell repartition
32 622 along these two parameters enables to identify cell sub-populations. The flow cytometry was
33 623 performed using a FACSCanto from BD Biosciences (RIO Imaging Platform, Montpellier, France).
34 624 For each sample, 10,000 events were counted. The results were analyzed with the FlowJo V 10.0.8
35 625 software. Between-group differences in the percent of proliferation were tested using the Mann-
36 626 Whitney U-test, with significance accepted at p value<0.05.

37 627

38 628 ***Histological procedures***

39 629 BgBRE snails (n=8 per group) were infected as described above with either SmBRE or SmVEN
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 630 parasites. For each experimental infection time point, at 24 and 96 h after infection, two snails were
4 631 fixed in Halmi's fixative (4.5% mercuric chloride, 0.5% sodium chloride, 2% trichloroacetic acid,
5 632 20% formol, 4% acetic acid and 10% picric acid-saturated aqueous solution). Embedding in paraffin
6 633 and transverse histological sections (3- μ m) were performed at the RHEM platform (Montpellier,
7 634 France). The slides were stained using Heidenhain's azan trichromatic staining solution as follows: (i)
8 635 serial re-hydration was performed in toluene followed by 95%, 70%, and 30% ethanol and then
9 636 distilled water; (ii) coloration was performed using azocarmine G (70% ethanol, 1% aniline, 1% acetic
10 637 alcohol, distilled water, 5% phosphotungstic acid, distilled water, Heidenhain's azan) and (iii) serial
11 638 dehydration was performed using 95% ethanol, absolute ethanol, and toluene. The preparations were
12 639 then mounted with Entellan (Sigma Life Science, St. Louis Missouri, USA) and subjected to
13 640 microscopic examination. The parasite sizes were measured with the ImageJ software (v2.0.0), and
14 641 between-group size differences were tested using the Mann-Whitney U-test with statistical
15 642 significance accepted at a p value < 0.05 based on the mean of the three largest serial cuts of each
16 643 sporocyst in sympatric or allopatric conditions.
17 644

25 645 ***Schistosoma intra-molluscal stage expression analysis***

26 646 - *Schistosoma* read subtractions

27 647 The *Biomphalaria* (v1) and *Schistosoma* (v5.2) genomes have been concatenated
28 648 (<https://www.vectorbase.org/organisms/biomphalaria-glabrata>;
29 649 <http://www.sanger.ac.uk/resources/downloads/helminths/schistosoma-mansoni.html>). High quality
30 650 reads (Phred score >29) were mapped against these concatenated genomes using Bowtie2 (v2.0.2),
31 651 which was run locally on the Galaxy project server. The reads that mapped only once and exclusively
32 652 to the *Schistosoma* genome were collected as corresponding to *Schistosoma* reads; reads that mapped
33 653 to the *Biomphalaria* genome or more than once to either genomes were excluded.

34 654 - *Gene analysis*

35 655 The above-selected *Schistosoma* reads were mapped against the concatenate genome to identify intra-
36 656 molluscal stage-specific *Schistosoma* genes. For selecting the relevant genes, the reads mapped in all
37 657 experimental conditions were summed. Solely genes with a minimal sum of 10 reads were kept for the
38 658 analysis. A heatmap was generated to analyse *Schistosoma* gene expression patterns using
39 659 Hierarchical Ascending Clustering (HAC) with Pearson correlation (uncentered) as applied by the
40 660 Cluster (v3.0) and Java TreeView (v1.1.6r4) software packages. Functional annotation of the genes
41 661 was assigned using BlastX with the cutoff set to e-value < $1e^{-3}$ (NCBI dataset, local cluster) and gene
42 662 ontology was performed using Blast2GO (v4.0.7) (supplementary table S3).
43 663

44 664 ***In silico characterization of Schistosoma miRNAs***

45 665 *S. mansoni* miRNAs were analyzed *in-silico* by comparing the relevant miRNA database (miRBase) to
46 666 our RNAseq libraries generated at the 24 h time point. *S. mansoni* precursor sequences were
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 667 downloaded from miRBase (<http://www.mirbase.org>, 03/09/2017), and high-quality reads from naive
4 668 (BgBRE) and 24 h post-infection samples (BB24, BV24, BR24) were aligned against a *S. mansoni*
5
6 669 sub-database of miRBase, as previously described (Zhao, et al. 2016). The identified precursors were
7
8 670 confirmed by alignment of high-scoring reads onto precursor and mature miRNAs from miRBase.
9 671 Solely reads with 100% identity were retained for analysis. The localization of each read against
10 672 miRNA sequence allowed us to identify either the precursor or just the mature miRNA. Precursors
11 673 found under both naive and infected conditions were excluded to avoid cross-species contamination.
12
13 674 Putative miRNA targets were predicted from among the differentially represented immune-related
14 675 transcripts (figure 1) using Miranda tools (`miranda input_miR input_Transcriptome -out results.txt -`
15 676 `quiet -sc 140 -en -15`) (Enright, et al. 2003). Because mature miRNAs may exist in two forms
16 677 depending on which strand (5'-3') of the precursor stem-loop is matured the predicted interactions
17 678 could involve the 5' and/or 3' forms, as noted. The results were extracted in line code using the awk
18 679 tool, compiled in supplementary files (supplementary table S4), and used to generate a Venn diagram.
19
20 680 To confirm the ability of a selected pre-miRNA to produce the stem-loop necessary to produce the
21 681 mature form, the secondary structures of precursor were predicted using RNAstructureWeb tool
22 682 (<http://rna.urmc.rochester.edu/RNAstructureWeb>, 03/09/2017) under default parameters.
23
24 683

25 684 *Snail survival analysis*

26 685 One hundred and sixty BgBRE snails were infected as described above with SmBRE or SmVEN
27 686 strains (n=50), and 60 non-infected BgBRE snails were retained as controls. The numbers of dead
28 687 snails were compiled weekly for 14 weeks. A Kaplan-Meier estimator was used to estimate the
29 688 survival function from lifetime data. Survival curves were generated using the xlstatsMac software and
30 689 the log-rank test was applied with significance accepted at $p < 0.05$.
31
32 690

33 691 *Acknowledgements and funding information:*

34 692 We thank Ms. Cécile Saint-Béat, Ms. Nathalie Arancibia, and Ms. Anne Rognon for their work and
35 693 diligence in helping generate some of the data described herein.

36 694 This work was supported by the French National Agency for Research (ANR) through a project
37 695 Invimory grant [ANR-13-JSV7-0009] to BG.
38
39 696
40 697

698 **References:**

- 699 Baeza Garcia A, Pierce RJ, Gourbal B, Werkmeister E, Colinet D, Reichhart JM, Dissous C,
700 Coustau C. 2010. Involvement of the cytokine MIF in the snail host immune response to the
701 parasite *Schistosoma mansoni*. *PLoS Pathog* 6:e1001115.
- 702 Basch PF. 1976. Intermediate host specificity in *Schistosoma mansoni*. *Exp Parasitol* 39:150-
703 169.
- 704 Bender RC, Goodall CP, Blouin MS, Bayne CJ. 2007. Variation in expression of *Biomphalaria*
705 *glabrata* SOD1: a potential controlling factor in susceptibility/resistance to *Schistosoma*
706 *mansoni*. *Dev Comp Immunol* 31:874-878.
- 707 Bonner KM, Bayne CJ, Larson MK, Blouin MS. 2012. Effects of Cu/Zn superoxide dismutase
708 (sod1) genotype and genetic background on growth, reproduction and defense in
709 *Biomphalaria glabrata*. *PLoS Negl Trop Dis* 6:e1701.
- 710 Bridger JM, Arican-Gotkas HD, Foster HA, Godwin LS, Harvey A, Kill IR, Knight M, Mehta IS,
711 Ahmed MH. 2014. The non-random repositioning of whole chromosomes and individual
712 gene loci in interphase nuclei and its relevance in disease, infection, aging, and cancer. *Adv*
713 *Exp Med Biol* 773:263-279.
- 714 Campbell G, Jones CS, Lockyer AE, Hughes S, Brown D, Noble LR, Rollinson D. 2000.
715 Molecular evidence supports an african affinity of the neotropical freshwater gastropod,
716 *Biomphalaria glabrata*, say 1818, an intermediate host for *Schistosoma mansoni*. *Proc Biol*
717 *Sci* 267:2351-2358.
- 718 Crellen T, Allan F, David S, Durrant C, Huckvale T, Holroyd N, Emery AM, Rollinson D,
719 Aanensen DM, Berriman M, et al. 2016. Whole genome resequencing of the human parasite
720 *Schistosoma mansoni* reveals population history and effects of selection. *Sci Rep* 6:20954.
- 721 DeJong RJ, Morgan JA, Paraense WL, Pointier JP, Amarista M, Ayeh-Kumi PF, Babiker A,
722 Barbosa CS, Bremond P, Pedro Canese A, et al. 2001. Evolutionary relationships and
723 biogeography of *Biomphalaria* (Gastropoda: Planorbidae) with implications regarding its role
724 as host of the human bloodfluke, *Schistosoma mansoni*. *Mol Biol Evol* 18:2225-2239.
- 725 Dheilly NM, Duval D, Mouahid G, Emans R, Allienne JF, Galinier R, Genthon C, Dubois E, Du
726 Pasquier L, Adema CM, et al. 2015. A family of variable immunoglobulin and lectin domain
727 containing molecules in the snail *Biomphalaria glabrata*. *Dev Comp Immunol* 48:234-243.
- 728 Dybdahl MF, Storfer A. 2003. Parasite local adaptation: Red Queen versus suicide king.
729 *TRENDS in Ecology and Evolution* 18:523-530.
- 730 Ebert D. 1998. Experimental evolution of parasites. *Science* 282:1432-1435.
- 731 Ebert D. 1994. Virulence and local adaptation of horizontally transmitted parasite. *Science*
732 265:1084-1086.
- 733 Enright AJ, John B, Gaul U, Tuschl T, Sander C, Marks DS. 2003. MicroRNA targets in
734 *Drosophila*. *Genome Biol* 5:R1.
- 735 Frank SA. 1996. Models of parasite virulence. *Q Rev Biol* 71:37-78.
- 736 Galinier R, Portela J, Mone Y, Allienne JF, Henri H, Delbecq S, Mitta G, Gourbal B, Duval D.
737 2013. Biomphalysin, a new beta pore-forming toxin involved in *Biomphalaria glabrata*
738 immune defense against *Schistosoma mansoni*. *PLoS Pathog* 9:e1003216.
- 739 Galinier R, Roger E, Mone Y, Duval D, Portet A, Pinaud S, Chaparro C, Grunau C, Genthon C,
740 Dubois E, et al. 2017. A multistrain approach to studying the mechanisms underlying
741 compatibility in the interaction between *Biomphalaria glabrata* and *Schistosoma mansoni*.
742 *PLoS Negl Trop Dis* 11:e0005398.

- 1
2
3 743 Gilges D, Vinit MA, Callebaut I, Coulombel L, Cacheux V, Romeo PH, Vigon I. 2000. Polydom:
4 744 a secreted protein with pentraxin, complement control protein, epidermal growth factor and
5 745 von Willebrand factor A domains. *Biochem J* 352 Pt 1:49-59.
- 6 746 Goodall CP, Bender RC, Brooks JK, Bayne CJ. 2006. *Biomphalaria glabrata* cytosolic
7 747 copper/zinc superoxide dismutase (SOD1) gene: association of SOD1 alleles with
8 748 resistance/susceptibility to *Schistosoma mansoni*. *Mol Biochem Parasitol* 147:207-210.
- 9 749 Greischar MA, Koskella B. 2007. A synthesis of experimental work on parasite local
10 750 adaptation. *Ecol Lett* 10:418-434.
- 11 751 Guillou F, Roger E, Mone Y, Rognon A, Grunau C, Theron A, Mitta G, Coustau C, Gourbal BE.
12 752 2007. Excretory-secretory proteome of larval *Schistosoma mansoni* and *Echinostoma*
13 753 *caproni*, two parasites of *Biomphalaria glabrata*. *Mol Biochem Parasitol*.
- 14 754 Hahn UK, Bender RC, Bayne CJ. 2001. Involvement of nitric oxide in killing of *Schistosoma*
15 755 *mansoni* sporocysts by hemocytes from resistant *Biomphalaria glabrata*. *J Parasitol* 87:778-
16 756 785.
- 17 757 Howard JC. 1991. Immunology. Disease and evolution. *Nature* 352:565-567.
- 18 758 Ittiprasert W, Knight M. 2012. Reversing the resistance phenotype of the *Biomphalaria*
19 759 *glabrata* snail host *Schistosoma mansoni* infection by temperature modulation. *PLoS Pathog*
20 760 8:e1002677.
- 21 761 Ittiprasert W, Miller A, Myers J, Nene V, El-Sayed NM, Knight M. 2010. Identification of
22 762 immediate response genes dominantly expressed in juvenile resistant and susceptible
23 763 *Biomphalaria glabrata* snails upon exposure to *Schistosoma mansoni*. *Mol Biochem Parasitol*
24 764 169:27-39.
- 25 765 Ittiprasert W, Nene R, Miller A, Raghavan N, Lewis F, Hodgson J, Knight M. 2009.
26 766 *Schistosoma mansoni* infection of juvenile *Biomphalaria glabrata* induces a differential stress
27 767 response between resistant and susceptible snails. *Exp Parasitol* 123:203-211.
- 28 768 Kalbe M, Eizaguirre C, Scharsack JP, Jakobsen PJ. 2016. Reciprocal cross infection of
29 769 sticklebacks with the diphylobothriidean cestode *Schistocephalus solidus* reveals consistent
30 770 population differences in parasite growth and host resistance. *Parasit Vectors* 9:130.
- 31 771 Kamiya T, Ona L, Wertheim B, van Doorn GS. 2016. Coevolutionary feedback elevates
32 772 constitutive immune defence: a protein network model. *BMC Evol Biol* 16:92.
- 33 773 Kawecki T, Ebert D. 2004. Conceptual issues in local adaptation. *Ecol Lett* 7:1225-1241.
- 34 774 Knight M, Elhelu O, Smith M, Haugen B, Miller A, Raghavan N, Wellman C, Cousin C, Dixon F,
35 775 Mann V, et al. 2015. Susceptibility of Snails to Infection with Schistosomes is influenced by
36 776 Temperature and Expression of Heat Shock Proteins. *Epidemiology (Sunnyvale)* 5.
- 37 777 Koskella B. 2014. Bacteria-phage interactions across time and space: merging local
38 778 adaptation and time-shift experiments to understand phage evolution. *Am Nat* 184 Suppl
39 779 1:S9-21.
- 40 780 Laine AL. 2005. Spatial scale of local adaptation in a plant-pathogen metapopulation. *J Evol*
41 781 *Biol* 18:930-938.
- 42 782 Larson MK, Bender RC, Bayne CJ. 2014. Resistance of *Biomphalaria glabrata* 13-16-R1 snails
43 783 to *Schistosoma mansoni* PR1 is a function of haemocyte abundance and constitutive levels of
44 784 specific transcripts in haemocytes. *Int J Parasitol* 44:343-353.
- 45 785 Li S, Shen L, Sun L, Xu J, Jin P, Chen L, Ma F. 2017. Small RNA-Seq analysis reveals microRNA-
46 786 regulation of the Imd pathway during *Escherichia coli* infection in *Drosophila*. *Dev Comp*
47 787 *Immunol* 70:80-87.
- 48 788 Lively CM. 1989. Adaptation by a Parasitic Trematode to Local Populations of Its Snail Host.
49 789 *Evolution* 43(8).

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

790 Lockyer AE, Emery AM, Kane RA, Walker AJ, Mayer CD, Mitta G, Coustau C, Adema CM,
791 Hanelt B, Rollinson D, et al. 2012. Early differential gene expression in haemocytes from
792 resistant and susceptible *Biomphalaria glabrata* strains in response to *Schistosoma mansoni*.
793 PLoS One 7:e51102.

794 Lockyer AE, Noble LR, Rollinson D, Jones CS. 2004. *Schistosoma mansoni*: resistant specific
795 infection-induced gene expression in *Biomphalaria glabrata* identified by fluorescent-based
796 differential display. *Exp Parasitol* 107:97-104.

797 Lockyer AE, Olson PD, Ostergaard P, Rollinson D, Johnston DA, Attwood SW, Southgate VR,
798 Horak P, Snyder SD, Le TH, et al. 2003. The phylogeny of the Schistosomatidae based on
799 three genes with emphasis on the interrelationships of *Schistosoma* Weinland, 1858.
800 *Parasitology* 126:203-224.

801 Lockyer AE, Spinks JN, Walker AJ, Kane RA, Noble LR, Rollinson D, Dias-Neto E, Jones CS.
802 2007. *Biomphalaria glabrata* transcriptome: identification of cell-signalling, transcriptional
803 control and immune-related genes from open reading frame expressed sequence tags
804 (ORESTES). *Dev Comp Immunol* 31:763-782.

805 Meninger T, Lerman G, Regev-Rudzki N, Gold D, Ben-Dov IZ, Sidi Y, Avni D, Schwartz E.
806 2017. Schistosomal MicroRNAs Isolated From Extracellular Vesicles in Sera of Infected
807 Patients: A New Tool for Diagnosis and Follow-up of Human Schistosomiasis. *J Infect Dis*
808 215:378-386.

809 Minchella DJ. 1985. Host life-history variation in response to parasitism. *Parasitology* 90:205-
810 216.

811 Mitta G, Adema CM, Gourbal B, Loker ES, Theron A. 2012. Compatibility polymorphism in
812 snail/schistosome interactions: From field to theory to molecular mechanisms. *Dev Comp*
813 *Immunol* 37:1-8.

814 Mone Y, Gourbal B, Duval D, Du Pasquier L, Kieffer-Jaquinod S, Mitta G. 2010. A large
815 repertoire of parasite epitopes matched by a large repertoire of host immune receptors in
816 an invertebrate host/parasite model. *PLoS Negl Trop Dis* 4.

817 Mone Y, Ribou AC, Cosseau C, Duval D, Theron A, Mitta G, Gourbal B. 2011. An example of
818 molecular co-evolution: reactive oxygen species (ROS) and ROS scavenger levels in
819 *Schistosoma mansoni*/*Biomphalaria glabrata* interactions. *Int J Parasitol* 41:721-730.

820 Morand S, Manning SD, Woolhouse ME. 1996. Parasite-host coevolution and geographic
821 patterns of parasite infectivity and host susceptibility. *Proc Biol Sci* 263:119-128.

822 Morgan JA, Dejong RJ, Adeoye GO, Ansa ED, Barbosa CS, Bremond P, Cesari IM, Charbonnel
823 N, Correa LR, Coulibaly G, et al. 2005. Origin and diversification of the human parasite
824 *Schistosoma mansoni*. *Mol Ecol* 14:3889-3902.

825 Morgan JA, Dejong RJ, Snyder SD, Mkoji GM, Loker ES. 2001. *Schistosoma mansoni* and
826 *Biomphalaria*: past history and future trends. *Parasitology* 123 Suppl:S211-228.

827 Myers J, Ittiprasert W, Raghavan N, Miller A, Knight M. 2008. Differences in cysteine
828 protease activity in *Schistosoma mansoni*-resistant and -susceptible *Biomphalaria glabrata*
829 and characterization of the hepatopancreas cathepsin B Full-length cDNA. *J Parasitol* 94:659-
830 668.

831 Nelson MK, Cruz BC, Buena KL, Nguyen H, Sullivan JT. 2016. Effects of abnormal temperature
832 and starvation on the internal defense system of the schistosome-transmitting snail
833 *Biomphalaria glabrata*. *J Invertebr Pathol*.

834 Nowacki FC, Swain MT, Klychnikov OI, Niazi U, Ivens A, Quintana JF, Hensbergen PJ, Hokke
835 CH, Buck AH, Hoffmann KF. 2015. Protein and small non-coding RNA-enriched extracellular

- 1
2
3 836 vesicles are released by the pathogenic blood fluke *Schistosoma mansoni*. *J Extracell Vesicles*
4 837 4:28665.
- 5 838 Nuismer SL, Dybdahl MF. 2016. Quantifying the coevolutionary potential of multistep
6 839 immune defenses. *Evolution* 70:282-295.
- 7
8 840 Osnas EE, Hurtado PJ, Dobson AP. 2015. Evolution of pathogen virulence across space during
9 841 an epidemic. *Am Nat* 185:332-342.
- 10 842 Osnas EE, Lively CM. 2005. Immune response to sympatric and allopatric parasites in a snail-
11 843 trematode interaction. *Front Zool* 2:8.
- 12 844 Osnas EE, Lively CM. 2004. Parasite dose, prevalence of infection and local adaptation in a
13 845 host-parasite system. *Parasitology* 128:223-228.
- 14 846 Penczykowski RM, Laine AL, Koskella B. 2015. Understanding the ecology and evolution of
15 847 host-parasite interactions across scales. *Evol Appl* 9:37-52.
- 16
17 848 Perrin C, Lepesant JM, Roger E, Duval D, Fneich S, Thuillier V, Alliene JF, Mitta G, Grunau C,
18 849 Cosseau C. 2012. *Schistosoma mansoni* mucin gene (*SmPoMuc*) expression: epigenetic
19 850 control to shape adaptation to a new host. *PLoS Pathog* 9:e1003571.
- 20 851 Pila EA, Gordy MA, Phillips VK, Kabore AL, Rudko SP, Hanington PC. 2016. Endogenous
21 852 growth factor stimulation of hemocyte proliferation induces resistance to *Schistosoma*
22 853 *mansoni* challenge in the snail host. *Proc Natl Acad Sci U S A* 113:5305-5310.
- 23 854 Pinaud S, Portela J, Duval D, Nowacki FC, Olive MA, Allienne JF, Galinier R, Dheilly NM,
24 855 Kieffer-Jaquinod S, Mitta G, et al. 2016. A Shift from Cellular to Humoral Responses
25 856 Contributes to Innate Immune Memory in the Vector Snail *Biomphalaria glabrata*. *PLoS*
26 857 *Pathog* 12:e1005361.
- 27
28 858 Portet A, Pinaud S, Tetreau G, Galinier R, Cosseau C, Duval D, Grunau C, Mitta G, Gourbal B.
29 859 2017. Integrated multi-omic analyses in *Biomphalaria-Schistosoma* dialogue reveal the
30 860 immunobiological significance of FREP-*SmPoMuc* interaction. *Dev Comp Immunol*. doi:
31 861 10.1016/j.dci.2017.02.025.
- 32
33 862 Prugnolle F, de Meeus T, Pointier JP, Durand P, Rognon A, Theron A. 2006. Geographical
34 863 variations in infectivity and susceptibility in the host-parasite system *Schistosoma*
35 864 *mansoni/Biomphalaria glabrata*: no evidence for local adaptation. *Parasitology* 133:313-319.
- 36 865 Raghavan M, Wijeyesakere SJ, Peters LR, Del Cid N. 2013. Calreticulin in the immune system:
37 866 ins and outs. *Trends Immunol* 34:13-21.
- 38
39 867 Richards CS. 1975. Genetic factors in susceptibility of *Biomphalaria glabrata* for different
40 868 strains of *Schistosoma mansoni*. *Parasitology* 70:231-241.
- 41
42 869 Robertson S, Bradley JE, MacColl AD. 2017. No evidence of local adaptation of immune
43 870 responses to *Gyrodactylus* in three-spined stickleback (*Gasterosteus aculeatus*). *Fish*
44 871 *Shellfish Immunol* 60:275-281.
- 45 872 Roger E, Grunau C, Pierce RJ, Hirai H, Gourbal B, Galinier R, Emans R, Cesari IM, Cosseau C,
46 873 Mitta G. 2008. Controlled Chaos of Polymorphic Mucins in a Metazoan Parasite (*Schistosoma*
47 874 *mansoni*) Interacting with Its Invertebrate Host (*Biomphalaria glabrata*). *PLoS Negl Trop Dis*
48 875 2:e330.
- 49
50 876 Roger E, Mitta G, Mone Y, Bouchut A, Rognon A, Grunau C, Boissier J, Theron A, Gourbal BE.
51 877 2008. Molecular determinants of compatibility polymorphism in the *Biomphalaria*
52 878 *glabrata/Schistosoma mansoni* model: New candidates identified by a global comparative
53 879 proteomics approach. *Mol Biochem Parasitol* 157:205-216.
- 54
55 880 Ryu SJ, Park SC. 2009. Targeting major vault protein in senescence-associated apoptosis
56 881 resistance. *Expert Opin Ther Targets* 13:479-484.
- 57
58
59
60

- 1
2
3 882 Sminia T, Barendsen L. 1980. A comparative morphological and enzyme histochemical study
4 883 on blood cells of the freshwater snails, *Lymnaea stagnalis*, *Biomphalaria glabrata*, and
5 884 *Bulinus truncatus*. Journal of Morphology 165:31-39.
6 885 Tack AJ, Thrall PH, Barrett LG, Burdon JJ, Laine AL. 2012. Variation in infectivity and
7 886 aggressiveness in space and time in wild host-pathogen systems: causes and consequences. J
8 887 Evol Biol 25:1918-1936.
9 888 Tennessen JA, Theron A, Marine M, Yeh JY, Rognon A, Blouin MS. 2015. Hyperdiverse gene
10 889 cluster in snail host conveys resistance to human schistosome parasites. PLoS Genet
11 890 11:e1005067.
12 891 Theron A. 2005. Les schistosomes et leurs hôtes : apport des marqueurs moléculaires à la
13 892 connaissance de leur phylogéographie, épidémiologie et écologie. . Bull Soc Zool Fr 130 205-
14 893 218.
15 894 Theron A, Pages JR, Rognon A. 1997. Schistosoma mansoni: distribution patterns of miracidia
16 895 among Biomphalaria glabrata snail as related to host susceptibility and sporocyst regulatory
17 896 processes. Exp Parasitol 85:1-9.
18 897 Theron A, Rognon A, Gourbal B, Mitta G. 2014. Multi-parasite host susceptibility and multi-
19 898 host parasite infectivity: a new approach of the Biomphalaria glabrata/Schistosoma mansoni
20 899 compatibility polymorphism. Infect Genet Evol 26:80-88.
21 900 Thrall PH, Burdon JJ, Bever JD. 2002. Local adaptation in the Linum marginale-Melampsora
22 901 lini host-pathogen interaction. Evolution 56:1340-1351.
23 902 Van Der Knaap WPW, Loker ES. 1990. Immune mechanisms in trematod-snail interactions.
24 903 Parasitology Today 6:175-182.
25 904 Walzer KA, Chi JT. 2017. Trans-kingdom small RNA transfer during host-pathogen
26 905 interactions: The case of P. falciparum and erythrocytes. RNA Biol:1-8.
27 906 WHO. 2010. Schistosomiasis Fact Sheet No. 115. World Health Organisation.
28 907 Zawada AM, Zhang L, Emrich IE, Rogacev KS, Krezdorn N, Rotter B, Fliser D, Devaux Y,
29 908 Ziegler-Heitbrock L, Heine GH. 2017. MicroRNA profiling of human intermediate monocytes.
30 909 Immunobiology 222:587-596.
31 910 Zhao X, Yu H, Kong L, Liu S, Li Q. 2016. High throughput sequencing of small RNAs
32 911 transcriptomes in two Crassostrea oysters identifies microRNAs involved in osmotic stress
33 912 response. Sci Rep 6:22687.
34 913
35 914
36 915
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 916 **Figure Legends**

4 917 Fig. 1: Dual-RNAseq of *Biomphalaria* immune-related transcripts

5 918 From among the differentially represented transcripts, Blast2GO functional annotation allowed us to identify 336
6 919 transcripts that appeared to be related to the *Biomphalaria* immune response. Abbreviations and colors: blue BB,
7 920 sympatric interaction between BgBRE and SmBRE; green BV, allopatric interaction between BgBRE and
8 921 SmVEN; and red BR, allopatric interaction between BgBRE and Srod. For each interaction 40 whole-snails are
9 922 used, 20 pooled at 24h and 20 at 96h post-infection. A) Venn diagram showing the relationships among the
10 923 immune transcripts found to be differentially expressed in the sympatric and allopatric interactions. B)
11 924 Clustering of differentially represented immune transcripts. Heatmap representing the profiles of the 336
12 925 differentially represented immune-related transcripts in the BB, BV, or BR interactions along the kinetic of
13 926 infection (at 24 and 96 h). Each transcript is represented once and each line represents one transcript. Colors:
14 927 yellow, over-represented transcripts; purple, under-represented transcripts; and black, unchanged relative to
15 928 levels in control naïve snails. C) Pie chart showing the distribution of the selected immune-related transcripts
16 929 across three immunological processes: immune recognition (pink), immune effectors (brown), and immune
17 930 signaling (blue). For each category and interaction, the respective proportion of transcripts and the direction of
18 931 the effect (over- or underexpression) are indicated.

19 932

20 933 Fig. 2: Differentially represented immune-related transcripts in sympatric and allopatric interactions.

21 934 Cumulative expression [Log2FC (fold change) from DESeq2 analysis] of the immune-related transcripts
22 935 identified as being differentially represented following sympatric or allopatric infection. Transcripts were
23 936 grouped into the three immunological groups described in Fig. 1, and from there into functional categories. The
24 937 yellow histograms correspond to cumulatively over-represented transcripts, while the purple histograms show
25 938 under-represented transcripts. The black (over-represented) and gray (under-represented) points correspond to
26 939 the number of transcripts analyzed in each functional category. Abbreviations: BB, BgBRE/SmBRE interaction;
27 940 BV, BgBRE/SmVEN interaction; and BR, BgBRE/Srod interaction. A. Immune transcript expression at 24 h
28 941 post-infection. B. Immune transcript expression at 96 h post-infection.

29 942

30 943 Fig. 3: Microscopic investigation of snail hemocyte proliferations

31 944 *In vitro* EdU labeling of hemocytes was conducted for sympatric and allopatric interactions A) Hemocytes were
32 945 collected at 24 h post-infection for *in vitro* analysis. Confocal microscopy of EdU-labeled hemocytes from snails
33 946 subjected to the allopatric BV interaction (BgBRE/SmVEN). Colors: blue, DAPI; and green, EdU. B)
34 947 Microscopic counting of EdU-labeled hemocytes from naïve control snails (BgBRE) (n=1,811) and those
35 948 subjected to the sympatric interaction (BB: BgBRE/SmBRE) (n=2,064) or an allopatric interaction (BV:
36 949 BgBRE/SmVEN) (n=1,366) recovered from 3 individual snails by condition. Colors: green, EdU-positive cells;
37 950 and blue, EdU-negative cells. Between-group differences in the percentage of proliferation were tested using a
38 951 Fisher exact test, with statistical significance accepted at $p < 0.05$. The “a” indicates a significant difference
39 952 between the naïve and infective conditions, while “b” indicates a significant difference between the infective
40 953 conditions.

41 954

42 955 Fig. 4: Flow cytometry investigation of the hemocyte response in sympatric and allopatric interactions

43 956

44 957

45 958

46 959

47 960

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

956 A) Flow cytometry was used to count *in vivo* EdU-labeled hemocytes at 24 h and 96 h after infection in
 957 sympatric and allopatric interactions. A total number of hemocytes of n=10,000 were recovered for 6 biological
 958 replicates of 3 snails. Control naïve snails (BgBRE, yellow) were compared to those subjected to the sympatric
 959 interaction (BB, BgBRE/SmBRE, blue) or an allopatric interaction (BV, BgBRE/SmVEN, green). B) The
 960 experiment described in A was repeated using the BgVEN snail strain. Control naïve snails (BgVEN, yellow)
 961 were compared to those subjected to the sympatric interaction (VV, BgVEN/SmVEN, green) or an allopatric
 962 interaction (VB, BgVEN/SmBRE, blue). C) FSC (forward-scattered light, representing cell size) and SSC (side-
 963 scattered light, representing cell granularity) circulating hemocyte patterns in BgBRE snails under the naïve
 964 condition (yellow) or 24 h and 96 h after infections in sympatry (BB24/96, BgBRE/SmBRE, blue) or allopatry
 965 (BV24/96, BgBRE/SmVEN, green). D) FSC and SSC circulating hemocyte patterns in BgVEN snails under the
 966 naïve condition (yellow) or 24 h and 96 h after infections in sympatry (VV24/96, BgVEN/SmVEN, blue) or
 967 allopatry (VB24/96, BgVEN/SmBRE, green). The red dots correspond to EdU-positive hemocytes. Between-
 968 group differences in the percentage of proliferation were tested using the Mann-Whitney U-test, with statistical
 969 significance accepted at p<0.05. The “a” indicates a significant difference between the naïve and infective
 970 condition, while “b” indicates a significant difference between the infective conditions.

971

972 Fig. 5: Development of parasites into snail tissues

973 A histological approach was used to monitor parasite size along the course of snail infection. The sympatric
 974 interaction (BgBRE x SmBRE) is shown in blue, and the allopatric interaction (BgBRE x SmVEN) is shown in
 975 green. For each experimental interaction, the parasite sizes were quantified at 24 and 96 h after infection.
 976 Morpho-anatomical aspects of the parasite are depicted to highlight a potential difference in parasite survival.
 977 n=7 to 10 sporocysts were used as indicated in the figure. Between-group parasite size differences were
 978 assessed using the Mann-Whitney U-test, with significance accepted at p<0.05 (indicated by “a” on the
 979 histograms).

980

981 Fig. 6: Clustering of intra-molluscal Schistosoma expression patterns

982 RNAseq library mapping enabled us to identify 351 genes expressed by *Schistosoma* parasites in *Biomphalaria*
 983 snail tissues. Colors: blue, *S. mansoni* Brazil (SmBRE); green, *S. mansoni* Venezuela (SmVEN); and red, *S.*
 984 *rodhaini* (Srod). The heatmap represents the profiles of the 351 genes expressed by the different parasites at 24 h
 985 after infection. Each transcript is represented once and each line represents one transcript. The expression level is
 986 highlighted by the different shades of blue.

987

988 Fig. 7: In-silico identification of parasite miRNAs

989 miRNAs were assessed using libraries obtained from naïve snails and snails infected for 24 h under the various
 990 interaction conditions (BB24, BgBRE x SmBRE; BV24, BgBRE x SmVEN; BR24, BgBRE x Srod). A) Table
 991 highlighting the precursor miRNAs that may have targets among the immune-related snail transcripts selected in
 992 the present work. They include eight precursors specifically recovered in BB24, two in BV24, and one shared
 993 across the three infected conditions. The total numbers of potential targets in each condition are indicated. B)
 994 Venn diagram showing the potential targets according to the sympatric or allopatric interactions. Shown is an
 995 example miRNA stem-loop precursor that presents the highest number of potential host targets.

996

997

Supplementary fig. S1: Clustering of all differential represented transcripts

998 Clustering of differentially represented transcripts. Heatmap representing the profiles of the 1,895 differentially
 999 represented immune-related transcripts in the BB, BV, or BR interactions along the time course of infection (at
 1000 24 and 96 h). Each transcript is represented once and each line represents one transcript. Colors: yellow, over-
 1001 represented transcripts; purple, under-represented transcripts; and black, unchanged relative to levels in control
 1002 naïve snails.

1003

1004

Supplementary fig. S2: Mortality of B. glabrata snails after infections

1005 The survival rates of *B. glabrata* subjected to infection by different *S. mansoni* strains were observed over 14
 1006 weeks. Kaplan Meier graphs were generated using xlstat, and the log-rank test ($p < 0.05$) was used to test for
 1007 significant between-group differences. Colored curves indicate the mortality rates of naïve snails (yellow)
 1008 ($n=60$), snails infected by the sympatric parasite (BB, BgBRE/SmBRE, blue) ($n=50$), and snails infected by the
 1009 allopatric parasite (BV, BgBRE/SmVEN, green) ($n=50$). The difference in mortality between naïve and infected
 1010 snails was significant ($p < 0.05$), whereas that between the two infected conditions was not ($p = 0.243$).

1011

1012 **Table 1:** Number of transcripts in each step of transcriptomic analysis

1013

Analysis of transcriptomic data		Analyses of Blast2GO	Analyses of annotations	
Full Transcriptome	Differentially expressed	Informative annotation	Immune Transcripts	Non Immune Transcripts
159,711	3,865	1,017	336	681

1014

1015

Supplementary table S1: Biomphalaria and Schistosoma compatibility between African and South-

1016

American strains

		<i>Biomphalaria</i>			
		<i>glabrata</i>		<i>pfeifferi</i>	
		BRE	VEN	SEN	
<i>Schistosoma mansoni</i>	BRE	P %	100	70	92
		I	3.6	3.5	2.9
	VEN	P %	100	95	100
		I	3.2	3.8	4
	SEN	P %	27	13	100
		I	1.3	1	2.5
<i>Schistosoma rodhaini</i>	P %	80	NA	NA	
	I	2	NA	NA	

1017

1018

1019

1020

1021

1022

The prevalence (P %: percentage of snail infected) and intensity (I: number of parasites per infected host) of infection are presented for each experimental infection. The indicated values correspond to 20 miracidia. Each pairwise combination of *Biomphalaria* (BgBRE, BgVEN, BpSEN) and *Schistosoma mansoni* (SmBRE, SmVEN, SmSEN) or *Schistosoma Rodhaini* (Srod) were tested for compatibility. NA: non-available data.

Discussion

Le polymorphisme de compatibilité est un phénomène notable dans l'interaction entre *Biomphalaria glabrata* et *Schistosoma mansoni*.

Un mollusque est dit résistant lorsqu'il est capable de reconnaître et éliminer le parasite et donc de bloquer son cycle de vie, nous sommes alors dans une interaction qui est incompatible. A l'inverse, un mollusque est dit susceptible lorsqu'il ne parvient pas à tuer et éliminer le parasite, ce qui entraîne alors une interaction dite compatible lors de laquelle le parasite continue son développement. L'infectivité du parasite reflète sa capacité à établir une infection durable et viable chez le mollusque.

Ce polymorphisme de compatibilité repose sur une concordance de déterminants moléculaires clés de l'interaction sur la base de l'hypothèse du « matching molecular variant ». Il repose sur l'interaction entre divers déterminants moléculaires ; côté mollusque sur des molécules de reconnaissance des pathogènes et les voies menant à l'élimination du parasite, et côté schistosome sur des facteurs de virulence visant à s'implanter et se développer chez l'hôte. Pour ce faire le parasite dispose de stratégies visant à contrer les défenses immunitaires de l'hôte (stratégie immunosuppressive) ou bien à passer inaperçu vis-à-vis du système immunitaire, en réalisant un mimétisme moléculaire (stratégie mimétique).

La relation entre *B. glabrata* et *S. mansoni* présente un haut niveau de complexité. En effet, le phénomène du "polymorphisme de compatibilité" reposant sur le modèle de "matching phenotype" fait appel à de multiples facteurs moléculaires de l'infectivité du parasite et de la résistance du mollusque qui sont en interaction. Ici nous proposons d'intégrer l'ensemble de ces facteurs dans une vision synthétique du polymorphisme de compatibilité à travers les études qui ont permis la caractérisation des FREP, des SmPoMuc et des facteurs clés impliqués dans la compatibilité et cela pour différentes souches de mollusques et de parasites. Enfin nous avons étudié cette compatibilité dans un contexte évolutif et plus particulièrement d'adaptation locale entre les deux partenaires issu d'interactions sympatriques ou allopatriques.

Les FREP et les SmPoMuc ont été mises en évidence dans un complexe immun entre l'hôte et le parasite (Moné, et al. 2010) et représentent des déterminants moléculaires clés dans les processus de compatibilité. Ici nous avons dans un premier temps fait la synthèse de toutes les techniques massives qui nous ont permis de mettre

Chapitre 1

en évidence le rôle de ces deux acteurs de l'interaction entre *B. glabrata* et *S. mansoni* avant d'examiner leurs profils d'expression ainsi que leurs polymorphismes à travers différentes souches d'hôtes et de parasites.

En 2008, des études de protéomique comparative, chez *Schistosoma mansoni*, ont permis de mettre en évidence que les SmPoMuc possèdent un domaine N-terminal très variable, avec de nombreuses répétitions en tandems et un domaine C-terminal conservé entre toutes les SmPoMuc (Roger, Gourbal, et al. 2008). C'est par une approche *in vitro* de co-immunoprecipitation de protéines de mollusque et de parasite que le complexe immun FREP/SmPoMuc a pu être identifié (Moné, et al. 2010). De plus, ces approches de protéomiques ont également permis de mettre en évidence un polymorphisme à la fois qualitatif et quantitatif des SmPoMuc ainsi que leur profil de glycosylation ont été mis en évidence (Roger 2008; Roger, Gourbal, et al. 2008; Roger, Grunau, et al. 2008). Un lien entre les forts taux de glycosylations des SmPoMuc et l'incompatibilité des parasites a également été mis en évidence par des approches de déglycosylation chimique (Roger, Gourbal, et al. 2008). Les SmPoMucs sont des protéines hautement glycosylées et hautement polymorphes, qui ne sont exprimées que par le stade du parasite infectant le mollusque ; elles sont produites et localisées dans la glande apicale du parasite ; puis sont sécrétées et libérées dans les produits d'excrétion-sécrétion (Roger, Gourbal, et al. 2008).

Les parasites présentent un répertoire individuel de SmPoMuc, chaque individu à son propre profil de SmPoMuc, mais toutefois au niveau des souches du parasite, il est possible de distinguer un profil spécifique de chaque souche lorsque on étudie un pool suffisamment important d'individus. Ces observations suggèrent que les SmPoMuc puissent jouer un rôle crucial dans le processus de compatibilité entre les parasites et leurs hôtes et puisse être le support d'une reconnaissance immunitaire par l'hôte intermédiaire mollusque (Roger, Mitta, et al. 2008). De plus, les mucines sont connues pour jouer un rôle plus général dans l'interaction hôte / pathogène en favorisant la pénétration des parasites dans les tissus de l'hôte (Mitta, et al. 2017). Toutes ces caractéristiques font des SmPoMuc des candidats importants dans la compréhension du polymorphisme de compatibilité.

Toujours dans le but d'expliquer les différences de résistances entre les souches de mollusques, une étude approfondie de la famille des lectines polymorphes et diversifiées, les FREP, a été réalisée. Les FREP ont dans un premier temps été identifiées comme glycoprotéines en solution dans le plasma de *Biomphalaria*, et présentant une capacité d'agglutination lors d'une exposition aux sporocystes de parasites et à leur produit

Chapitre 1

d'excrétion/sécrétion (Loker, et al. 1984; Loker, et al. 1994). Il semblerait donc que les FREP jouent un rôle dans la reconnaissance des pathogènes et puissent s'associer entre elles ou avec d'autres récepteurs afin d'augmenter le potentiel de reconnaissance immunitaire de l'individu (Adema and Loker 2015; Gordy, et al. 2015). Des études de génomiques sur les FREP, ont permis d'identifier 14 familles différentes, mais seulement 4 présentant une structure génomique complète (Léonard, et al. 2001; Zhang, et al. 2001; Zhang, et al. 2004). Une grande diversité structurale est observée chez ces molécules, elles peuvent contenir un ou deux domaines immunoglobuline (Dheilly, Duval, et al. 2015; Galinier, Roger, et al. 2017). Une diversité somatique des FREPs est mise en évidence (Loker, et al. 2004), ce qui suggère un grand potentiel de diversification. De plus suite à une infection l'expression de ces lectines augmente dans l'organisme (Dheilly, Duval, et al. 2015), suggérant qu'elles jouent un rôle dans la réponse immunitaire du mollusque. Finalement les FREP semblent aussi régulées par des mécanismes épigénétiques, car elles sont présentes dans des régions hypométhylées. Une absence de méthylation de l'ADN dans ces régions suggère une transcription aléatoire, qui peut expliquer une part de la diversification transcriptionnelle de ces molécules (Gavery and Roberts 2014; Portet, et al. 2017).

Nous sommes en présence des deux familles de molécules présentant de forts polymorphismes et/ou degrés de diversification, qui s'associent en complexe immunitaire lors de l'interaction entre *B. glabrata* et *S. mansoni*. Nous savons que chaque type de molécule présente un répertoire individuel et/ou populationnel, mais nous ignorons si elles jouent un rôle clé dans le polymorphisme de compatibilité entre le mollusque et le parasite. Pour cela, nous avons réalisé une étude complète des FREP et des SmPoMuc sur 4 souches de mollusques *B. glabrata* et 4 souches de parasites *S. mansoni*, provenant de Belo-Horizonte au Brésil (BgBAR-SmLE), de Recife au Brésil (BgBRE-SmBRE), de Guacara au Venezuela (BgVEN-SmVEN) et enfin de Lamentin en Guadeloupe (BgGUA-SmGH2). Les interactions sympatriques et allopatriques de ces 4 souches présentent des profils de compatibilité très différents. En effet, SmLE présente une infectivité très importante quelque soit le mollusque qu'on lui présente, alors que SmGH2 possède un faible pouvoir d'infectivité quelque soit l'hôte qu'il infecte, y compris son propre hôte sympatrique, quant à SmBRE et SmVEN, ils présentent des profils intermédiaires par rapport aux deux premiers, en étant toujours plus infectant chez leur hôte sympatrique (fig. 8). Ces résultats suggèrent une capacité intrinsèque du parasite,

Chapitre 1

qui pourrait être portée par des molécules telles que les SmPoMuc. Chez les mollusques, nous retrouvons les mêmes variations de susceptibilités, cette fois-ci, BgBAR présente les taux de susceptibilité les plus bas quelque soit le parasite qui l'infecte, y compris son parasite sympatrique, BgGUA, lui, présente la plus forte susceptibilité, il présentera des prévalences ainsi que des intensités d'infection très importantes. Les deux derniers mollusques, à savoir, BgBRE et BgVEN présentent des taux de susceptibilité intermédiaires qui varieront selon le parasite présenté (fig. 8). Ces résultats suggèrent, qu'il existe chez le mollusque une capacité intrinsèque de défense contre les parasites, qui pourrait être portée par des molécules de reconnaissances comme les FREP. Une analyse fonctionnelle de ces molécules dans chacune des souches sélectionnées, nous a permis de mettre en évidence un lien entre le polymorphisme de compatibilité présent entre les souches, et le rôle des FREP et des SmPoMuc.

Dans un premier temps, dans le but d'expliquer les différences de compatibilité, une analyse de la diversité et de l'expression de SmPoMuc est réalisée pour toutes les souches dans ce gradient de compatibilité. Il apparaît que les souches les plus compatibles, à savoir SmLE et SmVEN, présentent un grand nombre de variants de SmPoMuc différents et chacun faiblement exprimés. Ces arguments vont dans le sens du "matching phenotype", plus la population de parasite présente un grand nombre d'antigène différent moins il a de chance d'être reconnu par le système immunitaire du mollusque (fig. 8). Ici, nous avons une stratégie d'évitement de la reconnaissance par le système immunitaire de l'hôte qui est mise en place par le parasite. A noter, que SmVEN présente un profil un peu différent car il investit dans la diversification des SmPoMuc mais il présente tout de même une expression des variants plus importante que SmLE (fig. 8). A contrario, SmBRE et SmGH2 présentent un nombre de variants différents moins important que les deux autres souches de parasites. Pour ce qui est de l'expression de chaque variant, les deux souches se différencient. En effet, SmBRE, présente en plus d'une faible diversité une faible expression des variants, ici, nous pouvons penser qu'il n'a pas la capacité de diversifier ces mucines, donc il exprime faiblement chacun d'eux pour éviter d'être reconnu (fig. 8). Pour SmGH2, sa diversification de SmPoMuc est modérée voir faible et leur expression est importante, il apparaît donc qu'il sera plus facilement reconnu par le système immunitaire du mollusque. Il semblerait que le niveau d'expression des SmPoMuc puisse jouer un rôle dans la compatibilité avec le mollusque. En effet, nous pouvons imaginer que plus le parasite exprime de SmPoMuc, plus nous auront de complexe immun entre les FREP et les SmPoMuc entraînant alors un niveau de

Chapitre 1

reconnaissance accru et finalement l'élimination de ce dernier. Ceci pourrait expliquer pourquoi SmVEN qui exprime quasiment le même nombre de variants que SmLE, présente une compatibilité moins importante. De même, le niveau d'expression des SmPoMuc peut expliquer pourquoi SmGH2 est moins compatible que SmBRE, alors que le nombre de variant exprimé est proche. De plus il faut noter qu'il existe 4 groupes de SmPoMuc dont le nombre de variant et l'expression sont très différents. A noter, que le groupe 3.1(r1+r2), n'est exprimé que chez SmGH2, le mollusque le moins infectieux. Ces différences d'expression de groupes de SmPoMuc pourraient également jouer un rôle dans la compatibilité des parasites.

Dans un second temps, l'étude des capacités immunitaires des mollusques a été réalisée par une approche de transcriptomique massive avec un focus sur les FREP. Étant donné que la compatibilité semble dépendre des molécules exprimées constitutivement par l'hôte et son parasite, l'approche de transcriptomique massive a été réalisée sur des individus naïfs. BgBAR, qui correspond au mollusque le moins permissif, présente globalement une plus grande diversité de molécules impliquées dans la réponse immunitaire. De plus, BgBAR exprime aussi une plus grande quantité de FREP que les trois autres souches, et la majorité d'entre elles présentent un seul domaine immunoglobuline. Ceci suggère que cette classe de FREP puisse jouer un rôle clé dans la reconnaissance des parasites ainsi que dans la défense anti-schistosome. Il semblerait, pour ce qui est du mollusque, que la diversité des FREP ne soit pas corrélée avec le niveau de compatibilité. Un niveau d'expression plus important semble par contre requit pour améliorer la résistance du mollusque, ainsi que le type de FREP (à un ou deux domaine IgSF (**fig. 8**)). Il semblerait donc que les FREP et SmPoMuc soient des déterminants moléculaires de la compatibilité entre *B. glabrata* et *S. mansoni*. Cependant le processus de compatibilité est plus complexe et de nombreux autres gènes participent à la co-évolution ou l'adaptation entre ces deux partenaires.

Afin d'aller plus loin dans la compréhension de la mise en place de ces processus de compatibilité entre mollusques et schistosomes, nous avons également étudié la compatibilité dans un contexte d'adaptation locale par l'intermédiaire d'interaction sympatrique et allopatrique. Nous avons réalisé une infection de BgBRE par SmBRE ou SmVEN, ces deux interactions étant compatibles. Pour ces deux types d'interaction, le parasite arrive à se développer et à continuer son cycle de vie. Si les phénotypes d'infectivité sont les mêmes (même prévalence, même intensité parasitaire), en est-il de même pour les processus moléculaires et la réponse immunitaire mise en place ? Pour

Chapitre 1

répondre à cette question, nous avons tout d'abord réalisé une étude de transcriptomique massive. Cette dernière a permis de mettre en évidence une forte immunosuppression 24h post-infection, en condition sympatrique (BgBRE x SmBRE) alors que la réponse immunitaire du mollusque est activée 96h post-infection, en condition allopatrique (BgBRE x SmVEN) (fig. 8). Il semble donc que les interactions immunologiques entre le mollusque et le parasite en conditions sympatrique et allopatrique soient totalement différentes. L'étude de transcriptomique massive, nous a également permis de mettre en évidence un grand nombre de transcrits impliqués dans la réponse cellulaire. Lorsque l'on s'intéresse plus en détail à cette réponse nous observons une prolifération hémocytaire 24h post-infection en condition allopatrique plus importante qu'en sympatrie. A 96h post-infection, le taux de prolifération tend à revenir à un niveau basal, mais il reste plus élevé en condition allopatrique que sympatrique. De plus, au vu des données de transcriptomique des stades intramolluscaux du parasite, nous pouvions nous attendre à ce que le développement des parasites dans les tissus du mollusques se fasse différemment en condition sympatrique et allopatrique. En effet, grâce à des données histologiques, nous observons un développement du parasite allopatrique moins important 24h post-infection, qu'en condition sympatrique. Les parasites ont une taille plus petite. Ce retard de développement est rattrapé 96h post-infection. Il semble donc que les capacités intrinsèques des parasites allopatriques soient importantes. Malgré la réponse immunitaire du mollusque, le parasite arrive, avec un peu de retard, à se développer et à contourner/bloquer la réponse immunitaire qui s'est mise en place et qui est d'ailleurs inefficace. Finalement, dans le but de trouver des outils qui permettraient au parasite de détourner le système immunitaire du mollusque, une approche *in silico* de prédiction de microRNA (miRNA) a été réalisée et met en évidence un plus grand nombre de miRNA exprimé par le parasite sympatrique que par le parasite allopatrique, ce qui va dans le sens d'une co-évolution entre les systèmes d'infectivité du parasite et de défense du mollusque. Le parasite reconnaît son mollusque sympatrique, la co-évolution ou l'adaptation entre les deux lui a permis de développer des outils, parmi eux, les miRNA, avec lesquels il peut potentiellement cibler spécifiquement des candidats immunitaires clés de l'interaction et favoriser une immunosuppression de son hôte pour pouvoir contourner le système immunitaire de ce dernier et continuer son cycle de vie en se développant dans les tissus du mollusques.

Nous sommes donc en présence d'une adaptation locale entre le mollusque et son parasite au niveau moléculaire mais qui pourrait passer totalement inaperçue du fait des

Chapitre 1

capacités intrinsèques d'infectivité de certain parasite étant donné que nous avons ici deux interactions qui seront au final compatibles.

Il semblerait que les FREP et les SmPoMuc puissent donc jouer un rôle important dans la compatibilité entre *B. glabrata* et *S. mansoni*. De plus un phénomène d'adaptation est visible entre ces deux molécules, qui sont à la base du premier complexe immunitaire identifié entre un invertébré et un parasite métazoaire. Si nous essayons d'intégrer les deux aspects de ce travail et de voir comment peuvent être interprétés l'évolution du polymorphisme ou de la diversification des FREP et SmPoMuc dans un contexte sympatrique et allopatrique, que pouvons nous conclure ?

Dans le contexte sympatrique entre BgBRE et SmBRE, nous assistons à un phénomène d'adaptation locale y compris au niveau des FREP et SmPoMuc. En effet, BgBRE présentant un répertoire diversifié de FREP, la stratégie du parasite a été de limiter la diversification de ses SmPoMuc pour contourner le système immunitaire de son hôte. Nous pouvons imaginer qu'il serait difficile au parasite de générer une diversité de SmPoMuc plus grande encore que la diversité des FREP du mollusque dans cette interaction. Donc le parasite n'opte pas pour une stratégie visant à augmenter le polymorphisme par contre il réduit l'expression des SmPoMuc.

Dans l'interaction allopatrique entre BgBRE et SmVEN, cette fois le parasite présente une grande diversité de SmPoMuc qui sont en plus exprimés modérément, dans ce contexte le mollusque pourra reconnaître ce parasite allopatrique plus aisément. Cette stratégie chez SmVEN se justifie lorsque nous la replaçons dans le contexte sympatrique. En effet, BgVEN présente une faible diversité de FREP, et donc en conséquence cette pression de sélection modérée n'a pas poussé le parasite à sélectionner une grande diversité ou polymorphisme des SmPoMuc.

Maintenant si nous observons l'ensemble des interactions du continuum de compatibilité présenté précédemment, il apparaît que les répertoires de FREPs et de SmPoMuc du mollusque et du parasite reposent sur une adaptation locale. En effet, pour BgBAR vs SmLE, le mollusque présente une forte expression de FREP peu diversifiée, en conséquence le parasite diversifie beaucoup c'est SmPoMuc tout en les exprimant très faiblement afin de passer inaperçu.

A noter tout de même que l'interaction BgGUA vs SmGH2 ne semble pas suivre clairement cette règle d'adaptation locale entre les FREP et les SmPoMuc. En effet, le mollusque présente une diversification et expression modérées de ses FREP mais le

Chapitre 1

parasite lui présente une forte expression de ses SmPoMucs. Il faut noter que ce parasite présente des taux d'infectivités faibles, y compris sur sa souche sympatrique, mais aussi que ce mollusque présente des taux de susceptibilité très fort. Nous pouvons imaginer que le mollusque n'ayant pas de fortes capacités immunes intrinsèques n'oblige pas le parasite à développer de grande stratégie d'infectivité, en effet le parasite lui aussi semble posséder de faibles capacités intrinsèques (Moné, et al. 2011). Nous sommes peut-être en présence d'un effet insulaire ou d'un effet fondateur lors de la colonisation de la Guadeloupe par le Schistosome au cours de la traite négrière.

Il est cependant clair que l'interaction entre *B. glabrata* et *S. mansoni*, ne repose pas uniquement sur le complexe immunitaire FREP/SmPoMuc, mais que cette dernière est bien plus complexe que cela. Une étude plus approfondie des molécules de reconnaissances du mollusque, comme des molécules effectrices, permettrait une meilleure compréhension de ce système hôte / parasite.

Par ailleurs, parmi les mécanismes pouvant intervenir dans l'interaction complexe entre *Biomphalaria glabrata* et *Schistosoma mansoni*, en plus des phénomènes génétiques, nous retrouvons aussi, des phénomènes épigénétiques comme par exemple des modifications de marque chimique de l'ADN (i.e. phosphorylation, méthylation, etc.), des modifications du code des histones ou encore l'implication d'ARN non-codant (i.e. micro ARN, long ARN non-codant, etc.). Chez le parasite, des études épigénomiques ont révélé un changement de marque d'histone en amont des régions régulatrices des SmPoMuc suggérant que des bases épigénétiques puissent réguler l'expression de ces molécules (Perrin, et al. 2013) et générant ainsi une plasticité phénotypique et un polymorphisme accru durant les interactions avec l'hôte intermédiaire. Il serait donc intéressant de pousser plus en avant la question du rôle des processus épigénétiques dans l'évolution adaptative. En effet ces processus épigénétiques offrent des capacités d'adaptation rapide aux espèces et pourraient avoir un rôle très important permettant peut-être d'expliquer la rapidité de ces processus d'adaptation locale en particulier des parasites pour leurs hôtes.

Chapitre 1

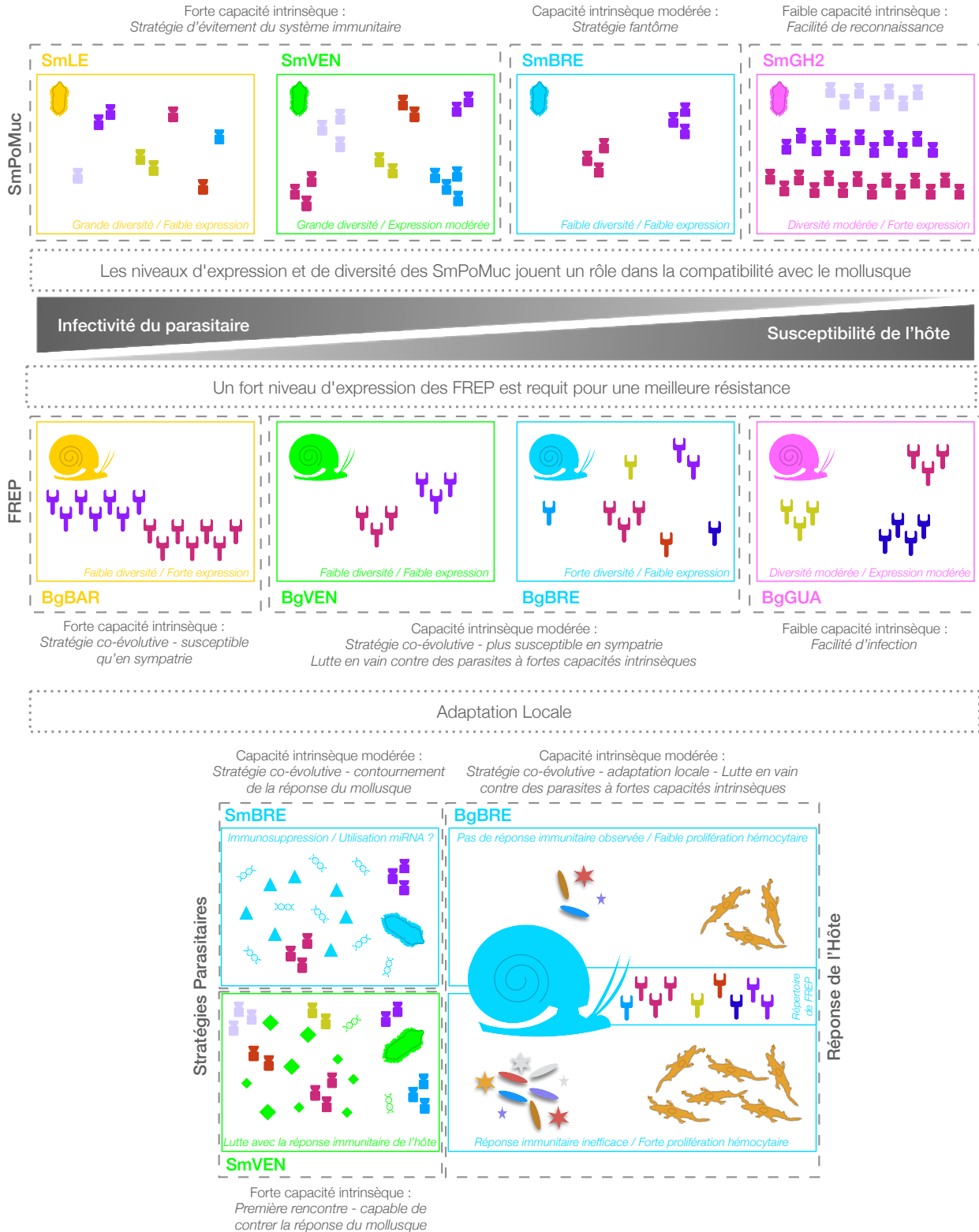


Figure 8 : Du Polymorphisme de Compatibilité à l'Adaptation Locale entre *B. glabrata* et *S. mansoni*

Figure adaptée d'après (Galinié, Roger, et al. 2017; Portet, et al. 2017). Diversité et expression des FREP et des SmPoMuc au travers de 4 combinaisons de souches de mollusques et de parasites présentant des niveaux d'infectivité et de susceptibilité graduels. Le nombre de symboles de FREP et SmPoMuc représente le niveau d'expression et leur couleur la diversité. Phénomène d'adaptation locale à l'échelle moléculaire entre le mollusque et son parasite. Le nombre d'hémocyte représente le taux de prolifération et les symboles, et le nombre, la réaction immunitaire du mollusque ou d'attaque du parasite.

Chapitre 2

Chapitre 2 : Du Polymorphisme de Compatibilité vers la compréhension d'un système immunitaire complexe.

Introduction

Le système immunitaire permet à l'hôte de détecter le pathogène et d'organiser une réponse de défense la plus appropriée. Confronté à une large gamme de pathogènes, allant des bactéries et virus jusqu'aux parasites métazoaires, le système immunitaire de l'hôte doit être capable de présenter une large gamme de récepteurs, pour répondre efficacement à ces multiples sollicitations.

Chez *Biomphalaria glabrata*, à partir d'expériences de co-immunoprécipitation (CoIP) réalisées au sein du laboratoire, un troisième partenaire a pu être mis en évidence dans le complexe immun FREP/SmPoMuc lors de l'interaction entre *B. glabrata* et *S. mansoni*. Ce troisième partenaire est une Thioester-containing Protein (TEP), nommée BgTEP (Moné 2010). C'est la première mise en évidence d'une interaction immunitaire entre une molécule de reconnaissance diversifiée d'un mollusque et d'un antigène polymorphe d'un parasite. Les TEP sont en particulier sécrétées par les hémocytes et clivées dans l'hémolymphe avant d'être stabilisée par un complexe protéique (Fraiture, et al. 2009). Elles ont été mises en évidence chez différents invertébrés et plus particulièrement chez une large variété d'espèces d'arthropodes (Levashina, et al. 2001; Blandin and Levashina 2004; Buresova, et al. 2011; Sekiguchi, et al. 2012; Urbanová, et al. 2015) ; de crustacés (Wu, et al. 2012; Li, et al. 2017) ou encore de mollusques (Zhang, et al. 2007) ; mais leur fonction a été majoritairement étudiée chez *Anopheles gambiae* (Levashina, et al. 2001). Chez ce dernier, la partie C-terminale de la protéine se fixe sur les bactéries ou les ookinètes de plasmodium grâce à son pont thioester ; l'implication de cette molécule complément-like dans l'immunité du moustique a été démontrée dans la lutte contre le plasmodium (Volohonsky, et al. 2010), mais également dans la réponse anti-bactérienne. En effet, suite à la reconnaissance dans l'hémolymphe des bactéries gram-positifs et gram-négatifs, AgTEP1 joue un rôle d'opsonine dans leur phagocytose (Levashina, et al. 2001; Yassine, et al. 2012). De plus, AgTEP1 est aussi capable de se fixer à la surface des parasites et de promouvoir leur mélanisation (Blandin, et al. 2008) et donc jouer un rôle dans la diminution des ookinètes de plasmodium (Smith, et al. 2015).

Chapitre 2

Elle est donc capable, à la fois de reconnaître des pathogènes mais aussi de jouer le rôle d'opsonine pour leur élimination.

Chez les invertébrés, un nombre croissant d'étude rapporte la présence de différents répertoires hautement diversifiés de récepteurs immunitaires. Ces molécules apparaissent comme essentielles dans le développement d'une réponse immunitaire fine et spécifique (Pasquier 2005). Nous pouvons retrouver des Dscam (Down syndrome cell adhesion molecule), diversifiées chez les arthropodes, générées par différents événements d'épissage, des FREP (Fibrinogen-Related Protein), chez les mollusques, générées par des hypermutations somatiques ou encore des C-Type Lectins, ou des Sp185/333 qui elles seront générées par édition de l'ARN ou modifications post-traductionnelles (Lemaitre and Hoffmann 2007; Armitage, et al. 2015). Chez *B. glabrata*, les FREP semblent jouer un rôle important dans le polymorphisme de compatibilité avec *S. mansoni* (Galinier, Roger, et al. 2017), mais ne permettent pas d'expliquer à elles seules la reconnaissance et la réponse immunitaire du mollusque. En effet, ce ne sont pas les seuls facteurs impliqués dans la reconnaissance des pathogènes, leur invalidation, par RNAi, met en évidence une diminution seulement partielle de la protection contre les pathogènes (Hanington, et al. 2012; Pinaud, et al. 2016). Ceci montre qu'ils sont bien impliqués dans la réponse immunitaire, mais que d'autres molécules manquent encore à être mises en évidence (Hanington, et al. 2012; Pinaud, et al. 2016).

De manière générale il est à noter un manque de connaissance sur le rôle de ces molécules impliquées directement ou indirectement dans la reconnaissance des pathogènes dans la réponse immunitaire des autres invertébrés. Dans ce contexte, nous nous proposons de réaliser une étude des mécanismes de reconnaissance de *Biomphalaria glabrata*, en réponse à une large gamme de pathogènes.

Pour cela, nous avons réalisé une étude sur la caractérisation structurale et fonctionnelle de BgTEP, Thioester-containing protein de *Biomphalaria glabrata*, précédemment identifiée dans le complexe immunitaire FREP/SmPoMuc/BgTEP (Moné 2010). Le but de cette étude était, dans un premier temps, de compléter la caractérisation structurale de cette protéine, avant de comprendre son rôle dans l'interaction avec les pathogènes. Pour cela nous avons non seulement étudié l'interaction de la BgTEP avec *S. mansoni*, mais nous avons aussi élargi l'étude à une plus large gamme de pathogènes, allant des bactéries jusqu'aux trématodes, dans le but d'avoir une vue globale du rôle de cette molécule dans la réponse immunitaire de *B. glabrata*.

Chapitre 2

Par la suite, étant donné que les FREP ne sont pas les seules molécules impliquées dans la reconnaissance immunitaire chez le mollusque, nous avons également étudié la capacité de la reconnaissance immunitaire de manière globale, par une approche protéomique d'interactome. Pour cela, nous avons utilisé la même gamme de pathogènes que précédemment, des bactéries aux trématodes. L'objectif étant alors d'identifier des molécules sentinelles utilisées par le mollusque dans la reconnaissance des déterminants de surfaces des pathogènes et leurs potentielles spécificités.

Ces études révèlent non seulement une implication de la BgTEP en réponse aux différents pathogènes, mais révèle en plus une certaine spécificité. De plus, l'étude protéomique met en évidence une large gamme de molécules de reconnaissances présentant des spécificités pour chaque pathogènes étudiés. Cependant leur fonction dans la réponse immunitaire du mollusque n'est pas démontrée. Une invalidation fonctionnelle, par RNAi (ARN interférants), permettrait de démontrer le recrutement et de valider au moins partiellement la fonction de ces molécules dans la reconnaissance des pathogènes, et dans la réponse immunitaire de *Biomphalaria*.

Dans ce cadre là, nous avons développé et mis au point une technique d'invalidation fonctionnelle par RNAi, en ciblant spécifiquement les hémocytes. Le but de cette étude est de pouvoir cibler des gènes exprimés dans les hémocytes, qui pourraient jouer un rôle dans la réponse immunitaire cellulaire et/ou la reconnaissance. Des premières études d'invalidation de la BgTEP semblent prometteuses pour comprendre au mieux son rôle fonctionnel dans la réponse immunitaire de *B. glabrata* face aux pathogènes.

1. Étude d'un facteur clé de la réponse immunitaire cellulaire : la BgTEP

Publication : The Thioester-containing protein, a key factor of the *Biomphalaria glabrata* immune cellular response?

PORTET Anaïs¹, GALINIER Richard¹, PINAUD Silvain, PORTELA Julien, NOWACKI Fanny, GOURBAL Benjamin and DUVAL David

*Article soumis à Frontiers in Immunology

BgTEP: a Complement-Related Protein with Dual Role in *Biomphalaria glabrata* Immune System.

Anaïs PORTET¹, Richard GALINIER¹, Silvain PINAUD¹, Julien Portela¹, Fanny NOWACKI², Benjamin Gourbal¹, David Duval^{1*}

¹Université de Perpignan Via Domitia, Interactions Hôtes Pathogènes Environnements UMR 5244, CNRS, IFREMER, Univ. Montpellier, F-66860 Perpignan, France, France, ²Institute for Biological and Environmental Sciences, University of Aberdeen, United Kingdom

Submitted to Journal:
Frontiers in Immunology

Specialty Section:
Molecular Innate Immunity

Article type:
Original Research Article

Manuscript ID:
314410

Received on:
26 Sep 2017

Frontiers website link:
www.frontiersin.org

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

Author contribution statement

AP performed interactome and immunoblotting, as well as quantitative RT-PCR experiments, FACS and microscopy analysis. RG designed the research, performed the phylogenetic analysis and participate to western blot experiments. SP performed interactome experiments. JP performed microscopy analysis. FN performed immunohistological experiment. BG substantially participated in conception and improvement of research. DD designed the research and performed genomic organization analysis of BgTEP gene. All authors participated to manuscript writing, editing, critical reviewing and they all approved the final draft.

Keywords

BgTEP, Complement-like protein, Biomphalaria glabrata, dual-role, opsonin

Abstract

Word count: 313

Insect Thioester-containing protein (iTEP) is the more recent defined group among the TEP superfamily. TEPs are key components of immune system, and iTEPs from fly and mosquito have been shown to be major immune weapons. Initially characterized from insects, TEP genes homologous to iTEP were further also described from several other invertebrates including arthropods, cnidarians and mollusks with few functional characterizations. In the freshwater snail *Biomphalaria glabrata*, vector of schistosomiasis disease, the presence of a TEP protein (BgTEP) has been previously described in a well-defined immune complex involving snail lectins (FREPs) and schistosome parasite mucins (SmPoMuc).

To study the potential role of BgTEP in the immune response of the snail, we first characterized its genomic organization and its protein predicted structure. A phylogenetic analysis clustered BgTEP in a well sustained subgroup of mollusk TEP. Then, we investigated the BgTEP expression profile in different snail tissues, as well as following immune challenges using different kind of pathogens along a kinetic of infection. Results revealed that BgTEP is particularly expressed in hemocytes, the immune-specialized cells in invertebrates, and is secreted into the hemolymph. Transcriptomic results also revealed an intruder-dependent differential expression pattern of BgTEP whilst interactome experiments showed that BgTEP is able to bind the surface of different pathogens whether in its full length form or in processed forms.

Immunolocalization approach during snail infection by the parasite *Schistosoma mansoni* revealed that BgTEP is solely expressed by a subtype of hemocytes, the blast-like cells. This hemocyte subtype is present in the hemocytic capsule surrounding the parasite, suggesting that BgTEP-positive cells must participate in the parasite clearance whole playing a complementary role in encapsulation.

Through this work we report the first characterization of an invertebrate TEP displaying an unexpected functional dual-role. Indeed, in addition to its previously characterized anti-protease activity, we show that BgTEP is also able to bind the pathogen surface membrane assuming an opsonin function.

Funding statement

Funding information:

This work was supported by the French National Agency for Research (ANR) through a project Invimory grant [ANR-13-JSV7-0009] to BG.

Ethics statements

(Authors are required to state the ethical considerations of their study in the manuscript, including for cases where the study was exempt from ethical approval procedures)

Does the study presented in the manuscript involve human or animal subjects: Yes

Please provide the complete ethics statement for your manuscript. Note that the statement will be directly added to the manuscript file for peer-review, and should include the following information:

- Full name of the ethics committee that approved the study

- Consent procedure used for human participants or for animal owners
- Any additional considerations of the study in cases where vulnerable populations were involved, for example minors, persons with disabilities or endangered animal species

As per the Frontiers authors guidelines, you are required to use the following format for statements involving human subjects: This study was carried out in accordance with the recommendations of 'name of guidelines, name of committee' with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the 'name of committee'.

For statements involving animal subjects, please use:

This study was carried out in accordance with the recommendations of 'name of guidelines, name of committee'. The protocol was approved by the 'name of committee'.

If the study was exempt from one or more of the above requirements, please provide a statement with the reason for the exemption(s).

Ensure that your statement is phrased in a complete way, with clear and concise sentences.

Our laboratory holds permit # A66040 for experiments on animals, which was obtained from the French Ministry of Agriculture and Fisheries and the French Ministry of National Education, Research, and Technology. The housing, breeding and care of the utilized animals followed the ethical requirements of our country. The experimenter possesses an official certificate for animal experimentation from both of the above-listed French ministries (Decree # 87-848, October 19, 1987). The various protocols used in this study have been approved by the French veterinary agency of the DRAAF Languedoc-Roussillon (Direction Régionale de l'Alimentation, de l'Agriculture et de la Forêt), Montpellier, France (authorization # 007083).

In review

1 **BgTEP: a Complement-Related Protein with Dual Role in**
2 ***Biomphalaria glabrata* Immune System.**
3

4
5
6 Anaïs PORTET, Richard GALINIER, Silvain PINAUD, Julien PORTELA, Fanny
7 NOWACKI, Benjamin GOURBAL and David DUVAL *

8
9
10
11 Univ. Perpignan Via Domitia, Interactions Hôtes Pathogènes Environnements UMR 5244,
12 CNRS, IFREMER, Univ. Montpellier, F-66860 Perpignan, France
13

14
15
16 Equal contributions : AP & RG
17

18
19
20
21 * corresponding author:
22 David Duval
23 david.duval@univ-perp.fr
24 UMR5244 IHPE, Université de Perpignan
25 58 avenue Paul Alduy, 66860 Perpignan
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50

51 **Abstract:**

52 Insect Thioester-containing protein (iTTEP) is the more recent defined group among the TEP
53 superfamily. TEPs are key components of immune system, and iTTEPs from fly and mosquito
54 have been shown to be major immune weapons. Initially characterized from insects, TEP
55 genes homologous to iTTEP were further also described from several other invertebrates
56 including arthropods, cnidarians and mollusks with few functional characterizations. In the
57 freshwater snail *Biomphalaria glabrata*, vector of schistosomiasis disease, the presence of a
58 TEP protein (BgTEP) has been previously described in a well-defined immune complex
59 involving snail lectins (FREP) and schistosome parasite mucins (SmPoMuc).

60 To study the potential role of BgTEP in the immune response of the snail, we first
61 characterized its genomic organization and its protein predicted structure. A phylogenetic
62 analysis clustered BgTEP in a well sustained subgroup of mollusk TEP. Then, we
63 investigated the BgTEP expression profile in different snail tissues, as well as following
64 immune challenges using different kind of pathogens along a kinetic of infection. Results
65 revealed that BgTEP is particularly expressed in hemocytes, the immune-specialized cells in
66 invertebrates, and is secreted into the hemolymph. Transcriptomic results also revealed an
67 intruder-dependent differential expression pattern of BgTEP whilst interactome experiments
68 showed that BgTEP is able to bind the surface of different pathogens whether in its full length
69 form or in processed forms.

70 Immunolocalization approach during snail infection by the parasite *Schistosoma mansoni*
71 revealed that BgTEP is solely expressed by a subtype of hemocytes, the blast-like cells. This
72 hemocyte subtype is present in the hemocytic capsule surrounding the parasite, suggesting
73 that BgTEP-positive cells must participate in the parasite clearance whole playing a
74 complementary role in encapsulation.

75 Through this work we report the first characterization of an invertebrate TEP displaying an
76 unexpected functional dual-role. Indeed, in addition to its previously characterized anti-
77 protease activity, we show that BgTEP is also able to bind the pathogen surface membrane
78 assuming an opsonin function.

79

80 **Keywords:** BgTEP, Complement-like protein, *Biomphalaria glabrata*, Dual-role, Opsonin

81

82

83

84

85

86

87

88

89

90

91

92

93

94

95

96

97

98

99

100 **Introduction:**

101 Thioester-containing proteins (TEPs) are large secreted glycoproteins characterized by the
102 presence of a unique intrachain thioester bond (Williams and Baxter, 2014). In native TEPs
103 this bond is unreactive, but following proteolytic activation, change in temperature or aqueous
104 conditions, the thioester bond becomes reactive and can bind closely accessible hydroxyl or
105 amine groups that are present at the surface of many biological entities including pathogens
106 (Shokal and Eleftherianos, 2017).

107 The unique intrachain thioester bond was originally described in the human alpha-2-
108 macroglobulin (A₂M), protease inhibitor and C3 protein, central components of the
109 complement system (Dodds and Law, 1998). Since then, members of the TEP superfamily
110 have been identified and characterized in numerous distant phyla from Ecdysozoans and
111 Lophotrochozoans to Deuterostomes. The TEP superfamily is divided in two subfamilies
112 displaying distinct functions, complement factors and A₂M (Shokal and Eleftherianos, 2017),
113 supported by the presence of anaphylatoxin (ANA) and C-terminal C345C domains only in
114 members of the complement factor subfamily. The complement factors subfamily contains
115 human C3, C4 and C5 proteins, and their orthologs. The A₂M subfamily comprises A₂M,
116 pregnancy zone protein-like (PZP), complement C3 and pregnancy zone protein-like (PZP)
117 A₂M domain-containing 8 (CPAMD8), and cell surface glycoprotein CD109. Besides
118 recently, two other classes of TEP were discovered in insects: the insect TEP (iTTEP) and the
119 macroglobulin/complement-related (MCR) (Sekiguchi et al., 2012), with iTTEP molecules
120 constituting clearly a third subfamily inside TEP superfamily, based on phylogenetic analysis
121 (Shokal and Eleftherianos, 2017).

122 TEP proteins are key components of innate immunity: Complement factors are deposited on
123 the pathogen surface, enhancing their phagocytosis (opsonisation), they can recruit phagocytic
124 cells at sites of infection (chemotaxis), and are also able of direct pathogen lysis; while A₂Ms
125 are pan-protease inhibitors that sequester pathogen proteases, inhibiting their activity, and
126 promoting their clearance (Williams and Baxter, 2014;Baxter et al., 2017).

127 TEPs seem to appear early in animal evolution: members of this family are present in a wide
128 variety of invertebrate species: in arthropods (Levashina et al., 2001;Blandin and Levashina,
129 2004;Buresova et al., 2011;Sekiguchi et al., 2012;Urbanová et al., 2015); crustaceans (Wu et
130 al., 2012;Li et al., 2017); cnidarians (Fujito et al., 2010) and mollusks (Zhang et al.,
131 2007;Yazzie et al., 2015).

132 In invertebrates, TEPs have been mostly studied in *Anopheles gambiae* and *Drosophila*
133 *melanogaster* (Vierstraete et al., 2004;Blandin et al., 2008). The *Anopheles gambiae* genome
134 encodes 19 homologues of invertebrate TEP (iTTEP) (AgTEP1-19), of which AgTEP1 is the
135 best functionally characterized (Blandin et al., 2008) and structurally the only crystallized
136 TEP from invertebrates (Baxter et al., 2007). Indeed, AgTEP1 is reported to play an opsonin
137 role in phagocytosis of gram-negative and gram-positive bacteria (Levashina et al.,
138 2001;Yassine and Osta, 2010;Yassine et al., 2012). Moreover, AgTEP1 is also able to bind
139 the plasmodium parasite surface and to promote its melanisation (Blandin et al., 2008), and so
140 plays an essential role in decreasing the plasmodium ookinetes load in mosquito's gut (Smith
141 et al., 2015). The *Drosophila melanogaster* genome encodes 6 homologues of iTTEP
142 (DmTEP1-6) (Blandin and Levashina, 2004). Except DmTEP5, DmTEPs are expressed in
143 potential immune tissues, i.e. hemocytes or fat body, and are up-regulated after immune
144 challenges with bacteria or yeast (Wertheim et al., 2005;Bou Aoun et al., 2010;Arefin et al.,
145 2017). DmTEP2, DmTEP3 and DmTEP6 bind to gram-negative, gram-positive bacteria and
146 fungi respectively, and play an opsonin role to promote the phagocytosis (Stroschein-
147 Stevenson et al., 2006;Bou Aoun et al., 2010). Furthermore a mutant fly line lacking the four
148 immune-inducible TEPs (TEP1-4) showed lower survival ability following either gram-
149 positive bacteria, fungi or parasitoid wasp immune challenges. This mutant fly line also

150 presented a reduced Toll pathway activation upon microbial infection, leading to reduced
151 antimicrobial peptide gene expressions (Dostálová et al., 2017). In this case the TEPs appear
152 like a real Pattern Recognition Receptor (PRR). More recently, a TEP has been identified in
153 the shrimp *Litopenaeus vannamei* (LvTEP1), and its protective role against both gram-
154 positive and gram-negative bacteria but also virus was highlighted by a knockdown approach
155 (Li et al., 2017). Also, the potential immune role of the *Chlamys farreri* TEP (CfTEP) has
156 been suggested as CfTEP transcripts expression is induced following bacterial challenges,
157 while CfTEP protein undergoes an apparent cleavage in a similar manner as the vertebrate's
158 C3 complement (Xue et al., 2017).

159 Herein, we report the functional characterization of TEP protein from the freshwater snail
160 *Biomphalaria glabrata* (BgTEP). In the last decade, *Biomphalaria glabrata* has attracted
161 particular attention due to its medical and epidemiological importance as a vector for
162 Schistosomiasis disease (King et al., 2005). Authors have invested considerable efforts to
163 investigate the molecular interactions and compatibility (susceptibility/resistance status)
164 between *B. glabrata* and its parasite *Schistosoma mansoni* (Mitta et al., 2012;Portet et al.,
165 2017) to help in the discovery of new ways to prevent and/or control Schistosomiasis disease
166 in the field (Tennessen et al., 2015). Co-immunoprecipitation (CoIP) experiments, using as
167 bait the previously characterized *S. mansoni* polymorphic mucins (SmPoMucs) (Roger et al.,
168 2008a;Roger et al., 2008b;Roger et al., 2008c), enabled us to identify putative SmPoMuc-
169 snail interacting immune receptors. An immune complex has been characterized associating 3
170 partners: 1) SmPoMucs parasite molecules, 2) Fibrinogen-related proteins (FREP) which is a
171 highly diversified lectin family from snail hemolymph secreted by hemocytes and considered
172 as pathogen recognition receptors (Adema et al., 1997;Zhang et al., 2004;Pila et al., 2017), 3)
173 and a third partner, the newly identified *B.glabrata* Thioester-containing Protein (TEP) named
174 BgTEP (Moné et al., 2010).

175 Structural modeling prediction displays a strong similarity between BgTEP and AgTEP1,
176 while the expression in different tissues reveals a wide distribution, showing a high
177 abundance in snail hemocytes (circulating immune cells). Moreover, BgTEP displays a
178 capacity to bind to diverse pathogens, followed by a biochemical processing of the protein.
179 Among hemocyte cells, BgTEP is secreted by a range of blast-like cells which are not
180 phagocytic cells, but which are involved in the formation of the capsule surrounding the
181 parasite. All these results suggest that BgTEP must play a role in innate immune response
182 against pathogens by assuming an opsonin function as evidenced in arthropods.

183

184 **Materials & Methods:**

185 Ethical statements

186 Our laboratory holds permit # A66040 for experiments on animals, which was obtained from
187 the French Ministry of Agriculture and Fisheries and the French Ministry of National
188 Education, Research, and Technology. The housing, breeding and care of the utilized animals
189 followed the ethical requirements of our country. The experimenter possesses an official
190 certificate for animal experimentation from both of the above-listed French ministries (Decree
191 # 87–848, October 19, 1987). The various protocols used in this study have been approved by
192 the French veterinary agency of the DRAAF Languedoc-Roussillon (Direction Régionale de
193 l'Alimentation, de l'Agriculture et de la Forêt), Montpellier, France (authorization # 007083).

194

195 Biological materials

196 One snail strain of *Biomphalaria glabrata* was used in this study: the albino Brazilian strain.
197 The snails were exposed to several pathogens, gram-negative bacteria culture of *Escherichia*
198 *coli*, gram-positive bacteria culture of *Micrococcus luteus* and yeast culture of *Saccharomyces*

199 *cerevisiae*. Snails were also exposed to one Guadeloupean parasite strain of *Schistosoma*
200 *mansoni*.

201 This last interaction was chosen for its incompatibility, which means, the snail immune
202 response is efficient and an encapsulation around the parasite is observed.

203

204 Antibody production

205 We used two types of antibody. The first is a purified polyclonal anti-BgTEP antibody
206 produced in rabbits and named: anti-BgTEP-PEP. The rabbit immune serum was purified on
207 an affinity column raised against immunogenic peptide produced by Eurogentec. The
208 sequence of the peptide is H2N - SSY GSK SFR PDT NIT C - CONH2. The second antibody
209 is a purified polyclonal anti-BgTEP antibody produced in chickens and named: anti-BgTEP-
210 RP. A truncated recombinant protein which corresponds to the C-terminal part of BgTEP
211 from nucleotides 1,036 to 1,445 corresponding to the amino-acid sequence [LIDRQ-
212 CLNCCP], was produced in *E. coli* and purified on nickel column (Agrobio manufactures).

213

214 Genomic, Proteic and Structural Organization of BgTEP

215 The cDNA sequence of BgTEP, was BLASTed against VectorBase to characterize the exon-
216 intron structure (Supp. Table 1 & Supp. Figure 1). The 5'UTR and 3'UTR ends were
217 determined using data of the Brazilian *B. glabrata* transcriptome (Dheilly et al., 2015)
218 Prediction of the BgTEP three-dimensional (3D) structure and alignment with AgTEP1
219 (2PN5 published in 2015 (Baxter et al., 2007) were performed using the I-Tasser and TM-
220 align servers (Zhang and Skolnick, 2005;Zhang, 2008). The 3D structure was obtained by
221 multiple threading using the I-Tasser server (available online), which combines two protein
222 structure prediction methods: threading and *ab initio* prediction (Roy et al., 2010). Structural
223 similarities between the functional domain of AgTEP1 and BgTEP were determined by
224 calculating a TM-score. A TM-score greater than 0.5 reveals significant alignment, whereas a
225 TM-score less than 0.17 indicates a random similarity.

226

227 Phylogenetic Organization of TEP family members

228 Homologous sequences were identified using BLAST searches against the GenBank non
229 redundant database (Bethesda, USA). For phylogenetic analyses, multiple protein sequence
230 alignments were performed with ClustalW using the BLOSUM62 substitution matrix model.
231 The neighbor-joining method (Poisson substitution model; uniform substitution rate;
232 gaps/missing data treatment: pairwise deletion) was used to generate the phylogenetic tree. A
233 bootstrap analysis of 2000 replications was carried out to assess the robustness of the tree
234 branches. A total of 125 full length sequences from TEP superfamily proteins (Supp. Table 2)
235 were chosen to construct the tree based on the best BLAST matches against the NCBI
236 database. The phylogenetic analysis was performed using MEGA 5 software (Tamura et al.,
237 2011).

238

239 BgTEP interactome and immunoblotting approaches

240 *B. glabrata* plasma preparation: The hemolymph was extracted from the head-foot
241 according to previously described procedures (Sminia and Barendsen, 1980). After recovering
242 the hemolymph, a first centrifugation for 5 min at 5,000 g and 4°C enables to eliminate
243 hemocytes then, an ultracentrifugation for 2.5h at 40,000 g at 4°C enables to eliminate the
244 hemoglobin. The plasma was only used for the study of the BgTEP profile in naive snail.

245 Interactome samples preparation: Two milliliters of fresh hemolymph was recovered
246 from naive snails and centrifuged 5 min at 5,000 g to eliminate hemocytes. In parallel, live
247 pathogen samples were recovered, centrifuged (5,000 g 5 min) and washed with CBSS (NaCl
248 2.8 g/L, KCL 0.15 g/L, Na₂HPO₄ 0.07 g/L, MgSO₄.7H₂O 0.45 g/L, CaCl₂.2H₂O 0.53 g/L,

249 NaHCO₃ 0.05 g/L) and kept at the bottom of the tube until used. Depending on the pathogen,
250 samples were recovered from 500 µL of bacteria-culture of *Escherichia coli* and *Micrococcus*
251 *luteus* (1.2x10⁷ part/mL), 500 µL of yeast-culture of *Saccharomyces cerevisiae* (7.5x10⁵
252 part/mL) and 500 miracidia (snail infective stage of *S. mansoni*) or 500 primary sporocysts
253 (first snail-stage of *S. mansoni*). Two interactome conditions were then tested for each
254 pathogen used: pathogens exposed either to (i) the host cell-free hemolymph fraction or (ii)
255 to CBSS that mimics the internal host environment. Samples were incubated at 26°C
256 (temperature of snail environment) for 30 min, to observe a rapid interaction between the
257 BgTEP and the pathogens, or 3 hours, to observe potential processing of BgTEP. Pathogen
258 samples incubated with hemolymph or CBSS buffer were then washed with CBSS as
259 described above and pellet pathogens were extracted using Laemmli buffer (Bio-Rad) with β-
260 mercaptoethanol. The biological material was used to study the BgTEP profile after
261 interaction between the cell-free hemolymph and pathogens.

262 Western-blot: For protein blot, 8 µL of snail plasma or 10 µL of interactome samples
263 were run on a 7.5% SDS-polyacrylamide precast gel (Mini Protean TGX Precast Gel Bio-
264 Rad) for bacteria and yeast samples and 12% SDS-polyacrylamide precast gel (Mini Protean
265 TGX Precast Gel Bio-Rad) for parasite samples and transferred onto a 0.2 µm PVDF
266 membrane with Trans-Blot Turbo Transfer Pack (Bio-Rad). After saturation during 1h at
267 37°C in TBSTM (1x TBS (500 mM Tris-HCl, 1.5 M NaCl, pH 7.5), 0.05% Tween-20, 5%
268 non-fat milk), the protein blots were incubated for 1.5h at room temperature in TBSTM, with
269 a 1:200 dilution of anti-BgTEP-PEP antibody for the western-blot on ultracentrifuged naive
270 snail plasma and a 1:2000 dilution of anti-BgTEP-RP antibody for the interactome
271 experiments. The blots were washed 3 times with TBST, and further incubated with 1:4000
272 dilution of the manufactured horseradish peroxidase-conjugated goat anti-mouse IgG
273 antibody (Pierce) for the western-blot on naive snail plasma, and 1:4000 dilution of the
274 manufactured horseradish peroxidase-conjugated goat anti-chicken IgY antibody (Southern
275 Biotech) for the interactome experiments, in TBSTM for 1h10 at room temperature. Blots
276 were finally washed 3 times with TBST, and then revealed in the presence of an enhanced
277 chemiluminescent substrate (Super Signal West Pico Chemiluminescent Substrate, Thermo
278 Scientist).

279

280 BgTEP Immunolocalisation in hemocyte populations:

281 In vivo pathogen infection: The Brazilian *Biomphalaria glabrata* strain was in vivo
282 injected with *Saccharomyces cerevisiae*, Alexa Fluor 488 conjugate (injection of 5 µL [2.10⁵
283 yeast/µL]) (Invitrogen, BioParticles Z23373). The hemolymph was recovered 3h post-
284 injection.

285 BgTEP immunolocalisation: The hemolymph was extracted from the head-foot
286 according to previously described procedures (Sminia and Barendsen, 1980). The hemocytes
287 were plated for 1h on polystyrene chamber-slides. Hemocytes were fixed using 4%
288 paraformaldehyde for 10 min, followed by a cell permeabilization step with 0.01% Triton X-
289 100 for 20 min. Cells were incubated with a 1:100 dilution of anti-BgTEP-PEP for 1h
290 followed by a 1:1000 dilution of the manufactured fluorescent goat anti-mouse IgG antibody
291 (Thermo Fisher Scientific – Alexa Fluor 594) for 50 minutes. After the BgTEP labelling, the
292 actin was labelled with (Alexa Fluor 488 or 594) Phalloidine (Thermo Fisher Scientific) for
293 20 min and the cell nucleus with Dapi (Biotum) for 30 sec. Observations were done on a
294 confocal microscopy using a Zeiss LSM 700 microscope, with 4 lasers (405, 488, 555 and
295 633 nm).

296

297 BgTEP tissue Expression Analysis

298 Tissue recovery: Tissues were collected from 10 snails under binocular microscope
299 dissection. Albumen gland, head-foot, hepato-pancreas, and ovotestis were recovered. For
300 hemocytes recovery, the hemolymph of 50 snails was collected, and cells were recovered
301 after centrifugation at 10,000g for 10 min.

302 Infection by multiple pathogens of whole snails: Contact with gram-positive and gram-
303 negative bacteria and yeast were executed according to previously described procedures
304 (Deleury et al., 2012); briefly snails were bathed with 10⁸ part/mL of pathogens for 1h, then
305 snails were washed. For the parasite infection, each snail was exposed for 6h to 10 miracidia
306 in 5 mL of pond water. For each pathogen stimulation, 36 snails were used: 4 replicates using
307 a pool of 3 snails were realised at 3 time points (6h, 12h and 24h after stimulation). As control
308 condition, 4 replicates using a pool of 3 snails were recovered for the evaluation of the basal
309 expression of BgTEP.

310 RNA extraction and Quantitative RT-PCR protocol: Snail total RNA was extracted
311 with TRIzol reagent (Sigma Life Science) according to manufacturer's instructions and
312 subsequently reverse transcribed to first strand cDNA using Maxima H Minus First Strand
313 cDNA Synthesis Kit with dsDNase (Thermo Scientific) according to manufacturer's
314 instructions.

315 Real-time RT-PCR analyses were performed with the LightCycler 480 System (Roche) in a
316 10 µL volume comprising 2 µL of cDNA diluted to 1:200 with ultrapure-water, 5 µL of No
317 Rox SYBR Master Mix blue dTTP (Takyon), 1 µL of ultrapure-water and 10 µM of each
318 primer. The primers used for the RT-QPCR are TEP-R:
319 ACCATTAGATCCACTGGAAGATA TEP-F: CTGACTTACCCTCGCTC for BgTEP, and
320 S19-R: CCTGTATTTGCATCCTGTT S19-F: TTCTGTTGCTCGCCAC for S19 ribosomal
321 protein gene used as housekeeping gene. The cycling program was: denaturation step at 95°C
322 for 2 minutes, 40 cycles of amplification (denaturation at 95°C for 10 sec, annealing at 58°C
323 for 20 sec and elongation at 72°C for 30 sec), with a final elongation step at 72°C for 5
324 minutes. QPCR was ended by a melting curve step from 65°C to 97°C with a heating rate of
325 0.11°C/sec and continuous fluorescence measurement. For each reaction, the cycle threshold
326 (Ct) was determined using the 2nd derivative method of the LightCycler 480 Software release
327 1.5 (Roche). PCR experiments were performed in triplicate (technical replicates) from four
328 biological replicates. The mean value of Ct was calculated. Corrected melting curves were
329 checked using the Tm-calling method of the LightCycler 480 Software release 1.5. Results
330 were normalized with respect to the S19 ribosomal protein gene, as previously described
331 (Galinier et al., 2013), and ΔCt values were calculated. BgTEP expression normalized by S19
332 housekeeping gene expression.

333

334 In situ histological localisation of BgTEP

335 Snail infection: Snail were infected with the parasite strain as previously described,
336 briefly each snail was exposed for 6h to 10 miracidia in 5 mL of pond water.

337 Immunocytochemistry procedures: Snails were fixed in Halmi's fixative (4.5%
338 mercuric chloride, 0.5% sodium chloride, 2% trichloroacetic acid, 20% formol, 4% acetic
339 acid and 10% picric acid-saturated aqueous solution). Embedding in paraffin and transverse
340 histological sections (10-µm) were performed. The slides were stained using Anti-BgTEP-
341 PEP antibody according to a previously developed protocol (Roger et al., 2008a).

342 Slides re-hydration was performed in serial toluene, 95%, 70%, and 30% ethanol and finally
343 PBS bathing (Saline Phosphate Buffer: pH 7,4-7,5; 8,41 mM Na₂HPO₄, 1.65 mM Na₂H₂PO₄,
344 45,34 mM NaCl; H₂O milliQ q.s.p.). The slides were immersed in a permeabilizing PBS
345 solution containing 0.5% Triton X-100 during 15 min. A saturation step was performed in
346 PBS buffer containing 1% gelatine hydrolysate (Bellon, France), 1% normal goat serum
347 (NGS, Sigma) and 0.1% NaN₃ (Sigma) during 1h at room temperature. Slides were then

348 successively incubated with the Anti-BgTEP-PEP antibody dilution 1:100 for 1h30 at room
349 temperature and with an Alexa Fluor 594 anti-rabbit IgG (Thermo Fisher Scientific) diluted
350 1:1000 for 1h at room temperature. Slides were mounted in Vectashield and stored in dark at
351 4°C. The slide observation was carried out by epifluorescence and light microscopy using a
352 Zeiss axioscope 2 microscope (Carl Zeiss AG) and a Leica DC350FX camera (Leica).

353

354 **Results:**

355 Organization of BgTEP

356 BgTEP presents all the characteristics of known iTep family members, including a signal
357 peptide for secretion, several predicted N-glycosylation sites, the canonical thioester motif
358 (GCGEQ), the complement component domain (pfam PF07678), the Alpha2 Macroglobulin
359 receptor binding domain (pfam PF07677) and finally numerous cysteines including the 6 C-
360 terminal ones which are a signature of iTeps (Moné et al., 2010). At the genomic level,
361 BgTEP is composed of 37 exons spreading on 10 scaffolds of the *Biomphalaria glabrata*
362 genome assembly (Adema et al., 2017) (Figure 1A). Exon-intron boundaries were conserved
363 along the sequence (Supp. Table 1). At the secondary structure level, BgTEP protein
364 possesses an N-terminal alpha helix characteristic of signal peptide for secreted proteins, like
365 other iTeps. As expected, BgTEP is composed of 8 MG (Macroglobulin) domains, a
366 succession of several β -sheets related to the related fibronectin type III (fnIII) domains, with
367 insertions of a LNK domain nested into MG6 and a CUB domain (β -sheet domain), and of a
368 TED domain (α -helical thioester domain) between MG7 and MG8. In the C-terminal part we
369 find a cluster of 6 cysteine residues (Figure 1A).

370 Furthermore, we have investigated the BgTEP protein tertiary structure with the structure
371 prediction software I-Tasser (Figure 1B), using AgTEP1 from *Anopheles gambiae* as
372 reference structure. The scores obtained for this prediction were highly significant (TM Score
373 = 0.98), indicating the robustness of the prediction, and also alignment structural conservation
374 with the AgTEP1 reference, despite a low similarity at the amino-acid level (29%) (Figure
375 1B).

376

377 Phylogenetic analysis of BgTEP

378 Since the first identification of BgTEP in 2010 (Moné et al., 2010), many new members of the
379 TEP superfamily were discovered and more particularly in the *Mollusca* phylum. Thus, we
380 conducted a new phylogenetic analysis in order to position BgTEP among the main TEP
381 family members. To do it, we used 125 amino acid sequences of full length TEP superfamily
382 proteins (see Supp Table 2), including complement-like factors, macroglobulin complement-
383 related proteins (MCR), A₂M, complement 3 and pregnancy zone protein-like A₂M domain-
384 containing 8 (CPAMD8), CD 109 glycoproteins and iTep proteins, to construct a
385 phylogenetic tree with the neighbor-joining method (Figure 2). Phylogenetic analysis
386 confirms the localization of BgTEP in the group of iTeps. This analysis segregates the
387 proteins in three major groups; the complement component group which includes complement
388 and complement-like factors as well as MCR (Figure 2, blue shades), the A₂M group
389 comprising A₂M and CPAMD8 (Figure 2, red shades), and the group formed by cell surface
390 TEP (CD 109) and iTep (Figure 2, green shades). However, the analysis reveals several
391 subgroups inside the group of iTep and CD 109. Indeed, vertebrate CD 109 and insect CD
392 109 form two distinct subgroups. Moreover, BgTEP is included in an additional well
393 supported subgroup constituted by both CD 109 and iTep from mollusk species, reflecting a
394 degree of similarity in the sequences of TEP proteins from *Mollusca* phylum. The mix of CD
395 109 and iTep in a same subgroup is probably the result of automatic and not manual
396 corrected annotation from Genbank without further characterization.

397

398 BgTEP Expression in snail tissues

399 A dissection of different organs (hemocytes, ovotestis, head-foot, hepatopancreas and
400 albumen gland) of *B. glabrata* has been done in order to identify the tissue distribution of the
401 BgTEP, by quantitative RT-PCR (Figure 3). BgTEP was expressed in all tissues examined
402 with different levels; a high expression is observed in hemocytes, ovotestis and head-foot,
403 while a lower expression is observed in hepatopancreas and albumen gland (Figure 3).

404 A western blot experiment was performed on ultracentrifuged plasma (obtained after
405 ultracentrifugation of cell-free hemolymph) from naïve snails with a polyclonal anti-BgTEP
406 antibody, designed from a C-terminal peptide and called anti-BgTEP-PEP. We detected 3
407 bands of BgTEP in naive plasma (Figure 4). The first band, abundantly detected, at
408 approximately 200 kDa corresponds to the full-length BgTEP (Figure 4). Two lower bands
409 were observed at 60 and 30 kDa, respectively, and were interpreted as BgTEP processed
410 forms, resulting from a fine regulation of proteolysis by endogenous proteases observed also
411 in the plasma of anopheles (Figure 4).

412

413 BgTEP expression after immune challenges

414 In some invertebrate models, the iTep has been suggested to play a role during immune
415 infections, most of the time assuming an opsonin function. To investigate this potential
416 function in our *B. glabrata* model, we have measured the relative expression ratio by RT-
417 QPCR of BgTEP transcripts in response to different immune challenges (Figure 5). The
418 expression varies greatly depending of pathogen immune challenges but also during the
419 infection kinetic. Indeed, BgTEP expression is decreased after both *E. coli* and *S. cerevisiae*
420 challenges, while it is up regulated after *M. luteus* and *S. mansoni* stimulations (Figure 5).
421 When challenging with *E. coli*, the expression is rapidly lowered nearly 5 fold, and returns to
422 basal expression level after 24 hours, while following the *S. cerevisiae* challenge, BgTEP
423 expression is only slightly decreased by 2 fold from 12h to 24h post-challenge. For the other
424 challenges, we observed a two-fold increase in expression from 6 to 12 hours after *M. luteus*
425 stimulation, while for the *S. mansoni* challenge, the BgTEP expression increases regularly
426 from 6 to 24 hours from 2 to 3.5-fold.

427 As the BgTEP is differently expressed following challenges with different pathogen types, we
428 wonder if this protein might be involved in pathogen recognition, like in other invertebrate
429 models, where TEPs were shown to play an opsonin role in immune recognition response.

430

431 BgTEP capacities to link pathogen surface

432 To investigate the BgTEP ability to bind to the pathogen surface during infections we have
433 performed interactions at 26°C between *B. glabrata* cell-free hemolymph and *M. luteus*, *E.*
434 *coli*, *S. cerevisiae* or *S. mansoni* parasites (Figure 6). As a comparison, pathogens alone were
435 incubated during the same time in CBSS buffer (negative control) (Figure 6), while naive
436 cell-free hemolymph alone was used as positive control of BgTEP detection (first lane from
437 Figure 6A). The experiments were performed with whole live pathogens allowing for two
438 interaction times (30 min and 3h) in order to see the binding and the potential processing of
439 BgTEP protein during the binding kinetic. At the end of the interactions, pathogens were
440 recovered by centrifugation, washed, and bound BgTEP was revealed by western blot using
441 the anti-BgTEP-RP antibody (Figure 6). The anti-BgTEP-RP antibody used is different from
442 the previously used anti-BgTEP-PEP. This antibody raised against the C-terminal part of the
443 BgTEP (from aa 200 to aa 500) revealed several additional bands in positive control among
444 which three more intense ones at 200, 100 and 30 kDa (Figure 6A, lane 1).

445 Interestingly we demonstrate that the full length of BgTEP (band of 200 kDa) binds all
446 bacteria, yeast (Figure 6A) or metazoan parasite (Figure 6B). Moreover and surprisingly we
447 observed that a short processed form of TEP (30 kDa) present in naïve hemolymph can also

448 bind to all pathogens. Minor other bands were detected by the anti-BgTEP-RP antibody from
449 75 to 150 kDa with *E. coli*, *M. luteus* and *S. cerevisiae*, that differed between 30 minutes and
450 3 hours interactions (Figure 6A). Multiple processed forms are able to bind the pathogen.

451 Besides, we investigated the interaction between BgTEP and two different development-
452 stages of *S. mansoni* parasite; the miracidia, a free-living and swimming larvae which infest the
453 host snail, and the primary sporocyst which is the first intra-molluscal stage of the parasite
454 after infestation (Figure 6B). For the interaction with *S. mansoni*, we demonstrate that the full
455 length of BgTEP (band of 200 kDa) also binds the parasite at the two development-stages, but
456 at a lower extent with miracidia stage (Figure 6B). We observed also that the short processed
457 form of BgTEP (30 kDa) binds the parasite but is no longer detected with miracidia stage at 3
458 hours interaction (Figure 6B). Moreover, minor bands were also detected from 100 to 150
459 kDa for primary sporocyst stage all along the interaction, whereas only weak one is observed
460 for miracidia stage and only at 30 minutes interaction (Figure 6B). This discrepancy between
461 the two profiles suggests different BgTEP binding and processing abilities for the two parasite
462 stages.

463 To conclude, we observe the binding of BgTEP to all the pathogens tested but also a
464 differential BgTEP pathogen-dependent processing occurring during opsonization. The
465 disappearance or the presence of new forms detected on the pathogen surface highly suggests
466 a fine proteolytic regulation of linked BgTEP potentially required for opsonization or/and
467 encapsulation.

468

469 BgTEP expression in hemocyte sub-populations

470 Since BgTEP can bind different pathogens and display an opsonin role in other models, we
471 have focused on immune cellular level. First, we performed an immune-labelling experiment
472 upon plated hemocytes to investigate which sub-type is expressing BgTEP (Figure 7A).
473 Immunolocalization shows that all hemocytes do not produce and secrete BgTEP and its
474 expression is only restricted to a subset of blast-like cells (Figure 7A). Moreover, to estimate
475 the BgTEP-positive blast-like cell proportion in hemocytes, we performed a BgTEP positive-
476 cell quantification by flow cytometry (Figure 7B). The BgTEP-positive-cells correspond to
477 20.7% of total hemocytes (Figure 7B). The blast-like cells corresponding to 47% of total
478 hemocytes, counting by May-Grünwald Giemsa coloration (Supp. Figure 2), among them
479 44% are BgTEP-positive-cells.

480

481 Role of BgTEP-positive hemocytes in phagocytosis and/or encapsulation processes

482 As BgTEP is also expressed by some immune cells, we have focused on its phagocytosis and
483 encapsulation role. A phagocytosis experiment has been performed using a strain of yeast of
484 *S. cerevisiae*, Alexa Fluor 488 conjugate. The yeasts were injected *in vivo* in snails, and after
485 3h the plasma was recovered to visualize yeast phagocytosis by confocal microscopy (Figure
486 8A). Interestingly, yeast phagocytosis was observed but only by some immune cells which do
487 not express BgTEP. Among immune cells, we have not observed phagocytosis in
488 granulocytes. The average number of yeast per phagocytic cell is between 4 and 5. By
489 immunolocalization, we have observed that BgTEP-positive cells, do not perform
490 phagocytosis (Figure 8A). The same approach was performed using *E. coli* or *S. aureus*
491 bacteria, and again no phagocytosis was observed in blast-like cells, expressing or not the
492 BgTEP (data not shown). In conclusion, hemocytes expressing BgTEP are not directly
493 implicated in the pathogen phagocytosis but secrete an opsonin factor able to bind pathogen
494 surface, facilitating its elimination by cooperation with other immune cell sub-types.

495 Finally, the role of BgTEP-positive hemocytes in parasite encapsulation was assessed through
496 *in-situ* immunocytochemistry localisation. Following infection of *B. glabrata* by an
497 incompatible *S. mansoni* parasite, the snail mounted a cellular immune response resulting in

498 the encapsulation of the parasite by the hemocytes. Histological sections of *S. mansoni*
499 infected snails were performed after 24h post exposure. Using red-labelled anti-BgTEP-PEP
500 antibody (Figure 8B), we observed a diffuse labelling around the parasite into the capsule.
501 This means that BgTEP get in contact with the parasite, which is consistent with the *in vitro*
502 binding demonstration of BgTEP to primary sporocyst surface (fig 6B). We also observed
503 numerous BgTEP-positive hemocytes migrating to the site of encapsulation, as well as
504 surrounding the capsule and also inside the capsule (Figure 8B). A less intense labelling is
505 also observed in head-foot cell wall, which is in accordance with the BgTEP expression
506 measured by Q-RT-PCR in this tissue. These results support the hypothesis of the opsonin
507 role of BgTEP, which could promote the recruitment of other hemocyte subtypes necessary to
508 mount an efficient phagocytosis or encapsulation immune response.
509

510 **Discussion:**

511 BgTEP characterization started 25 years ago with a first report of a proteinase inhibitory
512 activity in the plasma of *Biomphalaria glabrata* (Bender et al., 1992). In this paper, the
513 authors described a probable alpha macroglobulin with trypsin inhibitory activity that is
514 sensitive to methylamine treatment. They reported then inhibitory activities against serine-,
515 cysteine- and metallo-proteases, and demonstrated inhibitory activity against a cysteine
516 proteinase released from *S. mansoni* larval stage (Bender and Bayne, 1996; Fryer et al., 1996).
517 They finally succeeded in purifying the protein responsible for these activities and obtained
518 the first 18 amino acids by Edman degradation sequencing. This sequence corresponded to the
519 N-terminal part of BgTEP excluding its signal peptide. A detailed biochemical
520 characterization showed that the protein is structured as a tetramer, and undergoes a
521 conformational change after protease cleavage, leading to the activation of the thioester bond.
522 The foreign protease is then sequestered and cannot react with bulky substrates (Bender and
523 Bayne, 1996).

524 In 2010, we have characterized BgTEP for their ability to form an immune complex with a
525 snail plasmatic proteins, the FREP and parasite mucin, the SmPoMuc by a Co-
526 immunoprecipitation approaches (Moné et al., 2010). Interestingly, only peptides from the C-
527 terminal part of the protein were recovered in this immune complex by mass spectrometry
528 analysis, suggesting a possible processing of BgTEP. The protein sequence was characterized
529 and displayed all the features of invertebrate TEP proteins with 8 MG domains, with nested
530 insertion of a CUB domain, the canonical TED domain, several putative N-glycosylation
531 sites, and moreover the C-terminal signature composed by six cysteine residues (Figure 1).

532 Herein we explore the molecular involvement of BgTEP in the innate immune response of the
533 freshwater snail *Biomphalaria glabrata* face to a large panel of intruders. To elucidate the
534 nature of BgTEP regarding the TEP's superfamily, we performed a phylogenetic analysis
535 based on full-length amino acid sequences that clearly segregates the three main groups;
536 complement factors, A₂M and TEP/CD109 (Figure 2). BgTEP clearly clusters within the
537 iTEP/CD109 group, and so does not cluster with the A₂M group despite the previous report of
538 BgTEP proteinase inhibitory activity (Bender and Bayne, 1996). Furthermore, several
539 subgroups could be distinguished within the iTEP/CD109 group. Insect CD109, vertebrate
540 CD109, as well as iTEPs from other arthropod phyla than hexapods (crustaceans, chelicerates
541 and myriapods) form well-sustained subgroups, whereas iTEPs from insects were not all
542 clustered together (Figure 2) as also shown in previous studies (Bou Aoun et al., 2010).
543 Interestingly, a subgroup is formed by both iTEPs and CD109 from mollusks, which clusters
544 more distantly from iTEP and CD109 proteins of all other phyla. This may be related to the
545 primary sequence similarities between mollusk iTEPs, but it might also be correlated to
546 functional specificity. Among this mollusk subgroup, many proteins are predicted from
547 automatic genome annotations, which means that they were not previously characterized, with

548 the exception of clams TEP (Zhang et al., 2007) and bobtail squid CD109 (Yazzie et al.,
549 2015). This could explain why CD109 and TEP cluster together, as automatic annotations are
550 often inexact when based on partial or truncated sequences as found in draft genome
551 sequences. A deeper characterization and re-annotation of each of these molecules deserves
552 further investigation.

553 We then investigated the intron-exon structure of the BgTEP gene using the recently
554 published *B. glabrata* genome (Adema et al., 2017), and the VectorBase database to identify
555 37 exons distributed on 10 different scaffolds from the genome assembly (BglaB1 assembly)
556 (Figure 1). Such organization is consistent with the one of human TEP gene families like
557 human CD109, A₂M and C3 complement factor genes that respectively comprise 33, 36 and
558 41 exons (Prosper, 2011). In invertebrate, the genomic structure identification of *Chlamys*
559 *farreri* TEP highlights that its gene contains 40 exons, this results is very close to 37 exons of
560 BgTEP. Moreover, the phylogenetic analysis of the TEP superfamily reflecting a mollusk
561 TEP group close to vertebrate TEP and CD109 suggests a lower evolutionary divergence than
562 with other TEPs (Zhang et al., 2009). Indeed, the BgTEP gene is very different from the
563 AgTEP1 gene that is composed of only 11 exons (Personal communication from Vectorbase),
564 suggesting a strikingly different evolutionary story between snail and mosquito TEPs.
565 Despite these differences, it is not evident that phylogenetic proximity and genomic
566 organization may be linked to TEP function as TEP activity is more conditioned by its
567 quaternary structure than by its primary sequence (Williams and Baxter, 2014). A structural
568 protein prediction and alignment reveals a very close conformation between AgTEP1 and
569 BgTEP. This result reflects a potential common function between those two complement-like
570 components which otherwise display a low primary sequence similarity. Indeed, AgTEP1,
571 which is the most studied and the only crystallized invertebrate TEP, was shown to opsonize
572 Gram negative and positive bacteria, and to promote their phagocytosis (Levashina et al.,
573 2001). AgTEP1 was also shown to target Plasmodium parasites for lysis through a hemocyte
574 encapsulation process (Blandin and Levashina, 2004).

575 The high expression level of BgTEP transcripts in snail hemocytes, the immune specialized
576 circulating cells of *Biomphalaria* snails, correlates with results obtained for *Anopheles*
577 *gambiae* (Levashina et al., 2001), emphasizing its potential immune function. However, high
578 expression levels were also observed in others tissues like ovotestis. Ovotestis is the center of
579 eggs and spermatozooids production, and is of first importance for putative immune molecule
580 transmission to progeny. Indeed, it has been shown in a previous study that *B. glabrata*
581 invested in its offspring's protection (Baron et al., 2013) and that several immune factors
582 including BgTEP (called A2M when published) were recovered by proteomic analysis in egg
583 masses (Hathaway et al., 2010). Production of BgTEP transcripts in ovotestis is thus highly
584 relevant with this potential transfer of protection to eggs and progeny. Moreover, a recent
585 study showed in *Anopheles* that during spermatogenesis, AgTEP1 binds to and removes
586 damaged cells, increasing fertility rates (Pompon and Levashina, 2015).

587 In naïve snail, BgTEP is constitutively secreted in the hemolymph, and expressed at high
588 level in circulating immune cells. Western blot on plasma, using the anti-BgTEP-PEP
589 antibody, revealed the presence of full length but also of several processed forms of BgTEP.
590 This means that BgTEP certainly undergoes a cleavage by a, so far, undefined plasmatic
591 factor, as it is also the case for AgTEP1 (Levashina et al., 2001; Shokal et al., 2017). Indeed,
592 in *A. gambiae*, the AgTEP1 is found in full-length and also in a processed form named TEP-
593 cut, this allows to have a complex pattern of TEP prepared to answer to a pathogen attack
594 (Levashina et al., 2001). Moreover, in hemolymph, the AgTEP1 is maintained by a complex
595 of two proteins APL1 and LRIM1 to stabilize the processed form and avoid the unspecific
596 binding of the thioester domain to non relevant substrates (Levashina et al., 2001; Shokal et
597 al., 2017). In vertebrate, complement component pathway displays a major role in the innate

598 immune system. The complement component C3 activation is finely regulated by a serie of
599 proteolytic cleavage leading to the formation of different fragments of C3 such C3a, C3b,
600 iC3b, C3dg. All proteolytic fragments, such C3a, a small complement fragment, mediate
601 chemotaxis and local inflammation. C3b acts as an opsonin by enhancing cellular
602 phagocytosis by binding to the pathogen's surface. The C3b derived fragment, iC3b and C3dg
603 can bind to the pathogen and promote its uptake (Hamad et al., 2010;Feng et al., 2015;Foley
604 et al., 2015).

605 Three types of hemocytes have been described in *Biomphalaria glabrata* hemolymph; the
606 blast-like cells, the granulocytes and the hyalinocytes (Cavalcanti et al., 2012) (Supp. Figure
607 2). An immune-labelling of plated hemocytes using a fluorescent dye conjugated anti-BgTEP-
608 PEP antibody revealed that only blast-like cells are positive for BgTEP, but only for 44% of
609 them as determined by flow cytometry analysis (Figure 7A). This observation suggests that
610 more hemocyte subtypes would exist in the *B. glabrata* hemolymph than the ones previously
611 estimated solely based on cell morphology analysis. A functional hemocyte characterization
612 deserves further investigation.

613 We then developed an experimental approach to evaluate the BgTEP binding properties to
614 different pathogens: gram-positive and gram-negative bacteria, yeast and schistosome (Figure
615 6). Each of the four pathogens were incubated alive with cell-free hemolymph for 30 minutes
616 or 3 hours at 26°C, before being harvested, washed extensively and, denatured in Laemmli
617 buffer and analysed by western blot for the presence of BgTEP. After 30 minutes of
618 incubation, we observed that BgTEP is recovered bound to all pathogens tested in the present
619 study, in its full length as well as in processed form (30 kDa) (Figure 6). This suggests that
620 the binding of BgTEP may be not always mediated by the thioester bond. Indeed, as the
621 activation of the thioester bond needs a proteolytic cleavage as prerequisite, the presence of
622 bound full-length protein evidences that the thioester bond might be not systematically
623 required. These results are consistent with the previously observed binding of AgTEP1 to
624 bacteria that was shown to occur in both thioester-dependant and thioester-independent way
625 (Levashina et al., 2001 Cell). This could also indicate that BgTEP is probably able to bind
626 pathogen surfaces directly or indirectly associated with other immune relevant partners. After
627 3 hours of interaction, less full length and 30 kDa processed forms of BgTEP were recovered
628 bounded to the surface of *E. coli*, *S. cerevisiae* and *S. mansoni*, indicating a processing over
629 time (Figure 6). Interestingly new forms of BgTEP appeared after 3 hours interactions with
630 yeast and bacteria, which could result from the processing of full-length protein already
631 bound (Figure 6A). Another stiking result is the differences observed in the binding of BgTEP
632 between miracidia and sporocysts, which are two successive developmental-stages of *S.*
633 *mansoni* parasite (Figure 6B). Indeed, more processed forms of BgTEP are recovered bound
634 to sporocysts than miracidia. Moreover, all the bands are more intense with sporocysts all
635 along the kinetic, and no bands were detected with miracidia after 3 hours, suggesting a
636 higher specificity of BgTEP for sporocyste stage than for miracidia. Such a result is not
637 surprising as sporocyst is the consequence of miracidia transformation which consists mainly
638 in the loss of epidermal ciliated plates and tegument renewal. Several proteomic and glycomic
639 studies have highlighted differences from one developmental stage to another (Hokke et al.,
640 2007 Trends in Parasitol; Peterson et al., 2009 Int J of Parasitol). Thesz results would suggest
641 a subtle ability for the snail immune machinery to distinguish various developemental stages
642 of the parasite.

643 All put together, these results clearly indicate that BgTEP can be associated with the surface
644 of live intruders and could be differentially processed depending on the pathogen type. For
645 the first time, we approached also the dynamic of immune complex formation with a selective
646 processing of bound TEP between pathogens. So even pathogens were sensing by this
647 complement like factor (Tetreau et al., 2017), others maturation factors would be involved to

648 induce an appropriated immune response. Nevertheless, the binding mechanisms are still
649 unclear and need to be deeply characterized.

650 As the multiple forms of BgTEP can interact with several pathogens, we investigated the
651 relative expression of BgTEP transcript by quantitative RT-PCR following various immune
652 challenges (Figure 5). BgTEP expression is modulated, independently of the pathogens used
653 for the stimulation. *E. coli* and *S. cerevisiae* challenges decreased its expression, while *M.*
654 *luteus* and *S. mansoni* challenges up regulated it, like previously highlighted in transcriptomic
655 analysis of the snail immune response after bacterial and fungal infections (Deleury et al.,
656 2012). This suggests different immune response pathways toward all these different kinds of
657 pathogens. Interestingly, *S. mansoni* is the only one inducing a constant increase from 6 h to
658 24 h of BgTEP transcript expression suggesting a role of first importance in the interaction
659 between *S. mansoni* and *Biomphalaria glabrata*, which is in agreement with the first
660 identification of BgTEP in a host-parasite immune complex (Moné et al., 2010).

661 Study of the potential role of BgTEP in the immune response of the snail was completed by
662 performing an *in-situ* immunohistochemical analysis of *B. glabrata* snails infected with an
663 incompatible strain of *S. mansoni* (Figure 8B). The typical associated immune response
664 results in the encapsulation of the parasite by host hemocytes few hours post-infection. We
665 observed many BgTEP-positive hemocytes converging towards the encapsulated parasite,
666 surrounding the hemocyte capsule but also on head-foot. Moreover, we observed also a
667 diffuse labelling into the capsule around the parasite, suggesting the presence of BgTEP
668 protein at the parasite surface within the capsule. This observation suggests the participation
669 of BgTEP-positive hemocytes in the recruitment of capsule-forming hemocytes on the site of
670 infection, potentially via a putative α -2-macroglobulin receptor on their membrane (Coustau
671 et al., 2015; Paul et al., 2016; Pila et al., 2017). Besides, hemocytes converging to the site of
672 infection may also suggest a chemotaxis property of BgTEP due to a cleavage of bound TEP
673 into small fragments like an anaphylatoxin-like.

674 This study provides new insights about the potential immune function of BgTEP. We
675 demonstrate that its constitutive production by hemocytes must be modulated by immune
676 challenges, and that the full protein and its proteolytic fragments are able to bind at the
677 surface of different pathogens before and after specific cleavage maturing processes. Even if
678 the precise binding mechanism needs further characterization, our results suggest that BgTEP
679 displayed a potential role as opsonin by targeting pathogen surface.

680 Through this work we report the first characterization of an iTEP displaying a dual-role, as
681 possible existence was previously raised (Williams and Baxter, 2014), a role of A₂M and a
682 role of complement-like factor. Indeed, as described before BgTEP acts as a proteases
683 inhibitor (Bender and Bayne, 1996). But in the present study we also demonstrate that BgTEP
684 can bind different pathogens, including *S. mansoni* parasite and could participate in their
685 elimination.

686 Now, a more precise functional characterization is necessary to decipher the key role of the
687 BgTEP and its action dynamics during the immune response of the snail. For that, an
688 invalidation of the gene would be considered by CRISP/cas9 or a knock-down by RNAi.
689 Also, the nature and function of proteolytic products of BgTEP remain unknown and must be
690 explored to elucidate host pathogen interaction. Indeed, some pathogens circumvent the host
691 immune response by blocking or miscleaving complement components (Jusko et al.,
692 2014; Johnson et al., 2015; Luo et al., 2017).

693 In conclusion, progresses done in the recent years to depict the role of TEPs in the
694 antibacterial and antiparasite response for numerous distant unrelated phyla from primitive
695 Protostomes to Deuterostomes have shed light on the regulatory role of TEPs on the host
696 innate immune system. However, future studies are now timely needed to elucidate the
697 molecular processes by which TEPs are involved in host specific immune molecular response,

698 to ascertain their anti-pathogen immune function and their complex evolution in the animal
699 kingdom.

700

701 **Acknowledgements and funding information:**

702 We thank Mr. Chaparro Cristian to time use to read and improve the manuscript.

703 This work was supported by the French National Agency for Research (ANR) through a

704 project Invimory grant [ANR-13-JSV7-0009] to BG.

705

706 **References:**

707 Adema, C.M., Hertel, L.A., Miller, R.D., and Loker, E.S. (1997). A family of fibrinogen-
708 related proteins that precipitates parasite-derived molecules is produced by an
709 invertebrate after infection. *Proceedings of the National Academy of Sciences of the*
710 *United States of America* 94, 8691-8696.

711 Adema, C.M., Hillier, L.W., Jones, C.S., Loker, E.S., Knight, M., Minx, P., Oliveira, G.,
712 Raghavan, N., Shedlock, A., Do Amaral, L.R., Arican-Goktas, H.D., Assis, J.G.,
713 Baba, E.H., Baron, O.L., Bayne, C.J., Bickham-Wright, U., Biggar, K.K., Blouin, M.,
714 Bonning, B.C., Botka, C., Bridger, J.M., Buckley, K.M., Buddenborg, S.K., Lima
715 Caldeira, R., Carleton, J., Carvalho, O.S., Castillo, M.G., Chalmers, I.W.,
716 Christensens, M., Clifton, S., Cosseau, C., Coustau, C., Cripps, R.M., Cuesta-Astroz,
717 Y., Cummins, S.F., Di Stephano, L., Dinguirard, N., Duval, D., Emrich, S., Feschotte,
718 C., Feyereisen, R., Fitzgerald, P., Fronick, C., Fulton, L., Galinier, R., Gava, S.G.,
719 Geusz, M., Geyer, K.K., Giraldo-Calderón, G.I., De Souza Gomes, M., Gordy, M.A.,
720 Gourbal, B., Grunau, C., Hanington, P.C., Hoffmann, K.F., Hughes, D., Humphries,
721 J., Jackson, D.J., Jannotti-Passos, L.K., De Jesus Jeremias, W., Jobling, S., Kamel, B.,
722 Kapusta, A., Kaur, S., Koene, J.M., Kohn, A.B., Lawson, D., Lawton, S.P., Liang, D.,
723 Limpanont, Y., Liu, S., Lockyer, A.E., Lovato, T.L., Ludolf, F., Magrini, V.,
724 Mcmanus, D.P., Medina, M., Misra, M., Mitta, G., Mkoji, G.M., Montague, M.J.,
725 Montelongo, C., Moroz, L.L., Munoz-Torres, M.C., Niazi, U., Noble, L.R., Oliveira,
726 F.S., Pais, F.S., Papenfuss, A.T., Peace, R., Pena, J.J., Pila, E.A., Quelais, T., Raney,
727 B.J., Rast, J.P., Rollinson, D., Rosse, I.C., Rotgans, B., Routledge, E.J., Ryan, K.M.,
728 et al. (2017). Whole genome analysis of a schistosomiasis-transmitting freshwater
729 snail. *Nature Communications* 8, 15451.

730 Arefin, B., Kunc, M., Krautz, R., and Theopold, U. (2017). The Immune Phenotype of Three
731 <i>Drosophila</i> Leukemia Models. *G3: Genes/Genomes/Genetics*, g3.117.039487-g039483.039117.039487.

733 Baron, O.L., Van West, P., Industri, B., Ponchet, M., Dubreuil, G., Gourbal, B., Reichhart, J.-
734 M., and Coustau, C. (2013). Parental Transfer of the Antimicrobial Protein LBP/BPI
735 Protects *Biomphalaria glabrata* Eggs against Oomycete Infections. *PLoS Pathogens* 9,
736 e1003792-e1003792.

737 Baxter, R.H.G., Chang, C.-I., Chelliah, Y., Levashina, E.A., and Deisenhofer, J. (2007).
738 Structural basis for conserved complement factor-like function in the antimalarial
739 protein TEP1. 104.

740 Baxter, R.H.G., Contet, A., and Krueger, K. (2017). Arthropod Innate Immune Systems and
741 Vector-Borne Diseases. *Biochemistry* 56, 907-918.

742 Bender, R.C., and Bayne, C.J. (1996). Purification and characterization of a tetrameric alpha-
743 macroglobulin proteinase inhibitor from the gastropod mollusc *Biomphalaria glabrata*.
744 *Biochemical Journal* 316, 893-900.

745 Bender, R.C., Fryer, S.E., and Bayne, C.J. (1992). Proteinase inhibitory activity in the plasma
746 of a mollusc: evidence for the presence of alpha-macroglobulin in *Biomphalaria*

747 glabrata. *Comparative biochemistry and physiology. B, Comparative biochemistry*
748 102, 821-824.

749 Blandin, S., and Levashina, E.A. (2004). Thioester-containing proteins and insect immunity.
750 *Molecular Immunology* 40, 903-908.

751 Blandin, S.A., Marois, E., and Levashina, E.A. (2008). Antimalarial Responses in *Anopheles*
752 gambiae: From a Complement-like Protein to a Complement-like Pathway. *Cell Host*
753 & *Microbe* 3, 364-374.

754 Bou Aoun, R., Hetru, C., Troxler, L., Doucet, D., Ferrandon, D., and Matt, N. (2010).
755 Analysis of Thioester-Containing Proteins during the Innate Immune Response of
756 *Drosophila melanogaster*. *Journal of Innate Immunity* 3, 52-64.

757 Buresova, V., Hajdusek, O., Franta, Z., Loosova, G., Grunclova, L., Levashina, E.A., and
758 Kopáček, P. (2011). Functional Genomics of Tick Thioester-Containing Proteins
759 Reveal the Ancient Origin of the Complement System. *Journal of Innate Immunity* 3,
760 623-630.

761 Cavalcanti, M.G.S., Filho, F.C., Mendonça, A.M.B., Duarte, G.R., Barbosa, C.C.G.S., De
762 Castro, C.M.M.B., Alves, L.C., and Brayner, F.A. (2012). Morphological
763 characterization of hemocytes from *Biomphalaria glabrata* and *Biomphalaria*
764 straminea. *Micron (Oxford, England : 1993)* 43, 285-291.

765 Coustau, C., Gourbal, B., Duval, D., Yoshino, T.P., Adema, C.M., and Mitta, G. (2015).
766 Advances in gastropod immunity from the study of the interaction between the snail
767 *Biomphalaria glabrata* and its parasites: A review of research progress over the last
768 decade. *Fish & Shellfish Immunology*.

769 Deleury, E., Dubreuil, G., Elangovan, N., Wajnberg, E., Reichhart, J.M., Gourbal, B., Duval,
770 D., Baron, O.L., Gouzy, J., and Coustau, C. (2012). Specific versus non-specific
771 immune responses in an invertebrate species evidenced by a comparative de novo
772 sequencing study. *PLoS ONE* 7.

773 Dheilly, N.M., Duval, D., Mouahid, G., Emans, R., Allienne, J.-F., Galinier, R., Genthon, C.,
774 Dubois, E., Du Pasquier, L., Adema, C.M., Grunau, C., Mitta, G., and Gourbal, B.
775 (2015). A family of variable immunoglobulin and lectin domain containing molecules
776 in the snail *Biomphalaria glabrata*. *Developmental & Comparative Immunology* 48,
777 234-243.

778 Dodds, M.W., and Law, A.S.K. (1998). The phylogeny and evolution of the thioester bond-
779 containing proteins C3, C4 and α 2-macroglobulin. *Immunological Reviews* 166, 15-
780 26.

781 Dostálová, A., Rommelaere, S., Poidevin, M., and Lemaitre, B. (2017). Thioester-containing
782 proteins regulate the Toll pathway and play a role in *Drosophila* defence against
783 microbial pathogens and parasitoid wasps. *BMC Biology* 15, 79.

784 Feng, S., Liang, X., Kroll, M.H., Chung, D.W., and Afshar-Kharghan, V. (2015). von
785 Willebrand factor is a cofactor in complement regulation. *Blood* 125, 1034-1037.

786 Foley, J.H., Peterson, E.A., Lei, V., Wan, L.W., Krisinger, M.J., and Conway, E.M. (2015).
787 Interplay between fibrinolysis and complement: plasmin cleavage of iC3b modulates
788 immune responses. *Journal of Thrombosis and Haemostasis* 13, 610-618.

789 Fryer, S.E., Bender, R.C., and Bayne, C.J. (1996). Inhibition of cysteine proteinase from
790 *Schistosoma mansoni* larvae by alpha-macroglobulin from the plasma of *Biomphalaria*
791 glabrata. *The Journal of parasitology* 82, 343-347.

792 Fujito, N.T., Sugimoto, S., and Nonaka, M. (2010). Evolution of thioester-containing proteins
793 revealed by cloning and characterization of their genes from a cnidarian sea anemone,
794 *Haliplanella lineate*. *Developmental and Comparative Immunology* 34, 775-784.

795 Galinier, R., Portela, J., Moné, Y., Allienne, J.F., Henri, H., Delbecq, S., Mitta, G., Gourbal,
796 B., and Duval, D. (2013). Biomphalysin, a New β Pore-forming Toxin Involved in

797 Biomphalaria glabrata Immune Defense against Schistosoma mansoni. *PLoS*
798 *Pathogens* 9, e1003216-e1003216.

799 Hamad, O.A., Nilsson, P.H., Wouters, D., Lambris, J.D., Ekdahl, K.N., and Nilsson, B.
800 (2010). Complement Component C3 Binds to Activated Normal Platelets without
801 Preceding Proteolytic Activation and Promotes Binding to Complement Receptor 1.
802 *Journal of immunology (Baltimore, Md. : 1950)* 184, 2686-2692.

803 Hathaway, J.J.M., Adema, C.M., Stout, B.A., Mobarak, C.D., and Loker, E.S. (2010).
804 Identification of protein components of egg masses indicates parental investment in
805 immunoprotection of offspring by Biomphalaria glabrata (Gastropoda, Mollusca).
806 *Developmental and Comparative Immunology* 34, 425-435.

807 Johnson, J.B., Borisevich, V., Rockx, B., and Parks, G.D. (2015). A Novel Factor I Activity
808 in Nipah Virus Inhibits Human Complement Pathways through Cleavage of C3b.
809 *Journal of Virology* 89, 989-998.

810 Jusko, M., Potempa, J., Kantyka, T., Bielecka, E., Miller, H.K., Kalinska, M., Dubin, G.,
811 Garred, P., Shaw, L.N., and Blom, A.M. (2014). Staphylococcal proteases aid in
812 evasion of the human complement system. *Journal of innate immunity* 6, 31-46.

813 King, C.H., Dickman, K., and Tisch, D.J. (2005). Reassessment of the cost of chronic
814 helminthic infection: a meta-analysis of disability-related outcomes in endemic
815 schistosomiasis. *Lancet (London, England)* 365, 1561-1569.

816 Levashina, E.A., Moita, L.F., Blandin, S., Vriend, G., Lagueux, M., and Kafatos, F.C. (2001).
817 Conserved Role of a Complement-like Protein in Phagocytosis Revealed by dsRNA
818 Knockout in Cultured Cells of the Mosquito, Anopheles gambiae. *Cell* 104, 709-718.

819 Li, C., Li, H., Xiao, B., Chen, Y., Wang, S., L, K., Yin, B., Li, S., and He, J. (2017).
820 Identification and functional analysis of a TEP gene from a crustacean reveals its
821 transcriptional regulation mediated by NF- κ B and JNK pathways and its broad
822 protective roles against multiple pathogens. *Developmental and Comparative*
823 *Immunology* 70, 45-58.

824 Luo, S., Dasari, P., Reiher, N., Hartmann, A., Jacksch, S., Wende, E., Barz, D., Niemiec,
825 M.J., Jacobsen, I., Beyersdorf, N., Hünig, T., Klos, A., Skerka, C., and Zipfel, P.F.
826 (2017). The secreted Candida albicans protein Pra1 disrupts host defense by broadly
827 targeting and blocking complement C3 and C3 activation fragments. *Molecular*
828 *Immunology*.

829 Mitta, G., Adema, C.M., Gourbal, B., Loker, E.S., and Theron, A. (2012). Compatibility
830 polymorphism in snail/schistosome interactions: From field to theory to molecular
831 mechanisms. *Developmental and Comparative Immunology* 37, 1-8.

832 Moné, Y., Gourbal, B., Duval, D., Du Pasquier, L., Kieffer-Jaquinod, S., and Mitta, G.
833 (2010). A large repertoire of parasite epitopes matched by a large repertoire of host
834 immune receptors in an invertebrate host/parasite model. *PLoS Neglected Tropical*
835 *Diseases* 4.

836 Paul, L.M., Carlin, E.R., Jenkins, M.M., Tan, A.L., Barcellona, C.M., Nicholson, C.O.,
837 Michael, S.F., and Isern, S. (2016). Dengue virus antibodies enhance Zika virus
838 infection. *Clinical & Translational Immunology* 5, e117-e117.

839 Pila, E.A., Li, H., Hambrook, J.R., Wu, X., and Hanington, P.C. (2017). Schistosomiasis from
840 a Snail's Perspective: Advances in Snail Immunity. *Trends in Parasitology*.

841 Pompon, J., and Levashina, E.A. (2015). A New Role of the Mosquito Complement-like
842 Cascade in Male Fertility in Anopheles gambiae. *PLoS Biology* 13, 1-17.

843 Portet, A., Pinaud, S., Tetreau, G., Galinier, R., Cosseau, C., Duval, D., Grunau, C., Mitta, G.,
844 and Gourbal, B. (2017). Integrated multi-omic analyses in Biomphalaria-Schistosoma
845 dialogue reveal the immunobiological significance of FREP-SmPoMuc interaction.
846 *Developmental & Comparative Immunology*.

- 847 Prosper, J.Y.A. (2011). *Characterization of CD109*. University of Toronto.
- 848 Roger, E., Gourbal, B., Grunau, C., Pierce, R.J., Galinier, R., and Mitta, G. (2008a).
849 Expression analysis of highly polymorphic mucin proteins (Sm PoMuc) from the
850 parasite *Schistosoma mansoni*. *Molecular and Biochemical Parasitology* 157, 217-
851 227.
- 852 Roger, E., Grunau, C., Pierce, R.J., Hirai, H., Gourbal, B., Galinier, R., Emans, R., Cesari,
853 I.M., Cosseau, C., and Mitta, G. (2008b). Controlled Chaos of Polymorphic Mucins in
854 a Metazoan Parasite (*Schistosoma mansoni*) Interacting with Its Invertebrate Host
855 (*Biomphalaria glabrata*). *PLoS Neglected Tropical Diseases* 2, e330-e330.
- 856 Roger, E., Mitta, G., Moné, Y., Bouchut, A., Rognon, A., Grunau, C., Boissier, J., and
857 Théron, A. (2008c). Molecular determinants of compatibility polymorphism in the
858 *Biomphalaria glabrata*/*Schistosoma mansoni* model: new candidates identified by a
859 global comparative proteomics approach. *Molecular and biochemical parasitology*
860 157, 205-216.
- 861 Roy, A., Kucukural, A., and Zhang, Y. (2010). I-TASSER: a unified platform for automated
862 protein structure and function prediction. *Nature protocols* 5, 725-738.
- 863 Sekiguchi, R., Fujito, N.T., and Nonaka, M. (2012). Evolution of the thioester-containing
864 proteins (TEPs) of the arthropoda, revealed by molecular cloning of TEP genes from a
865 spider, *Hasarius adansoni*. *Developmental and Comparative Immunology* 36, 483-489.
- 866 Shokal, U., and Eleftherianos, I. (2017). Evolution and Function of Thioester-Containing
867 Proteins and the Complement System in the Innate Immune Response. *Frontiers in*
868 *Immunology* 8, 1-9.
- 869 Shokal, U., Kopydlowski, H., and Eleftherianos, I. (2017). The distinct function of
870 *Tep2* and *Tep6* in the immune defense of *Drosophila*
871 *melanogaster* against the pathogen *Photorhabdus*. *Virulence* 5594, 1-15.
- 872 Sminia, T., and Barendsen, L. (1980). A comparative morphological and enzyme
873 histochemical study on blood cells of the freshwater snails *Lymnaea stagnalis*,
874 *Biomphalaria glabrata*, and *Bulinus truncatus*. *Journal of Morphology* 165, 31-39.
- 875 Smith, R.C., Barillas-Mury, C., and Jacobs-Lorena, M. (2015). Hemocyte differentiation
876 mediates the mosquito late-phase immune response against *Plasmodium* in *Anopheles*
877 *gambiae*. *Proc Natl Acad Sci U S A* 112, E3412-3420.
- 878 Stroschein-Stevenson, S.L., Foley, E., O'farrell, P.H., and Johnson, A.D. (2006).
879 Identification of *Drosophila* Gene Products Required for Phagocytosis of *Candida*
880 *albicans*. *PLoS Biology* 4, e4.
- 881 Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5:
882 Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary
883 Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution* 28,
884 2731-2739.
- 885 Tennessen, J.A., Théron, A., Marine, M., Yeh, J.-Y., Rognon, A., and Blouin, M.S. (2015).
886 Hyperdiverse Gene Cluster in Snail Host Conveys Resistance to Human Schistosome
887 Parasites. *PLoS Genetics* 11, e1005067.
- 888 Tetreau, G., Pinaud, S., Portet, A., Galinier, R., Gourbal, B., and Duval, D. (2017). Specific
889 Pathogen Recognition by Multiple Innate Immune Sensors in an Invertebrate. *Front*
890 *Immunol.*
- 891 Urbanová, V., Šíma, R., Šauman, I., Hajdušek, O., and Kopáček, P. (2015). Thioester-
892 containing proteins of the tick *Ixodes ricinus*: Gene expression, response to microbial
893 challenge and their role in phagocytosis of the yeast *Candida albicans*. *Developmental*
894 *& Comparative Immunology* 48, 55-64.
- 895 Vierstraete, E., Verleyen, P., Baggerman, G., D'hertog, W., Van Den Bergh, G., Arckens, L.,
896 De Loof, A., and Schoofs, L. (2004). A proteomic approach for the analysis of

- 897 instantly released wound and immune proteins in *Drosophila melanogaster*
898 hemolymph. *Proceedings of the National Academy of Sciences of the United States of*
899 *America* 101, 470-475.
- 900 Wertheim, B., Kraaijeveld, A.R., Schuster, E., Blanc, E., Hopkins, M., Pletcher, S.D., Strand,
901 M.R., Partridge, L., and Godfray, H.C.J. (2005). Genome-wide gene expression in
902 response to parasitoid attack in *Drosophila*. *Genome Biology* 6, R94-R94.
- 903 Williams, M., and Baxter, R. (2014). The structure and function of thioester-containing
904 proteins in arthropods. *Biophysical Reviews* 6, 261-272.
- 905 Wu, C., Noonin, C., Jiravanichpaisal, P., Söderhäll, I., and Söderhäll, K. (2012). An insect
906 TEP in a crustacean is specific for cuticular tissues and involved in intestinal defense.
907 *Insect Biochemistry and Molecular Biology* 42, 71-80.
- 908 Xue, Z., Wang, L., Liu, Z., Wang, W., Liu, C., Song, X., Wang, L., and Song, L. (2017). The
909 fragmentation mechanism and immune-protective effect of CfTEP in the scallop
910 *Chlamys farreri*. *Dev Comp Immunol* 76, 220-228.
- 911 Yassine, H., Kamareddine, L., and Osta, M.A. (2012). The Mosquito Melanization Response
912 Is Implicated in Defense against the Entomopathogenic Fungus *Beauveria bassiana*.
913 *PLOS Pathogens* 8, e1003029.
- 914 Yassine, H., and Osta, M.A. (2010). *Anopheles gambiae* innate immunity. *Cellular*
915 *Microbiology* 12, 1-9.
- 916 Yazzie, N., Salazar, K.A., and Castillo, M.G. (2015). Identification, molecular
917 characterization, and gene expression analysis of a CD109 molecule in the Hawaiian
918 bobtail squid *Euprymna scolopes*. *Fish & Shellfish Immunology* 44, 342-355.
- 919 Zhang, H., Song, L., Li, C., Zhao, J., Wang, H., Gao, Q., and Xu, W. (2007). Molecular
920 cloning and characterization of a thioester-containing protein from Zhikong scallop
921 *Chlamys farreri*. *Molecular Immunology* 44, 3492-3500.
- 922 Zhang, H., Wang, L., Song, L., Zhao, J., Qiu, L., Gao, Y., Song, X., Li, L., Zhang, Y., and
923 Zhang, L. (2009). The genomic structure, alternative splicing and immune response of
924 *Chlamys farreri* thioester-containing protein. *Developmental and Comparative*
925 *Immunology* 33, 1070-1076.
- 926 Zhang, S.-M., Adema, C.M., Kepler, T.B., and Loker, E.S. (2004). Diversification of Ig
927 superfamily genes in an invertebrate. *Science (New York, N.Y.)* 305, 251-254.
- 928 Zhang, Y. (2008). I-TASSER server for protein 3D structure prediction. *BMC Bioinformatics*
929 9, 40-40.
- 930 Zhang, Y., and Skolnick, J. (2005). TM-align: a protein structure alignment algorithm based
931 on the TM-score. *Nucleic Acids Research* 33, 2302-2309.

932
933
934

935 **Figure 1: BgTEP organization**

- 936 **A. Schematic representation of BgTEP sequence organization. The top black lines correspond**
937 **to *Biomphalaria glabrata* genome scaffolds, with the corresponding scaffold numbers. The**
938 **center part is an Exon-Intron schematic representation, the squares are exons, the lines are**
939 **introns, the number corresponds to the exon number, and finally the exon colors correspond**
940 **to the protein domain colors. The lower part corresponds to the schematic domain**
941 **arrangement of BgTEP protein. It starts from N-terminal by a signal peptide (SP) domain**
942 **(blue) followed by macroglobulin domain 1 (MG1) (yellow), MG2 (magenta), MG3 (orange),**
943 **MG5 (magenta), MG6 (yellow), linker domain (LNK) (green), MG7 (magenta), CUB (grey),**
944 **thioester domain (TED) (black) and MG8 (red).**
- 945 **B. Overview of three-dimensional BgTEP structure predicted by I-Tasser software, using**
946 ***Anopheles gambiae* TEP1 (AgTEP1). The colored domains correspond to the colors of the**

947 schematic structure (A). Comparison of BgTEP and AgTEP1; despite a low similarity at the
948 amino-acid level (29%), a high conserved spatial conformation (TM Score = 0.98) was
949 observed between BgTEP and TEP1 of *Anopheles gambiae*.

950

951 Figure 2: Phylogenetic Tree of TEP superfamily

952 Phylogenetic analysis of the TEP superfamily full length protein sequences from 125
953 members. Complement factor groups are colored in blue shades, alpha-2 macroglobulin
954 groups are colored in red shades, and iTEP and CD 109 groups are colored in green shades.
955 BgTEP is indicated in bold type. A bootstrap analysis of 2000 replications was carried out on
956 the tree inferred from the neighbor joining method and the values are shown at each branch
957 of the tree.

958

959 Figure 3: Tissue Expression of BgTEP

960 Transcription profile of BgTEP in different tissues extracted from a pool of 10 snails.
961 Quantitative RT-PCR was performed on RNA from tissues dissected from snail. Five snail
962 tissues are dissected: Albumen gland, Hepatopancreas, Head-foot, Ovotestis and finally
963 Hemocytes. The BgTEP expression is normalised by S19 housekeeping transcript expression,
964 then the relative tissues-specific expression of BgTEP is compared to the non-immune-related
965 tissue: the albumen gland.

966

967 Figure 4: BgTEP Secretion

968 Eight microliters of *B. glabrata* ultracentrifugated plasma and a monoclonal anti-BgTEP
969 antibody were used for Western-Blot. The negative control includes an Ab+pep (anti-BgTEP
970 antibody and BgTEP peptide) pre-incubation. Arrows correspond to putative full-length and
971 two processed forms of the protein present in naive hemolymph.

972

973 Figure 5: Immune-Inducible Transcription of BgTEP

974 Relative expression ration of BgTEP transcripts compared to ribosomal protein S19
975 transcripts. Quantitative RT-PCR was performed from whole snails, and relative expression
976 ratio were normalized compared to the one obtained in naïve conditions (unchallenged
977 snails). BgTEP expression after challenges with *M. luteus* (decreasing hatching), *E. coli*
978 (increasing hatching), *S. cerevisiae* (rectangle) and *S. mansoni* (square) was monitored at 3
979 time points (6, 12 and 24 hours) post challenge and compared to naive expression (black).

980

981 Figure 6: Immunoblotting analysis of BgTEP

982 Western blot analysis of living pathogens having interacted or not with *B. glabrata* cell-free
983 hemolymph, using anti-BgTEP-RP antibody. Ultracentrifuged cell-free hemolymph from
984 naïve snail was used as positive control of BgTEP detection (Naïve). For each interaction
985 condition, negative control (C-) corresponds to the pathogen alone, interactome (Int.)
986 corresponds to the interaction between the pathogen and *B. glabrata* cell-free hemolymph.
987 Red stars correspond to putative full-length (250 kDa) and cleaved protein (30 kDa)
988 observed in all interactions and black stars indicate bands between 75 and 150 kDa
989 corresponding to BgTEP alternative processed forms. A. Comparison of BgTEP binding and
990 time-dependent processing after 30 minutes and 3 hours of interaction between cell-free
991 hemolymph and *M. luteus*, *E. coli*, *S. cerevisiae*, and comparison with naive hemolymph
992 profile. B. Comparison of BgTEP binding and time-dependent processing after 30 minutes
993 and 3 hours of interaction between snail cell-free hemolymph and *S. mansoni* at two
994 development-stages (miracidia and primary sporocyst).

995

996 Figure 7: Cellular Expression of BgTEP

997 *A. Immunolocalization of BgTEP in hemocyte populations by confocal microscopy. Three cell*
998 *types are represented in Biomphalaria glabrata hemolymph; the hyalinocytes with*
999 *cytoplasmic projections, the granulocytes with granules into the cytoplasm and the blast-like*
1000 *cells, the smallest cells determined with May-Grünwald Giemsa coloration (Supp. Figure 2),*
1001 *with a proportion expressing the BgTEP. The green labelling corresponds to the actin, the*
1002 *blue is cell nucleus and the red is the BgTEP. B. Flow cytometry profile of BgTEP-positive*
1003 *hemocytes in hemolymph. A total number of hemocytes of n=13,785 were recovered.*
1004 *Comparison of hemocytes labelled with anti-BgTEP antibody and hemocytes labelled only*
1005 *with secondary anti-body (fluorescent antibody) as negative control. The total hemocytes are*
1006 *shown according to FSC (forward-scattered light, representing cell size) and SSC (side-*
1007 *scattered light, representing cell granularity). The red dots correspond to BgTEP-positive*
1008 *hemocytes. C. Flow cytometry quantification of BgTEP-positive hemocytes in hemolymph.*
1009 *Comparison of the hemocyte fluorescence between the hemocytes labelled with anti-BgTEP*
1010 *antibody (red) and hemocytes labelled only with secondary anti-body (fluorescent antibody)*
1011 *as negative control (bleu). A fluorescent cut-off was determined and 20.7% of hemocytes are*
1012 *BgTEP-positive.*

1013

1014 Figure 8: Opsonin Role of BgTEP

1015 *A. Co-immunolocalisation of phagocytic and BgTEP-positive hemocytes by confocal*
1016 *microscopy. Labelling has been performed on a hemolymph fraction. On picture 1, green*
1017 *fluorescent-yeast phagocytosis by hemocytes is shown, for that the hemocyte nucleus are*
1018 *labelled in blue (dapi) and the actin is labelled in red. On picture 2, green fluorescent-yeast*
1019 *are phagocytosed, hemocyte nucleus are labelled in blue and the BgTEP is labelled in red*
1020 *with an anti-BgTEP antibody coupled with red fluorescent-secondary-antibody. B. Histologic*
1021 *slide of a S. mansoni parasite encapsulation by hemocytes 24h post-infection. White arrows*
1022 *show the BgTEP-positive cells around and in the capsule and also in the head-foot. The*
1023 *BgTEP is labelled by anti-BgTEP antibody coupled with red fluorescent-secondary-antibody,*
1024 *on histological slide.*

1025

1026 *Supplementary Figure 1:*

1027 *Web-logo representation of the consensus sequence of intron-exon junction in BgTEP gene.*

1028

1029 *Supplementary Figure 2:*

1030 *May-Grünwald Giemsa staining of B. glabrata hemocytes revealing the three hemocyte sub-*
1031 *types; hyalinocytes, granulocytes and blast-like cells.*

1032

Figure 1.TIFF

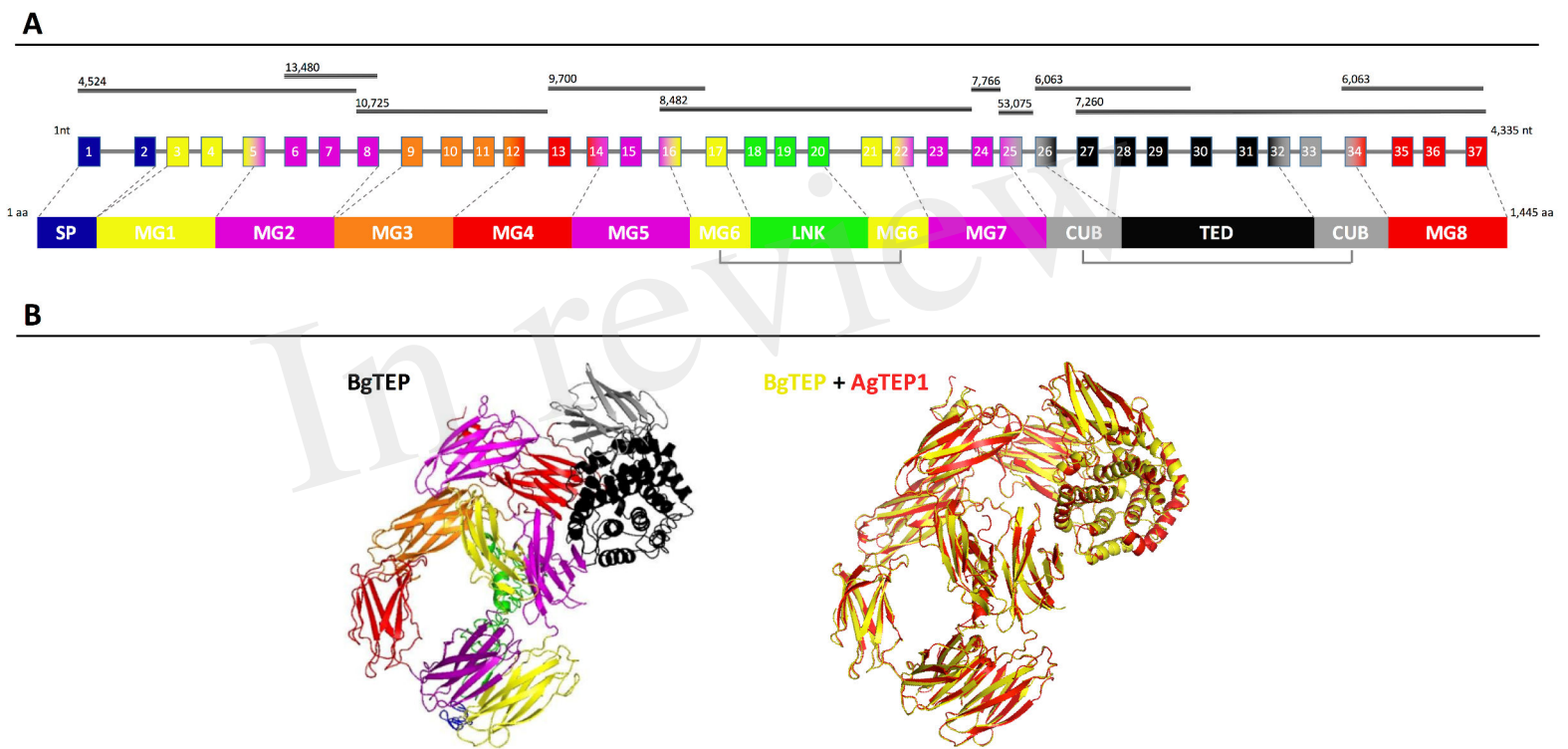


Figure 2.TIFF

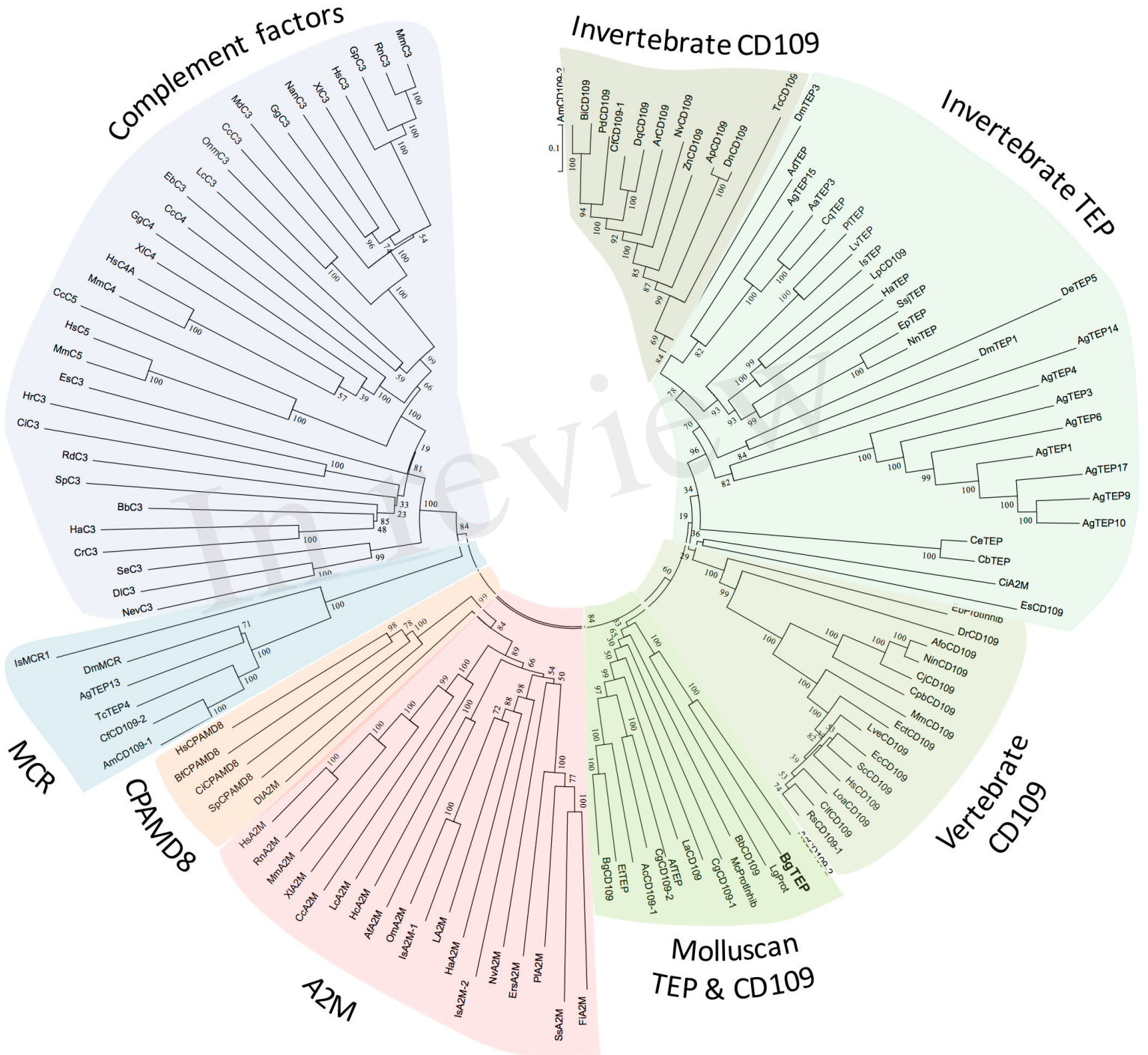


Figure 3.TIFF

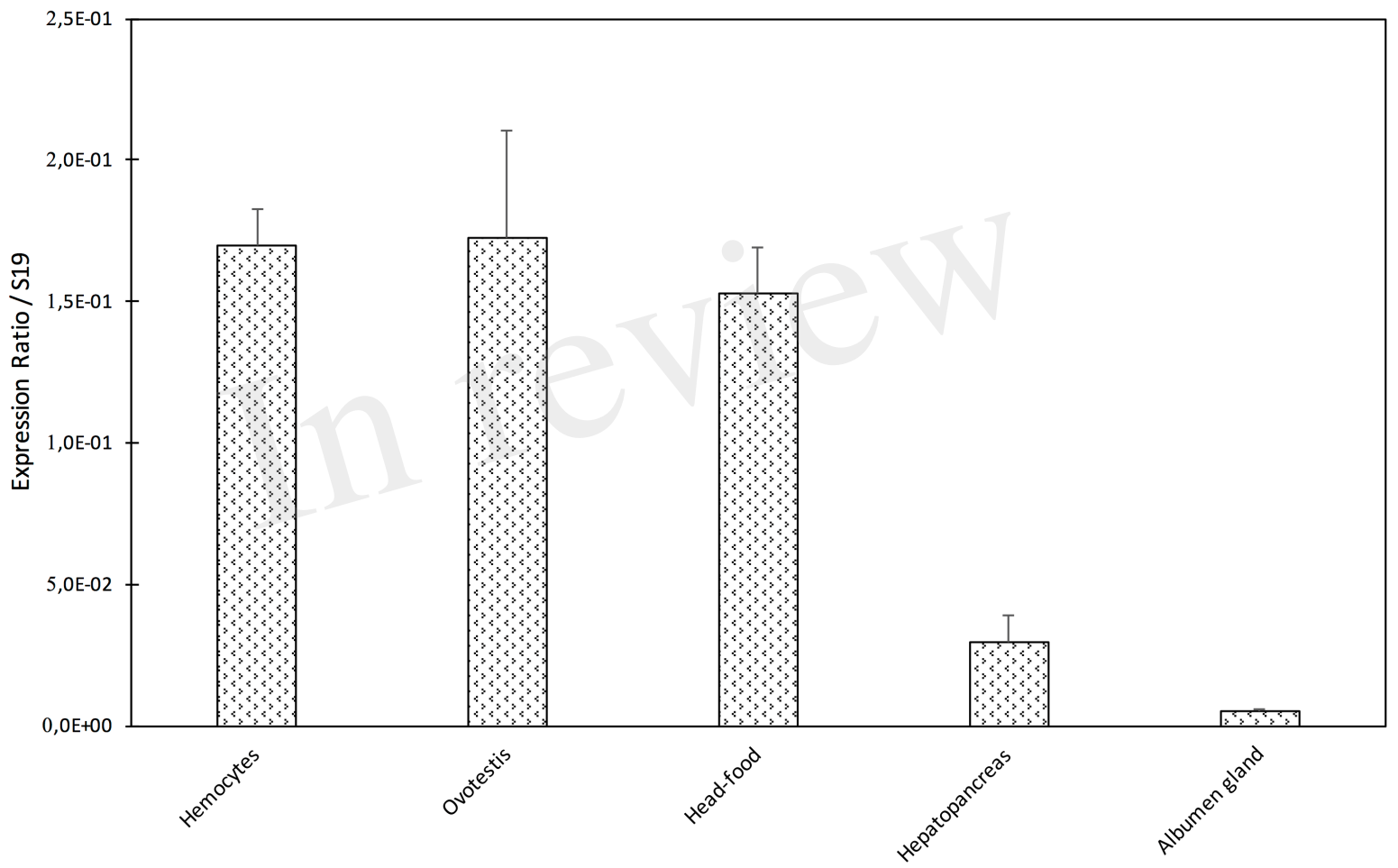


Figure 4.TIFF

Plasma



Ab

Ab + pep

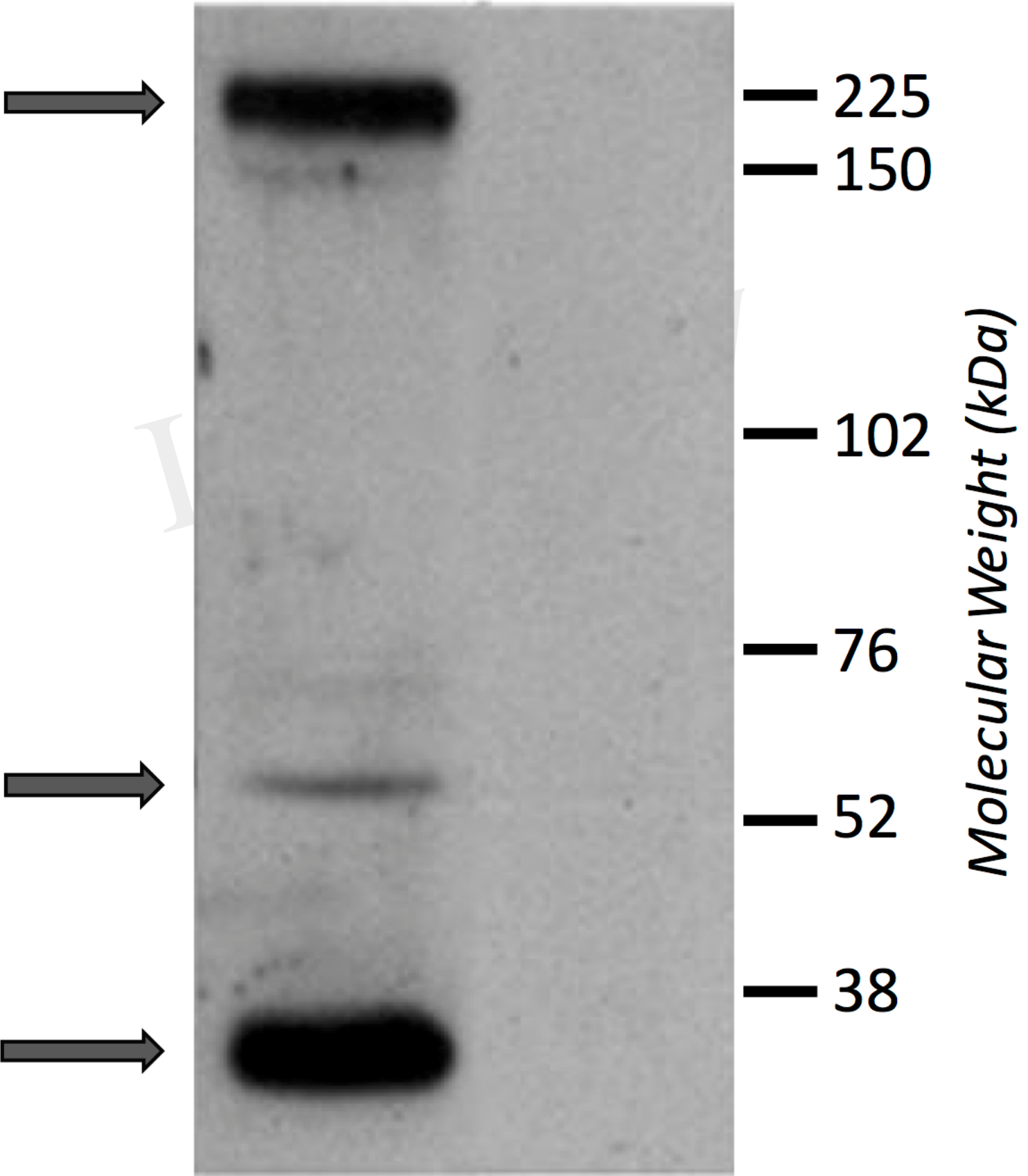
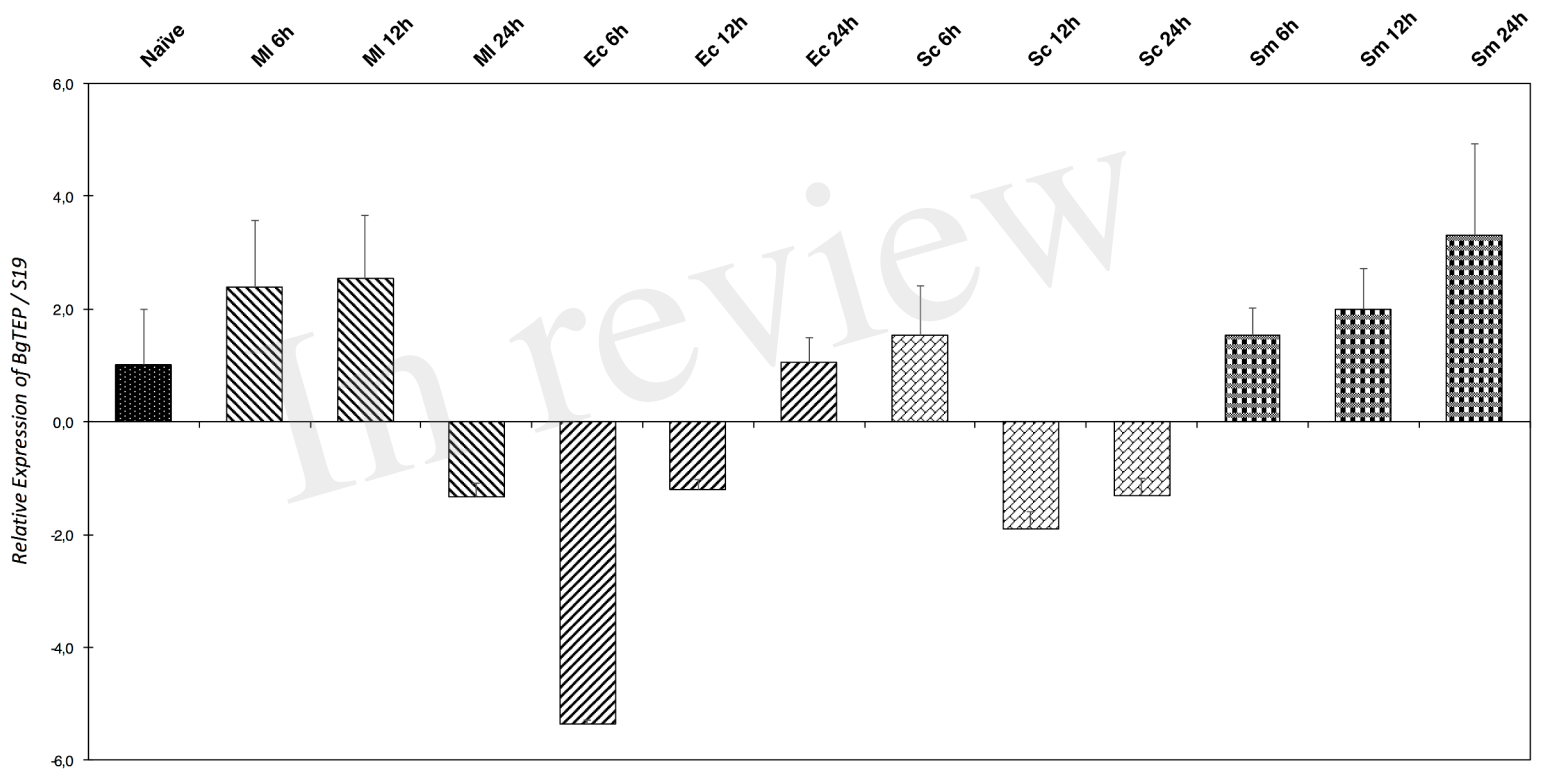


Figure 5.TIFF



A

Figure 6.TIFF

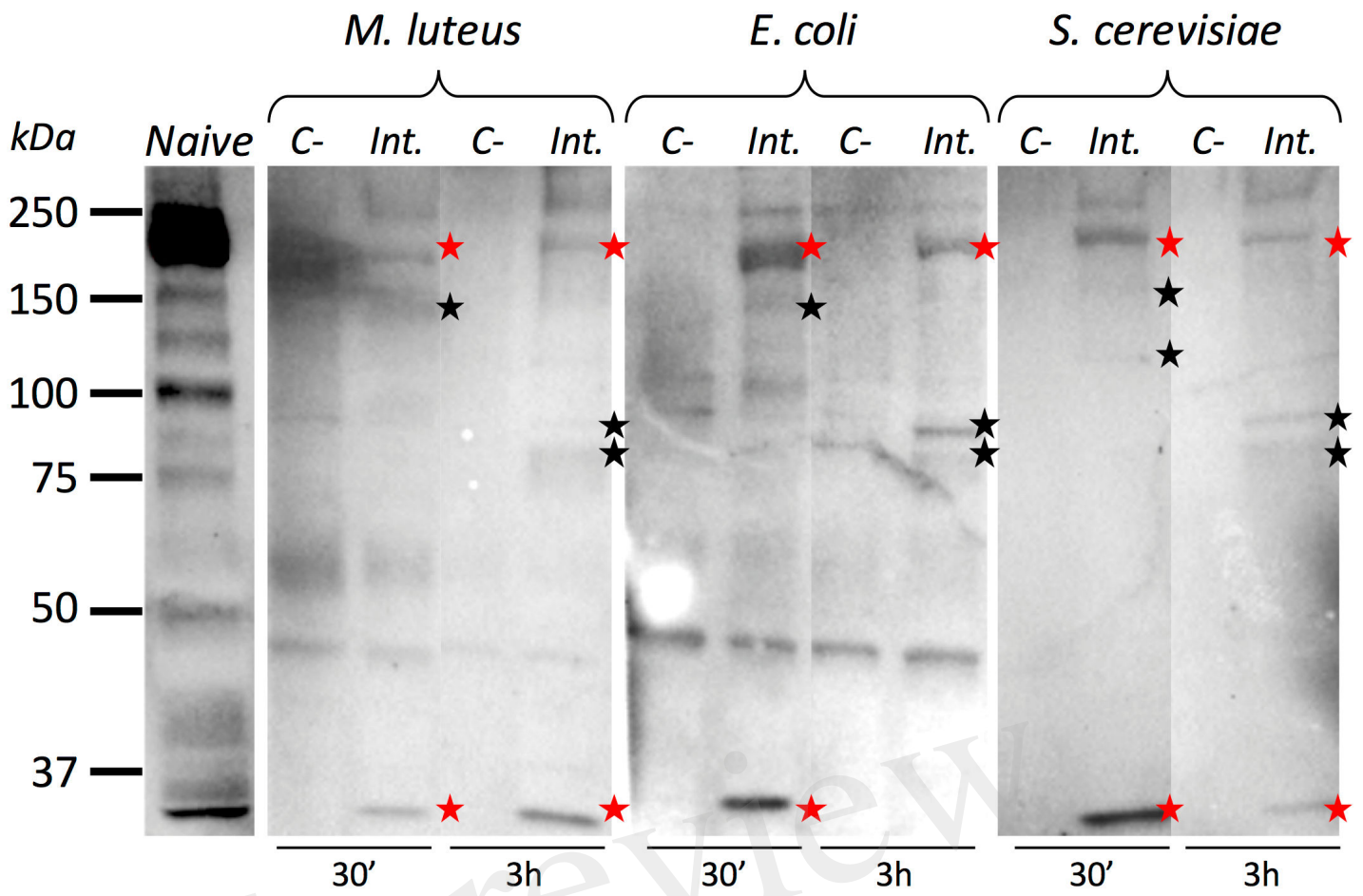
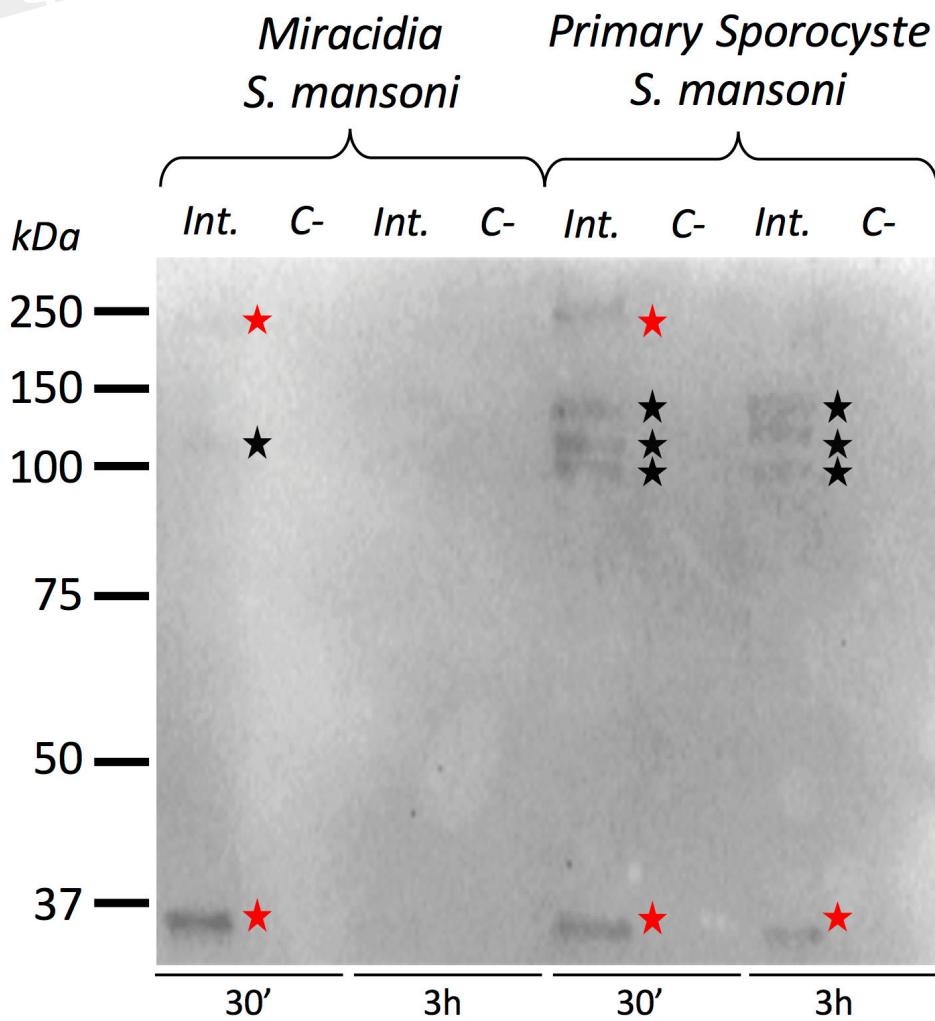
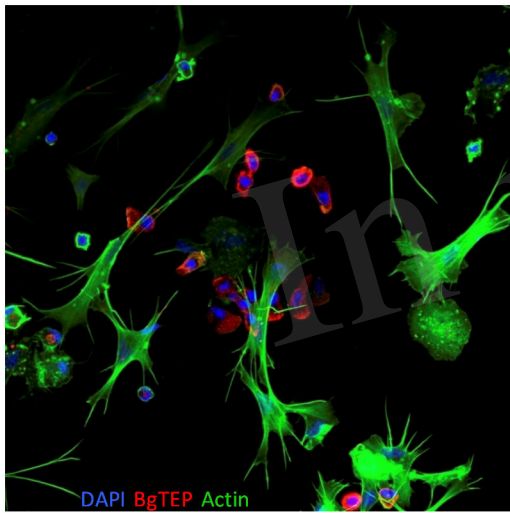
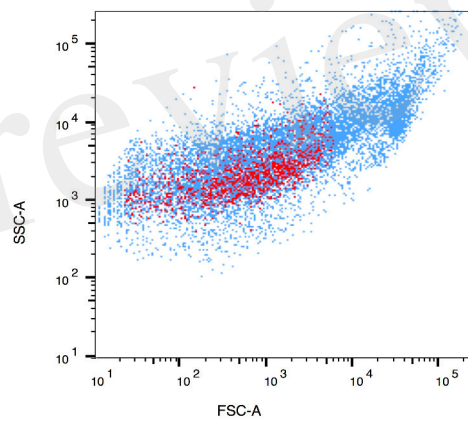
**B**

Figure 7.TIFF

A



B



C

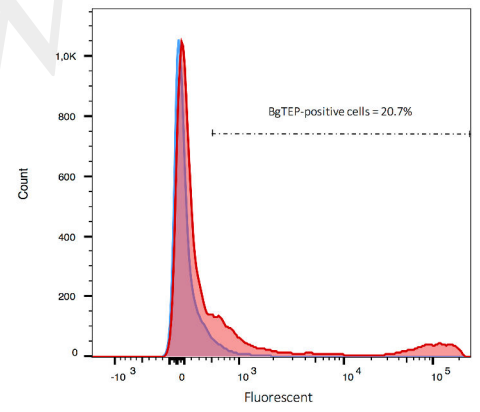
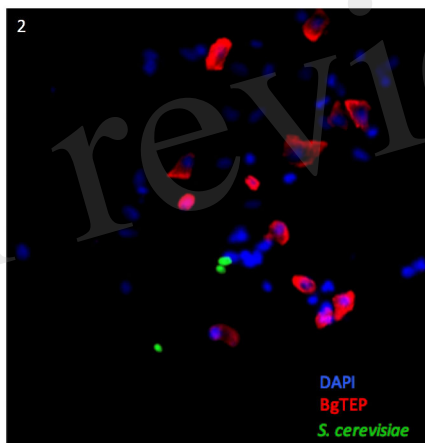
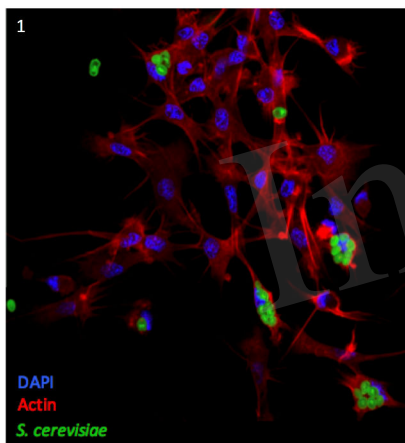
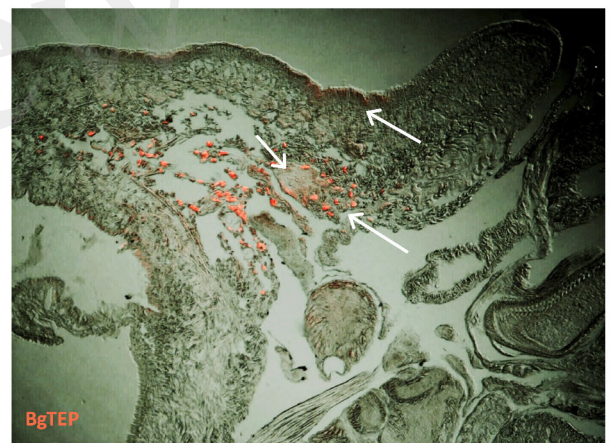


Figure 8.TIFF

A



B



2. Mise en évidence d'effecteurs immunitaires chez *Biomphalaria glabrata* en réponse à divers pathogènes

Publication: Specific pathogen recognition by multiple innate immune sensors provides support to Medzithov's cooperative concept in a non-model invertebrate.

TETREAU Guillaume¹, PINAUD Silvain¹, PORTET Anaïs¹, GALINIER Richard, GOURBAL Benjamin and DUVAL David

*Article accepté dans *Frontiers in Immunology*



Specific Pathogen Recognition by Multiple Innate Immune Sensors in an Invertebrate

Guillaume **Tetreau**^{*†}, Silvain **Pinaud**[‡], Anaïs **Portet**[‡], Richard **Galinier**, Benjamin **Gourbal** and David **Duval**^{*}

University of Perpignan, IHPE UMR 5244, CNRS, IFREMER, University of Montpellier, Perpignan, France

OPEN ACCESS

Edited by:

Larry J. Dishaw,
University of South Florida St.
Petersburg, United States

Reviewed by:

Katherine Buckley,
George Washington University,
United States
Robert Braidwood Sim,
University of Leicester, United
Kingdom

*Correspondence:

Guillaume Tetreau
guillaume.tetreau@gmail.com;
David Duval
david.duval@univ-perp.fr

†Present address:

Guillaume Tetreau,
University of Grenoble Alpes, CNRS,
CEA, IBS, Grenoble, France

[‡]These authors have contributed
equally to this work.

Specialty section:

This article was submitted to
Molecular Innate Immunity,
a section of the journal
Frontiers in Immunology

Received: 07 July 2017

Accepted: 20 September 2017

Published: xx September 2017

Citation:

Tetreau G, Pinaud S, Portet A,
Galinier R, Gourbal B and Duval D
(2017) Specific Pathogen
Recognition by Multiple Innate
Immune Sensors in an Invertebrate.
Front. Immunol. 8:1249.
doi: 10.3389/fimmu.2017.01249

Detection of pathogens by all living organisms is the primary step needed to implement a coherent and efficient immune response. This implies a mediation by different soluble and/or membrane-anchored proteins related to innate immune receptors called PRRs (pattern-recognition receptors) to trigger immune signaling pathways. In most invertebrates, their roles have been inferred by analogy to those already characterized in vertebrate homologs. Despite the induction of their gene expression upon challenge and the presence of structural domains associated with the detection of pathogen-associated molecular patterns in their sequence, their exact role in the induction of immune response and their binding capacity still remain to be demonstrated. To this purpose, we developed a fast interactome approach, usable on any host-pathogen couple, to identify soluble proteins capable of directly or indirectly detecting the presence of pathogens. To investigate the molecular basis of immune recognition specificity, different pathogens (Gram-positive bacterium, *Micrococcus luteus*; Gram negative, *Escherichia coli*; yeast, *Saccharomyces cerevisiae*; and metazoan parasites, *Echinostoma caproni* or *Schistosoma mansoni*) were exposed to hemocyte-free hemolymph from the gastropod *Biomphalaria glabrata*. Twenty-three different proteins bound to pathogens were identified and grouped into three different categories based on their primary function. Each pathogen was recognized by a specific but overlapping set of circulating proteins in mollusk's hemolymph. While known PRRs such as C-type lectins were identified, other proteins not known to be primarily involved in pathogen recognition were found, including actin, tubulin, collagen, and hemoglobin. Confocal microscopy and specific fluorescent labeling revealed that extracellular actin present in snail hemolymph was able to bind to yeasts and induce their clotting, a preliminary step for their elimination by the snail immune system. Aerolysin-like proteins (named biomphalysins) were the only ones involved in the recognition of all the five pathogens tested, suggesting a sentinel role of these horizontally acquired toxins. These findings highlight the diversity and complexity of a highly specific innate immune sensing system. It paves the way for the use of such approach on a wide range of host-pathogen systems to provide new insights into the specificity and diversity of immune recognition by innate immune systems.

Keywords: invertebrate innate immunity, interactome, pathogen sensing, *Biomphalaria glabrata*, pathogen-recognition receptor, proteomic profiling, immune specificity, hemocyte-free hemolymph

INTRODUCTION

The innate immune system allows the host to sense pathogens and mount an appropriate anti-pathogenic defense. Confronted with a large variety of pathogens, ranging from viruses to multicellular parasites, the animals' immune systems did not converge to a unique system with shared features but they emerged independently to provide an optimal protection of the host from infection (1). However, they all tend toward the genesis of a restricted repertoire of pathogen recognition molecules, named pattern-recognition receptors (PRRs), allowing to identify a determined diversity of pathogens (2). In vertebrates, pathogens recognition ability can be complemented by somatic recombination and hypermutation of a large repertoire of genes encoding immune receptors that lead to the production of soluble or membrane-bound antibodies (3, 4). Twelve years ago, Hargreaves and Medzhitov described the innate immune system in vertebrates as a complex of several recognition molecules capable of triggering one or more pathways to eliminate a given pathogen (1). Concepts highlighting the cooperation and complementation between the different recognition molecules leading to the activation of immune responses have since been supported by functional studies in vertebrates and in some model species (5, 6).

In invertebrates, and despite the lack of a vertebrate-like adaptive immunity, an increasing number of studies reported different repertoires of surprisingly highly diversified immune receptors within the innate immune system. This molecular diversity appears to be an essential basis for developing a fine and specific immune response against a large range of pathogens (7). The diversified arthropods' Down syndrome cell adhesion molecule (Dscam) generated by different splicing events, the somatic hypermutated snail fibrinogen-related proteins (FREPs), the C-type lectins, or the sea urchin 185/333 proteins whose diversity is generated by RNA editing and post-translational modifications are the most well-known diversified immune molecules (8–10). However, they are not the only critical factors involved in pathogen recognition since their knock-out by RNA interference did not result in a complete lack of protection (11, 12).

Many additional actors have been characterized with the increasing use of high-throughput sequencing. Their annotation as “immune-like receptors” was based on the induction of their gene expression following infectious challenges and/or on the presence in their gene sequence of homologous domains already characterized in known immune receptors. Indeed, most immunological processes in invertebrates are extrapolated based on protein sequence homology with other model species (13–15). Moreover, many transcriptomic experiments performed in invertebrates following challenges with different pathogens resulted in a list of differentially expressed immune genes, supposedly involved in pathogen recognition, for which the interaction with pathogens and the potential roles in immune recognition have never been validated (16–18). As a consequence, many molecular functions still remain to be clarified, particularly their real contribution in the effective host immune response and the nature of the pathogen and/or molecular target with whom they interact.

To solve these questions, we investigated the immune sensing ability for a wide range of pathogens, from bacteria to trematodes, by the schistosomiasis vector snail, *Biomphalaria glabrata*. The objective of this study was to identify which molecules from the snail host interacted with pathogen's surface determinants and their potential role in the specificity of the innate immune system. In this study, we report the repertoire of sensors from innate immunity constituted of previously characterized immune recognition factors (IRF) and of proteins involved in non-canonical immune pathways. These diverse and complementary molecules display a sentinel role by their constitutive expression in naïve animals. This circulating activity brings clues about the specificity and the mechanisms of pathogen detection in the host plasma. These results provide insights into the evolutionary selection of such factors and their role in specificity of invertebrate innate immunity that ultimately trigger an appropriate immune response, from inflammation to targeted clearance mechanisms.

MATERIALS AND METHODS

Snail Rearing

An albino strain of the freshwater snail *B. glabrata* originated from Recife, Brazil (BgBRE2) was used as the invertebrate host (19). The snail strain was maintained in rearing chambers at 26°C, 12/12 h light/dark period.

Hemolymph Extraction and Interaction with Pathogens

The interactome procedure used in this study consists in comparing the proteomic profile of the pathogen alone with the proteomic profile of the pathogen that was in contact with the cell-free hemolymph from the snail (Figure 1). This allows identifying the native proteins from the hemolymph that interact with outer proteins from the entire living pathogen. Hemolymph was collected from the head-foot region of twenty 9- to 10-mm snails (Figure 1, 1) as previously described (20). 5 and 2 mL of hemolymph from a pool of snails were used for each replicate for interactome with bacteria and yeast and with metazoan parasites, respectively. Hemolymph was centrifuged at 2,000 × g for 10 min and the supernatant, constituting the cell-free hemolymph, was recovered for further interaction (Figure 1, 2). All plasma preparations were used immediately after their collection.

Integrity of the cells was verified by confocal microscopy to ensure that the procedure was not damaging the hemocytes, which could bias downstream analyses. Three conditions were tested: 1. freshly collected hemocytes were centrifuged at 2,000 × g for 10 min and used as a control for intact cells; 2. hemocytes vortexed and centrifuged at 2,000 × g for 10 min corresponded to the hemolymph preparation procedure of the interactome; 3. hemolymph sonicated (70% for 5 s) and then centrifuged (2,000 × g for 10 min) was the control of disrupted cells. Hemolymphatic cells were deposited on microscope slides to check their integrity and adhesion to surface. Cells were labeled with DAPI, which labels the DNA, and phalloidin, which labels the actin, by incubation for 20 and 2 min at 26°C in dark, respectively. Preparation was observed under a Zeiss LSM 700 microscope with two lasers at

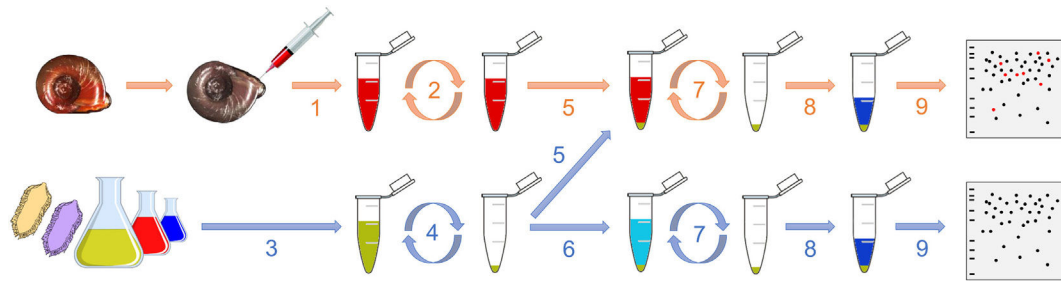


FIGURE 1 | Graphical representation of the interactome procedure. The hemolymph is collected (1) and then centrifuged (2). Meanwhile, the pathogen is also collected (3) and centrifuged (4). The cell-free hemolymph is put in contact with the pellet of pathogen (5; “pathogens + hemolymph”). A control is also performed consisting in adding a buffer that mimics the internal snail osmolarity to the pellet of pathogen (6; “pathogens only”). After 20 min, the suspension is centrifuged (7), the pellet is washed and proteins are extracted (8) for their proteomic profiling by 2D-PAGE (9).

wavelengths of 405 and 488 nm for detection of DAPI and phalloidin labeling, respectively.

Five pathogens from three different kingdoms were used: the Gram-positive bacteria *Micrococcus luteus*, the Gram-negative bacteria *Escherichia coli*, the yeast *Saccharomyces cerevisiae*, and the two parasitic trematodes *Echinostoma caproni* and *Schistosoma mansoni*. *S. mansoni* and *E. caproni* have been maintained in the laboratory on *B. glabrata* BgBRE2 snails as previously described (12, 21).

The bacteria were plated and isolated on LB-agar Petri dishes. For each bacterium, one colony was introduced into a LB liquid medium and cultured overnight. Then, 150 μ L of culture media, which contained approximately 35 million of bacteria, was sampled (Figure 1, 3) and centrifuged at $5,000 \times g$ for 10 min (Figure 1, 4). This quantity of bacterial cells was based on studies previously published (22, 23) and it was shown to be above the detection threshold of the 2D-SDS-PAGE approach by preliminary tests (data not shown), which ensured a proper analysis of the interactome profiles. The supernatant was discarded and the pellet was washed twice with 1 mL of Chernin’s balanced salt solution (CBSS); NaCl, 48 mM; KCl, 2 mM; Na_2HPO_4 , 0.5 mM; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.8 mM; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 3.6 mM; NaHCO_3 , 0.6 mM; pH 7.4. This buffer was chosen to mimic the internal snail osmolarity (24). The pellet was then resuspended in 1 mL of cell-free hemolymph and incubated on a rotating agitator for 20 min at 26°C (snail rearing chamber temperature) (Figure 1, 5). As a control, the bacterial pellet was incubated with 1 mL of filtered-CBSS in the same conditions (Figure 1, 6). After the incubation, the suspension was centrifuged at $5,000 \times g$ for 10 min and the pellet was washed twice with 1 mL of CBSS (Figure 1, 7). Three biological replicates of each condition (“pathogen alone” and “pathogen + hemolymph”) were performed.

The yeast culture was performed on a unique colony in Sabouraud liquid medium (dextrose, 20 g L^{-1} ; pancreatic digest of casein, 5 g L^{-1} ; peptic digest of animal tissue, 5 g L^{-1} , pH 5.6) at 26°C for 4 days. One hundred microliters of culture media, which contained approximately 30 million yeast cells, was collected as described above for bacteria.

Schistosoma mansoni eggs were recovered as previously described (12), then exposed to water and light for 2 h to let miracidia hatch. *E. caproni* adults were recovered on the

digestive tracts of mice, cultured *in vitro* in RPMI solution supplemented with penicillin and streptomycin (SP4458, Sigma) at 37°C for 2 days. Eggs were recovered, washed, and stored in water in the dark at 26°C with air injector. Twenty days later, eggs were put in fresh water and exposed to light for 2 h for miracidium hatching. One thousand five hundred miracidia from *S. mansoni* and *E. caproni* were individually counted by using a glass pipette and processed as described for bacteria until protein extraction.

Protein Extraction and 2D-SDS-PAGE Profiling

Proteins were extracted by resuspending the pellet of CBSS-washed pathogens in 70 μ L of denaturing UTTC buffer (urea, 7 M; thiourea, 2 M; Tris, 30 mM; CHAPS, 4%; pH 8.5) (Figure 1, 8). After 2 h incubation at room temperature on a rocking agitator, the sample was centrifuged at $10,000 \times g$ for 5 min and the supernatant was transferred to a low protein binding tube for its analysis by 2D-electrophoresis (Figure 1, 9).

Then, 280 μ L of rehydration buffer (urea, 7 M; thiourea, 2 M; CHAPS, 4%; DTT, 65 mM) containing 0.2% of Bio-Lyte 3/10 ampholyte (Bio-Rad) was added. The sample was then loaded on a tray channel for 5 h of passive rehydration followed by 14 h of active rehydration (50 V) of a 17 cm ReadyStrip IPG strip with a non-linear 3–10 pH gradient (Bio-Rad). Focusing was performed using the following program: 50 V for 1 h, 250 V for 1 h, 8,000 V for 1 h, and a final step at 8,000 V for a total of 90,000 V h with a slow ramping voltage (quadratically increasing voltage) at each step. Rehydration and focusing were both performed on a Protean IEF Cell system (Bio-Rad). Focused proteins were reduced by incubating the strip twice with equilibration buffer (Tris, 1.5 M; urea, 6 M; SDS, 2%; glycerol, 30%; bromophenol blue; pH 8.8) containing DTT (130 mM) at 55°C and they were alkylated by an incubation with equilibration buffer containing iodoacetamide (135 mM) on a rocking agitator (400 rpm) at room temperature protected from light.

Proteins were separated in function of their molecular weight on a 12/0.32% acrylamide/piperazine diacrylamide gel run at 25 mA/gel for 30 min followed by 75 mA/gel for 8 h using a Protean II XL system (Bio-Rad). Protein standards were loaded with Whatman paper impregnated with 3 μ L of Unstained

Precision Plus Protein Standards (Bio-Rad) on the left part of the gels. Gels were stained following a regular silver staining procedure: sensitizing using sodium acetate (68 g L^{-1}) and sodium thiosulfate (2 g L^{-1}), marking with 2.5 g L^{-1} of silver nitrate, and then developing with sodium carbonate (25 g L^{-1}) in a 7.5% formaldehyde solution. Staining was stopped by replacing the developing solution by a solution of glycine (5 g L^{-1}) in 0.1% acetic acid. Gels were scanned using a ChemiDoc MP Imaging System (Bio-Rad) associated with Image Lab software version 4.0.1 (Bio-Rad). The qualitative comparative analysis of digitized proteome maps was conducted using the image analysis software PDQuest 7.4.0 (Bio-Rad). Only spots present in all the three replicates of “pathogens + hemolymph” samples and absent from all the profiles of pathogens alone were selected and picked in a mass spectrometry (MS)-compatible silver stained gel for further identification.

Spot Picking and Trypsin Digestion

Spots were excised from the gels using a Onetouch Plus Spot Picker Disposable (Harvard Apparatus), equipped with specific 1.5-mm methanol-washed tips. The gel plug containing the spot was disposed into a methanol-washed low protein binding tube and stored at -80°C until further processing. Gel plug was first destained by incubating it in $150 \mu\text{L}$ of a solution of potassium ferricyanide (15 mM) and sodium thiosulfate (50 mM) at room temperature for 10 min on a rocking agitator (500 rpm). The destaining solution was discarded and this step was repeated once. Then, the plug was washed twice by adding $150 \mu\text{L}$ of ammonium bicarbonate (25 mM) and it was incubated at room temperature for 30 min on a rocking agitator (500 rpm). Finally, $150 \mu\text{L}$ of a solution of ammonium bicarbonate (12.5 mM) and acetonitrile (50%) was added to the spot. After an incubation at room temperature for 10 min on a rocking agitator (500 rpm), the solution was discarded and the gel plug lyophilized for 30 min. The plug was rehydrated with $50 \mu\text{L}$ of sequencing grade modified trypsin (Promega) and incubated on ice for 30 min. The excess of trypsin was discarded and $50 \mu\text{L}$ of ammonium bicarbonate (25 mM) was added. Digestion was performed overnight at 30°C . The $50 \mu\text{L}$ of solution were put in a new methanol-washed low-protein binding tube and the peptides were extracted from the plug by washing it three times with $100 \mu\text{L}$ of a solution of formic acid (1%) and acetonitrile (50%) and by incubating 15 min at room temperature on a rocking agitator (500 rpm). The solution was collected at each washing step and mixed together in the same tube (final volume: $350 \mu\text{L}$). The solution was flash-frozen in liquid nitrogen, lyophilized for 3 h and stored at -80°C until further processing.

MS/MS Identification

Peptides were resuspended in $10 \mu\text{L}$ of 3% (v/v) acetonitrile and 0.1% (v/v) formic acid, and then analyzed with a nano-LC1200 system coupled to a Q-TOF 6550 mass spectrometer equipped with a nanospray source and an HPLC-chip cube interface (Agilent Technologies). A 34-min linear gradient ($3\text{--}75\%$ acetonitrile in 0.1% formic acid), at a flow rate of 350 nL min^{-1} , was used to separate peptides on a polaris-HR-Chip C18 column ($150 \text{ mm long} \times 75 \mu\text{m}$ inner diameter). Full autoMS1 scans from 290 to 1700 m/z and autoMS2 from 59 to 1700 m/z were recorded.

In every cycle, a maximum of five precursors sort by charge state ($2+$ preferred and single-charged ions excluded) were isolated and fragmented in the collision cell that was automatically adjusted depending on the m/z . Active exclusion of these precursors was enabled after 1 spectrum within 0.2 min, and the absolute threshold for precursor selection was set to 1,000 counts (relative threshold 0.001%). For protein identification, peak lists were extracted (merge MSn scans with the same precursor at $\pm 30 \text{ s}$ retention time window and $\pm 50 \text{ ppm}$ mass tolerance) and compared with specific databases by using the PEAKS studio 7.5 proteomics workbench (Bioinformatics Solutions Inc., build 20150615). The searches were performed with the following specific parameters: enzyme specificity, trypsin; three missed cleavages permitted; fixed modification, carbamidomethylation (C); variable modifications, oxidation (M), pyro-glu from E and Q; monoisotopic; mass tolerance for precursor ions, 20 ppm ; mass tolerance for fragment ions, 50 ppm ; MS scan mode, quadrupole; and MS/MS scan mode, time of flight. For each interactome experiment, each spot identification was performed against the *B. glabrata* translated transcriptome (12, 25) and against the corresponding pathogen proteome. Only significant hits with a false discovery rate ($\text{FDR} \leq 1$) for peptide and protein cutoff ($-\log P \geq 20$ and number of unique peptides ≥ 2) were considered. For ensuring a proper identification of the proteins found by the interactome approach, a BLAST search against NCBI nr database was performed and the conserved domains of the sequence were retrieved using the NCBI CD-search available at <https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi> (26). For each protein, pI and molecular mass were also calculated with the ExpASY Compute pI/Mw tool (available at http://web.expasy.org/compute_pi) to compare with their location on the gel and provide an additional confirmation of their proper identification.

Validation of Actin As an Extracellular Immune Factor

Integrity of the cells was verified by confocal microscopy prior to actin localization in the plasma to ensure that the preparative procedure was not damaging the hemocytes, which could bias downstream analyses. The same three samples of hemolymph used for cell integrity (centrifuged hemolymph, vortexed and centrifuged hemolymph, and sonicated and centrifuged hemolymph) were used. $40 \mu\text{L}$ of hemolymph from each sample were extracted in Laemmli buffer (Bio-Rad) containing β -mercaptoethanol and denaturated at 99°C for 5 min. Proteins were separated in a 12% acrylamide gel using the Mini-Protean Tetra Cell machinery (Bio-Rad) powered by PowerPac HC (Bio-Rad) at 110 V for 80 min. Proteins were then transferred onto a $0.2 \mu\text{m}$ PVDF membrane using Trans-Blot Turbo Transfer Pack for 3 min at 25 V and 2.5 A (Bio-Rad). After saturation during 1 h at 37°C in TBSTM [$1 \times \text{TBS}$ (500 mM Tris-HCl , 1.5 M NaCl , $\text{pH } 7.5$), 0.05% Tween20, 5% non-fat milk], the membrane was incubated for 90 min at RT in TBSTM containing a mouse actin monoclonal antibody (mAbG_{Ea}, ThermoFisher) at a $1:1,000$ dilution. The membrane was washed three times with TBST (TBSTM without milk), and further incubated for 70 min at RT with manufactured horseradish peroxidase-conjugated goat anti-mouse IgG antibody

(Agrisera) at a 1:4,000 dilution. The membrane was washed three times with TBST. Actin presence was revealed by incubating the membrane in an enhanced chemiluminescent reagent (Super Signal West Pico Chemiluminescent Substrate, ThermoScientist) for 30 min at RT. The membrane was scanned using a ChemiDoc MP Imaging System (Bio-Rad) associated with Image Lab software version 4.0.1 (Bio-Rad).

450

451 Yeast Clotting by Incubation with Cell-Free 452 Hemolymph

453 Yeast cells were cultured in Sabouraud medium as described
454 above. They were washed twice with CBSS. Yeasts were then
455 resuspended either in CBSS or in cell-free hemolymph for 20 min
456 or 3 h. Preparations were deposited on microscope slides for plat-
457 ting and were then labeled with DAPI and phalloidin as described
458 above. They were observed using a Zeiss LSM 700 microscope.
459

460

461 RESULTS AND DISCUSSION

462

463 An Original and Simple Method

464 Generally, the identification of host molecules that can bind or
465 recognize a set of pathogen determinants is performed by global
466 pull-down assays. Such global interactome approach consists in
467 the incubation of native or denatured protein extracts from both
468 the host and the pathogen. The resulting interacting protein
469 complexes are then separated through differential centrifuga-
470 tion steps, revealed by SDS-PAGE and identified by MS (27–29).
471 Although powerful, this strategy suffers from several flaws,
472 mainly associated with the extraction procedure itself which
473 might (i) affect the nature of protein interactions by changing
474 their conformation and (ii) promote forced interaction between
475 proteins that would not encounter each other *in vivo*. Therefore,
476 a part of the interactions observed can be essentially artificial and
477 experimentally biased. To bypass these problems, we propose a
478 new and simple interactome procedure in a cell-free hemolymph
479 context that tends to mimic biological interactions between
480 pathogens and soluble host proteins (Figure 1). Indeed, entire
481 living pathogens were exposed to circulating humoral factors
482 already present in cell-free hemolymph freshly extracted from
483 naïve snails and they were incubated at 26°C, which corresponds
484 to the environmental and internal temperature of this ectother-
485 mic organism. Therefore, only proteins present at the surface of
486 the pathogen are recognized in a biologically realistic context.
487 Moreover, the short time (20 min) chosen allows focusing
488 exclusively on the very first step of innate immune response and
489 avoiding the pathogen to respond to the attack from the immune
490 factors, which could affect pathogens' proteomic profiles and
491 bias the analysis. As a control, only spots that were present in
492 the three “pathogen + hemolymph” replicates and absent in the
493 three “pathogen only” replicates were considered for the analysis
494 of each pathogen studied. Each MS/MS profile was confronted
495 to both the databases of the host and of the pathogen. This
496 ensured that the approach reliably enabled the identification
497 of host's interacting molecules while limiting the risk of false
498 positives. No significant matches were observed against any of
499 the pathogen databases, which confirms that all spots exclusively

identified in the analysis of “pathogen + hemolymph” samples
and not in the “pathogens only” gels were proteins from the snail's
plasma. The benefit of this approach relies on its universality: it
can be used with most host and parasite systems and gives rise
to reliable qualitative differences within just few hours, which
represents a great step forward for studies focusing on model
and non-model systems.

500

501 Identification of a Large Variety 502 of Interacting Proteins

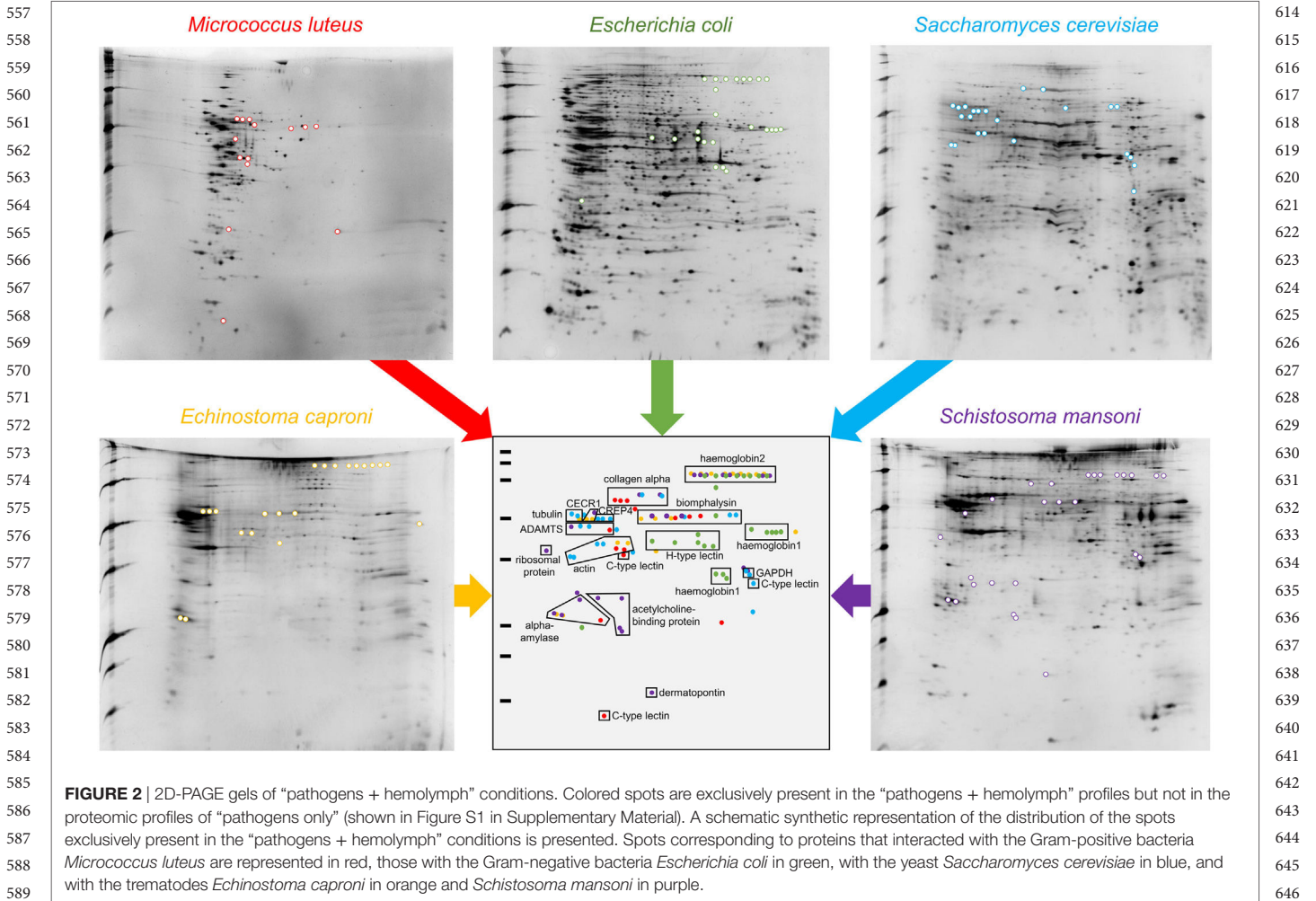
503 This approach allowed the identification of a total of 109 spots
504 exclusively identified in “pathogen + hemolymph” samples for
505 the five pathogens tested (Figure 2; Figure S1 in Supplementary
506 Material). These spots provided a significant match to 34 unique
507 accession numbers, referring to 23 different proteins (Table S1
508 in Supplementary Material). Each pathogen was recognized by a
509 specific, but overlapping, set of circulating proteins in mollusk's
510 hemolymph (Figure 3). Specific recognition proteins to a given
511 pathogen must be expected since each class of pathogen express
512 at their surface specific and different structural motif also called
513 pathogen-associated molecular patterns (PAMPs). The best
514 known PAMPs are lipopolysaccharide from Gram-negative
515 bacteria, lipoteichoic acid or peptidoglycan from Gram-positive
516 bacteria, mannan-derived molecules or glycan from fungi, and
517 fucosylated or glycoprotein receptors from *Schistosoma* sp. (30).
518 Surprisingly, we identified numerous proteins not known to be
519 involved in pathogen recognition and/or killing (extracellular
520 matrix proteins, protease, and carbohydrase enzyme). Considering
521 that some of these proteins are generally considered as intracel-
522 lular molecules, a possible explanation for their presence could be
523 that host's cells were damaged during the hemolymph collection
524 (although non-invasive) and/or during the centrifugation step.
525 A dual-staining with DAPI and phalloidin of hemocytes revealed
526 no difference between fresh hemocytes and vortexed ones that
527 were intact, as compared to sonicated hemocytes that were totally
528 disrupted (Figure 4). This confirmed that the procedure of prepa-
529 ration of cell-free hemolymph did not damage the cells and that
530 all interacting proteins from the snails were naturally present in
531 the extracellular compartment of the hemolymph.
532

533 We, thus, propose to classify the snail interacting proteins iden-
534 tified into three different categories based on their nomenclature
535 and known primary function: (i) molecules previously described
536 as primary pathogen recognition molecules able to trigger an
537 immunological response, with potential additional lytic activity
538 (IRF), (ii) proteins whose primary role is not pathogen sensing
539 but are involved in other physiological functions [non-canonical
540 proteins interacting with pathogens (NCIP)], and (iii) enzymes
541 implicated in the metabolism of a wide range of molecules
542 enzymes interacting with pathogens (EIP).
543

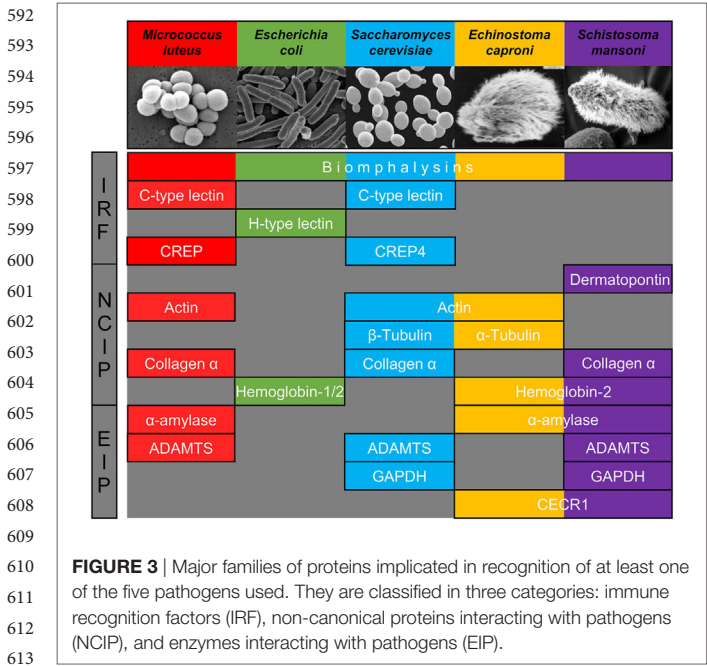
544 Pathogen Sensing by Soluble Immune 545 Receptors and Atypical Toxins (IRF)

546 Among the IRE, two different families of proteins are identified:
547 lectins and biomphalysin (Figure 2). Lectins represent a large
548 family with a wide variety of evolutionarily conserved structures
549 and some of them have been described as involved in immune
550

501
502
503
504
505
506
507
508
509
510
511
512
513
514
515
516
517
518
519
520
521
522
523
524
525
526
527
528
529
530
531
532
533
534
535
536
537
538
539
540
541
542
543
544
545
546
547
548
549
550
551
552
553
554
555
556



590
591
592



610
611
612
613

614
615
616
617
618
619
620
621
622
623
624
625
626
627
628
629
630
631
632
633
634
635
636
637
638
639
640
641
642
643
644
645
646
647
648
649
650
651
652
653
654
655
656
657
658
659
660
661
662
663
664
665
666
667
668
669
670

recognition (7, 31). Among them, calcium-dependent (C-type) lectins were considered the most promising pattern-recognition proteins involved in the specific recognition of pathogens in the invertebrate immune system. This specificity is due to their high level of polymorphism and/or diversification to face up pathogens' antigenic diversity (31). In addition to their role as soluble receptors, they can also limit the spreading of the pathogen in the host's tissues and participate to its elimination (32, 33). Two different C-type lectins were interacting with the bacterium *M. luteus* and the yeast *S. cerevisiae* but not with the three other pathogens (Figure 2; Table S1 in Supplementary Material). Another C-type lectin-related protein (CREP4), recently characterized in *B. glabrata* from transcriptomic data (25), was apparently able to bind to *S. cerevisiae*. By contrast, the recognition of the bacterium *E. coli* involved a totally different category of lectin, the hyal-adherins (H-type), which are also carbohydrate-binding proteins but data are missing concerning their role in pathogen recognition. Among the lectins, FREPs are proteins containing immunoglobulin-like domains whose role in the interaction between snails and metazoan parasites has been suggested (34, 35). Surprisingly, FREPs were not identified in the interaction with both metazoan parasites in

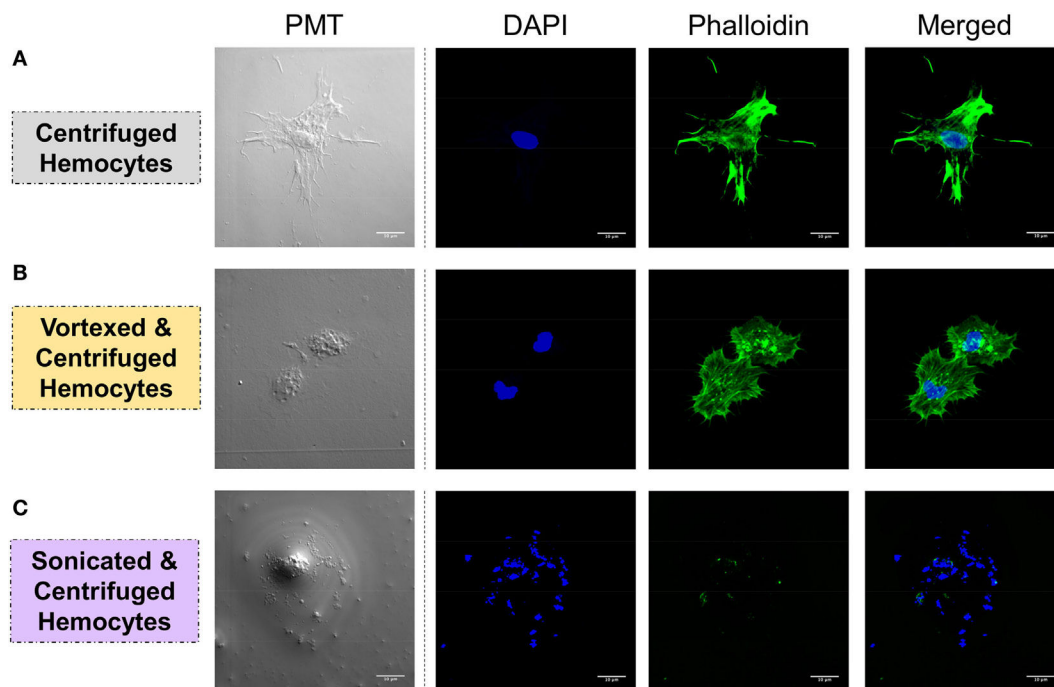


FIGURE 4 | Hemocyte integrity was tested by analyzing the spreading capacity and by observing the nuclear/cytoplasmic ratio. Hemolymph was carefully collected and were either **(A)** slowly centrifuged, **(B)** vortexed and centrifuged, or **(C)** sonicated and centrifuged. Hemocytes were stained with DAPI, which colors nuclei acids contained in the nucleus in blue, and with phalloidin, which colors f-actin in green. White bar = 10 μ m.

our study while they were evidenced in previous transcriptomic and proteomic studies (27, 29). Such discrepancy with previous results likely comes from the different developmental stage of the parasites used in the different studies, i.e., miracidia herein and sporocysts in other studies. Several proteomic and glycomic studies showed that the glycan elements harbored by *Schistosoma*, to which FREPs bind, differ from one developmental stage to another (36, 37). This would suggest a subtle ability for the snail immune machinery to distinguish various intramolluscal developmental stages of the parasite (miracidium to primary and secondary sporocysts or even cercariae) and FREPs might not be involved in the recognition of all stages. Moreover, FREPs were previously identified by interactome experiments after 2.5 h of contact between protein extracts from sporocyst and snail cell-free hemolymph (27) while our procedure includes a 20-min contact of outer pathogen membrane proteins with circulating snail hemolymph proteins. Of note, it has been observed that some FREPs can form multimers and that they can interact with other proteins such as thioester containing proteins (TEPs), which could both modulate their recognition ability (27, 34, 38). It is, therefore, possible that these processes are mandatory for the recognition by FREPs of the pathogens used in this study. A longer exposure time between pathogens with proper membrane-bound glycan antigens and the cell-free hemolymph would then be required for the complexes to form and for their detection by our interactome approach.

The second class of IRF identified is the biomphalysin toxin, which is an aerolysin-like protein that has been acquired by

a putative horizontal gene transfer from a bacterium (39) (Figure 3). This protein is constituted of two domains: one large domain that shares structure similarities with β -pore-forming toxins whose role is to perforate cell membranes by forming transmembrane pores and a small domain potentially involved in pathogens' carbohydrate motifs recognition (39). Biomphalysin is a dual protein: it has recently been shown to directly bind to *S. mansoni* sporocysts and to have a lytic activity enhanced by snail plasmatic factors (39). Herein, we demonstrate for the first time that this anti-schistosome toxin is also able to interact with other pathogens and suggest a role in bacterial clearance. One (*E. coli*) and three (*M. luteus*, *S. cerevisiae*, *E. caproni*, and *S. mansoni*) spots were identified as biomphalysins in 2D gels (Figure 2; Table S1 in Supplementary Material). Even if they were all of the same size (65–70 kDa), the expected size of biomphalysin (39), they exhibited a large range of isoelectric points, from slightly acid/neutral for *E. caproni* and *S. mansoni* to basic for *E. coli* and *S. cerevisiae* (Figure 2). Altogether, this suggests that different protein isoforms of biomphalysins must be involved in the recognition/clearance of the same pathogen but also of different pathogens. Interestingly, different biomphalysin genes were predicted in the recently sequenced genome of *B. glabrata* (BioProject: PRJNA290623 on NCBI database) (40), which suggests that they might be different genes rather than different isoforms (39). This biomphalysin family could be a major player of the specificity of the *Biomphalaria* innate immune response together with lectins.

Biomphalysins were the only proteins that interacted with all pathogens. There is a growing number of evidence that

aerolysin-like proteins have been horizontally transferred within many different invertebrate phyla acquiring in the same time potentially new and varied functions but details of their involvement in the invertebrate immunity remain largely unknown (41). The interactome approach developed herein suggests that biomphalysins might be a key component of the pathogen sensing system, and potentially of its specificity. Indeed, heterogeneous assembly from these different monomeric isoforms to the heptameric biomphalysin pore complex may generate a high degree of pathogen-binding specificity. In *Anopheles gambiae*, two C-type lectins, CTL4 and CTLMA2, form a disulfide-linked heterodimer to specifically kill *E. coli* (42). The ability to form heterodimers could greatly expand the repertoire of recognition molecules (43, 44). Further experiments are now required to understand how biomphalysin gene expression is regulated in response to exposure with different pathogens and how the different proteins are recruited to respond to a specific pathogen encounter.

Pathogen Sensing by Major Extracellular Matrix Components (NCIP)

The category of NCIP includes proteins whose primary function is not immunity, such as cell-matrix junction proteins (dermatopontin, collagen) and cytoskeleton extracellular matrix proteins (actin, tubulin). Concerning the dermatopontin, its gene expression can be increased after immune challenge with *E. caproni* (21, 45) and *S. mansoni* (45) but not with *E. coli*, *B. cereus*, and *S. cerevisiae* (46). While its role was unknown at this time, our results suggest that it might be involved in a hemolymph coagulation-like system to prevent parasite establishment through the tissue of the host (Figure 3).

The same type of molecular process is expected for other extracellular proteins such as actin. Western blot analyses of cell-free hemolymph using anti-actin antibodies revealed its presence in the extracellular compartment of the hemolymph (Figure 5). Considering that the procedure of hemolymph collection and preparation did not damage the cells (Figure 4), this actin must be considered as a real extracellular actin (ECA) present in snail hemolymph. Interestingly, the amount of ECA present in hemolymph was similar between the three conditions tested in western blot, which suggests that ECA is an important component of hemolymph released by a process still unknown in mollusk. In insects, some isoforms are secreted from cells through an exosome-independent pathway (47) while monocyte cells can release some extracellular vesicles (ectosome) containing b-actin and actinin in vertebrates (48). Observation of yeasts by confocal microscopy shows that in CBSS buffer, some actin is located inside the yeast, revealed as small precisely localized green dots (Figure 6). In the presence of cell-free hemolymph, these intra-yeast dots of actin are still visible but there is a large amount of ECA surrounding the yeast cells, which appears as early as 20 min and seems even more intense after 3 h of incubation (Figure 6). Considering that yeasts were still intact after 20 min of contact with cell-free hemolymph, this actin surrounding the yeasts is likely the ECA from snail that is able to bind and participate to yeast clotting prior to its elimination. The triggering of the destruction of yeast cells by these immune complexes is indicated

by their nuclear destructuration visible at 3 h (Figure 6). This finding is consistent with recent studies that demonstrated an active role of actin in extracellular trap for pathogens clotting, facilitating their elimination by phagocytosis in the mosquito *A. gambiae* for example (47). Until now, these soluble molecules were considered as damage-associated molecular patterns (DAMPs) potentially involved in the “danger theory” where self-constituents could trigger an immune response (49). Based on our results and particularly on the short time of our interaction that prevents the pathogen from circumventing host immune factors, these molecules must be considered as soluble immune sensing factors rather than just DAMPs.

The case of hemoglobin is particularly interesting. Two different classes of hemoglobin were identified against *E. coli* (hemoglobin-1 and -2) while only hemoglobin-2 was interacting with *E. caproni* and *S. mansoni* (Figure 2). Many different isoforms were identified (same size, different isoelectric points) but they were at a much lower size (55–60 and 100–120 kDa for hemoglobin-1 and -2, respectively) than the predicted full-size hemoglobin protein predicted from *B. glabrata* genome (514 and 582 kDa, respectively) (Figure 2). Such peptides with enhanced or alternative functionality that can be liberated from larger proteins are named cryptides. Those derived from

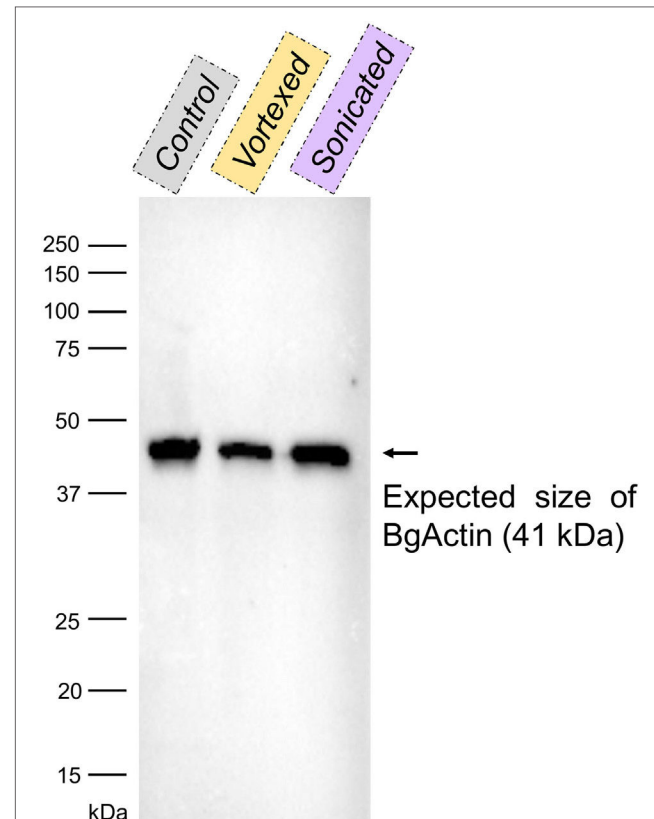


FIGURE 5 | Western blot with anti-actin antibodies of the cell-free hemolymphs prepared by (A) slow centrifugation (“control”), (B) vortexing and centrifugation (“vortexed”) or (C) sonication and centrifugation (“sonicated”). The band corresponding to the size of actin from *Biomphalaria glabrata* (~41 kDa; BgActin) is indicated by an arrow.

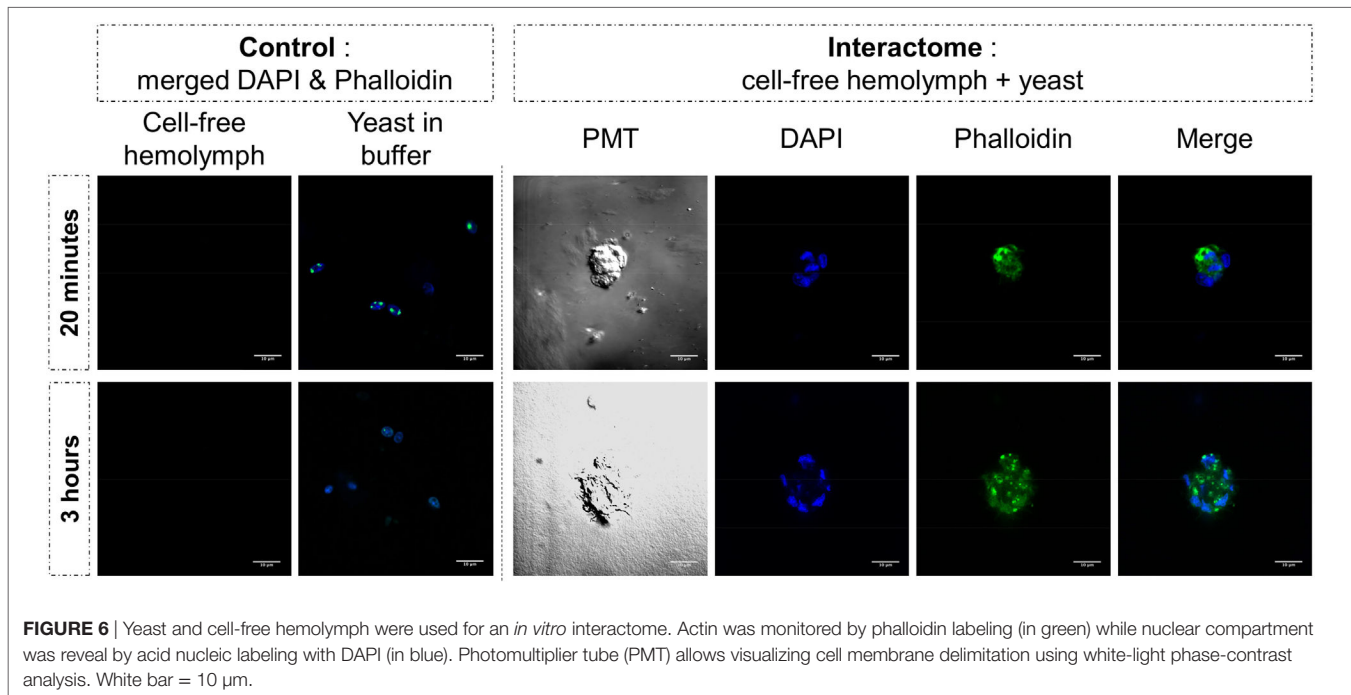


FIGURE 6 | Yeast and cell-free hemolymph were used for an *in vitro* interactome. Actin was monitored by phalloidin labeling (in green) while nuclear compartment was revealed by acid nucleic labeling with DAPI (in blue). Photomultiplier tube (PMT) allows visualizing cell membrane delimitation using white-light phase-contrast analysis. White bar = 10 μm .

hemoglobin have already been associated with immune modulation, hematopoiesis, signal transduction, and microbicidal activities in metazoans (50). Although identified as differentially expressed upon *S. mansoni* exposure in *B. glabrata* (45), these highly abundant proteins were excluded from previous interactome approaches by ultracentrifugation of plasma as they were thought to interfere with pathogen recognition and not be directly implicated in it (27). Also, the role of this major protein in hemolymph has been largely neglected as its function was expected to be mostly pleiotropic. Hemoglobin and/or hemoglobin cryptides could directly interfere with the pathogen and limit its growth, as it has been shown for the “classical swine fever virus” (51), and/or they could reinforce the interaction between pathogen and extracellular matrix proteins, as it has been shown between human fibronectin and the pathogenic yeast *Candida albicans* (52). The binding of hemoglobin to the major virulence factor of *Salmonella typhi* has also been shown to promote the production of proinflammatory cytokines from monocytes (53).

Host Plasmatic Enzymes Involved in Pathogen Surface Binding (EIP)

Many different EIPs were identified in this interactome approach (Figure 3). α -amylases have already been identified after co-immunoprecipitation of *B. glabrata* plasmatic proteins with *S. mansoni* protein extracts but they were considered as mucus contamination at this time (27). Present data challenge this contamination hypothesis since α -amylase was only detected after interaction of hemolymph with *M. luteus*. α -amylases would, thus, be critical for the host’s specific response to certain pathogens. For the other EIPs, reports on the involvement of ADAMTS, GAPDH, and CECR1 in invertebrate immunity are scarce.

However, GAPDH has been demonstrated to modulate immune responses against bacteria in plants (54) and metalloproteases have been characterized as key actors of many diverse immune and inflammatory processes in vertebrates (55). Results obtained in this study demonstrate that their binding to the pathogen surface can no longer be considered as artifactual. Further experiments are now required to understand if EIPs can bind directly to surface pathogens’ factors or if their involvement is related to their enzyme activities to mediate the maturation of immune complexes after association with other IRFs and/or NCIPs.

Experimental Support to Theoretical Concepts Opens New Perspectives for Studying Pathogen Sensing by Invertebrates

Although extensively investigated and well documented in vertebrates, the factors involved in invertebrate immune recognition rather constitute a black-box in which many different proteins with a wide range of functions, often referred to as PRRs, can be found (56, 57). Some responses have arisen from model species essentially from insects such as *Drosophila* for which the Gram-negative bacteria-specific Imd pathway and the fungi and Gram-positive bacteria-specific Toll pathway have been first identified (8). However, data remain scarce in non-model species mostly due to the absence of reliable knock-out technology, which may fail in demonstrating the full richness and the role of invertebrate pathogen recognition molecules (7, 58).

In this study, we developed a simple interactome approach to identify soluble plasmatic molecules that bind directly or indirectly to pathogen surfaces and to gain access rapidly to the biological functions of the candidate proteins. Here, we focused on the sentinel

1013 role of molecules that interacted with pathogens since they were
 1014 constitutively present in hemolymph of uninfected (naïve) snails.
 1015 Indeed, most of the studies are based on the differential analysis
 1016 (i.e., uninfected vs infected, or infected by different pathogens) of
 1017 the host immune response (efficient or not) leading to a list of genes
 1018 whose immunological function is rarely demonstrated. Moreover,
 1019 if functional invalidation (gene knock-out, siRNA-mediated gene
 1020 silencing, and mutants) already demonstrated the requirement
 1021 of such molecules during the immune response, the first step of
 1022 pathogens binding is still rarely studied (11, 12, 33).

1023 Each pathogen was recognized by a specific, although partially
 1024 overlapping set of interacting proteins from the mollusk (**Figure 3**;
 1025 Table S1 in Supplementary Material). Most of pathogens' percep-
 1026 tion involved at least three different families of proteins from
 1027 two of the three protein categories described (**Figure 3**). Such
 1028 contrasting sets of binding proteins, in terms of diversity and
 1029 quantity, suggest that specificity of immune detection quickly
 1030 occurs at a fine scale. The recognition of the same pathogen by
 1031 several different sensors with a high degree of specificity suggests
 1032 that these molecules are part of different host defense pathways
 1033 that can interact with each other (1). Such interactions can
 1034 take three different forms: by cooperation, leading to the more
 1035 efficient engagement of the same effector mechanism, by comple-
 1036 mentation, allowing to trigger different complementing effector
 1037 mechanisms or by compensation, where one pathway compen-
 1038 sates the deficiency of another one (59). The real involvement of
 1039 these proteins in pathogen recognition, as expected in parasite
 1040 antigen/host receptor interaction, is still not demonstrated and
 1041 will require specific investigation of downstream process for each
 1042 candidate identified. Thus, these pathways might contribute to
 1043 assess the danger for which they have been exposed and lead-
 1044 ing *in fine* to discriminate symbiotic organisms from pathogens
 1045 (60). Simultaneous activation of distinct recognition pathways
 1046 would enable a concerted and appropriate response to tolerate or
 1047 eliminate such or such intruder. Another aspect of the molecular
 1048 interaction not yet described and evaluated in invertebrates is the
 1049 temporal dynamic of pathogen perception by soluble immune
 1050 factors. Can this recognition be immediate and frozen once and
 1051 for all, or require gradual biochemical and structural maturation
 1052 to recruit other more specialized immune factors? The dynamic
 1053 of structuration of soluble immune complexes by analyzing
 1054 interactome at different times must be explored to answer this
 1055 question. In this study, we show that different biochemical
 1056 interactions between the external surface of pathogens and host
 1057 molecules occur within just 20 min of interaction. This supports
 1058 the idea of a first wave of pathogen detection that we called
 1059 "sensing," a prerequisite for the subsequent activation of immune
 1060 system. This sensing step appears additive but also epistatic by
 1061 the number of various biological functions involved and suggests
 1062 a cooperative crosstalk for a specific immune response (1). The
 1063 relative function of the IRF, NCIP, and EIP, whether they are
 1064 implicated in pathogen recognition, immune complex maturation,
 1065 and/or triggering of immune response, will require further
 1066 investigation. The method developed herein allowed reaching the
 1067 early step of pathogen sensing, validating the binding ability of
 1068 several IRF, and opening opportunity in model systems to deeper
 1069 study their activity in the immune response pathways.

1070 In summary, the present data constituted an important step
 1071 toward a better understanding of the pathogen sensing and
 1072 immune specificity in invertebrates. It clearly demonstrates
 1073 that innate immune response in invertebrate is not supported
 1074 by a unique class of immune factors but rather by a panel of
 1075 molecules involved in diverse biological functions and able to
 1076 bind specifically to a range of distinct pathogens. Notably, it
 1077 involves some dual immune proteins able to play a role in both
 1078 pathogen binding and clearance. This work does not intend to
 1079 provide an extensive description of all sensing molecules but
 1080 it definitely opens the way to a better integrative biological
 1081 overview of molecules necessary to initiate an orchestrated
 1082 immune response against pathogens in both model and non-
 1083 model organisms.

1084 AUTHOR CONTRIBUTIONS

1085 GT, SP, and DD designed the research; AP, RG, and BG substan-
 1086 tially participated in conception and improvement of research; SP,
 1087 AP, and DD performed interaction experiments; GT performed
 1088 the 2D-SDS-PAGE experiments and qualitative analysis; SP and
 1089 AP performed the Western blots, fluorescent labeling, and micro-
 1090 scope observations; all authors contributed to the analysis and
 1091 interpretation of the results; GT, SP, and DD led the manuscript
 1092 writing; all authors participated to manuscript writing, editing,
 1093 and critical reviewing; and they all approved the final draft.

1094 ACKNOWLEDGMENTS

1095 The authors want to thank Philippe Chan, Marie-Laure Walet-
 1096 Balieu and David Vaudry from PISSARO Proteomic Platform for
 1097 2D spots protein identification and Nathalie Arancibia, Cécile
 1098 Saint-Beat and Anne Rognon for the animal breeding facilities.
 1099 The authors would like to thank the members of EcoEvl's groups
 1100 of IHPE laboratory for helpful discussions.

1101 FUNDING

1102 This work was funded by ANR JCJC INVIMORY (number ANR-
 1103 13-JSV7-0009) from the French National Research Agency (ANR).

1104 SUPPLEMENTARY MATERIAL

1105 The Supplementary Material for this article can be found online at
 1106 [http://journal.frontiersin.org/article/10.3389/fimmu.2017.01249/
 1107 full#supplementary-material](http://journal.frontiersin.org/article/10.3389/fimmu.2017.01249/full#supplementary-material).

1108 **FIGURE S1** | 2D-PAGE gels of "pathogens + hemolymph" and "pathogens only"
 1109 for each of the five pathogens used. Arrows are indicating spots exclusively
 1110 present in the "pathogens + hemolymph" profiles but not in the proteomic
 1111 profiles of "pathogens only," which represents proteins from *Biomphalaria*
 1112 *glabrata* hemolymph that participated in the recognition of pathogen's proteins.

1113 **TABLE S1** | Protein identification of the 109 spots revealed only in
 1114 "pathogens + hemolymph" gels as compared to "pathogens only" gels. For each
 1115 spot, the $-10\log P$ values of proteins and peptides are indicated, together with
 1116 the top BLAST hit in NCBI nr database, the conserved domains of the sequence
 1117 retrieved (performed with NCBI CD-search available at [https://www.ncbi.nlm.nih.
 1118 gov/Structure/cdd/wrpsb.cgi](https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi)) and the pI and molecular mass (calculated with the
 1119 ExPASy "Compute pI/Mw tool" available at http://web.expasy.org/compute_pi).

REFERENCES

- 1127
1128
1129
1130
1131
1132
1133
1134
1135
1136
1137
1138
1139
1140
1141
1142
1143
1144
1145
1146
1147
1148
1149
1150
1151
1152
1153
1154
1155
1156
1157
1158
1159
1160
1161
1162
1163
1164
1165
1166
1167
1168
1169
1170
1171
1172
1173
1174
1175
1176
1177
1178
1179
1180
1181
1182
1183
1. Hargreaves DC, Medzhitov R. Innate sensors of microbial infection. *J Clin Immunol* (2005) 25:503–10. doi:10.1007/s10875-005-8065-4
 2. Janeway CA Jr, Medzhitov R. Innate immune recognition. *Annu Rev Immunol* (2002) 20:197–216. doi:10.1146/annurev.immunol.20.083001.084359
 3. Matthews AJ, Zheng S, Dimenna LJ, Chaudhuri J. Regulation of immunoglobulin class-switch recombination: choreography of noncoding transcription, targeted DNA deamination, and long-range DNA repair. *Adv Immunol* (2014) 122:1–57. doi:10.1016/B978-0-12-800267-4.00001-8
 4. Methot SP, Di Noia JM. Molecular mechanisms of somatic hypermutation and class switch recombination. *Adv Immunol* (2017) 133:37–87. doi:10.1016/bs.ai.2016.11.002
 5. Delaney JR, Stöven S, Uvell H, Anderson KV, Engström Y, Mlodzik M. Cooperative control of *Drosophila* immune responses by the JNK and NF- κ B signaling pathways. *EMBO J* (2006) 25:3068–77. doi:10.1038/sj.emboj.7601182
 6. Slack E, Hapfelmeier S, Stecher B, Velykoredko Y, Stoel M, Lawson MA, et al. Innate and adaptive immunity cooperate flexibly to maintain host-microbiota mutualism. *Science* (2009) 325:617–20. doi:10.1126/science.1172747
 7. Du Pasquier L. Germline and somatic diversification of immune recognition elements in Metazoa. *Immunol Lett* (2006) 104:2–17. doi:10.1016/j.imlet.2005.11.022
 8. Lemaitre B, Hoffmann J. The host defense of *Drosophila melanogaster*. *Annu Rev Immunol* (2007) 25:697–743. doi:10.1146/annurev.immunol.25.022106.141615
 9. Ghosh J, Buckley KM, Nair SV, Raftos DA, Miller C, Majeske AJ, et al. Sp185/333: a novel family of genes and proteins involved in the purple sea urchin immune response. *Dev Comp Immunol* (2010) 34:235–45. doi:10.1016/j.dci.2009.10.008
 10. Armitage SA, Peuss R, Kurtz J. Dscam and pancrustacean immune memory – a review of the evidence. *Dev Comp Immunol* (2015) 48:315–23. doi:10.1016/j.dci.2014.03.004
 11. Hanington PC, Forsys MA, Dragoo JW, Zhang SM, Adema CM, Loker ES. Role for a somatically diversified lectin in resistance of an invertebrate to parasite infection. *Proc Natl Acad Sci U S A* (2010) 107:21087–92. doi:10.1073/pnas.1011242107
 12. Pinaud S, Portela J, Duval D, Nowacki FC, Olive MA, Allienne JF, et al. A shift from cellular to humoral responses contributes to innate immune memory in the vector snail *Biomphalaria glabrata*. *PLoS Pathog* (2016) 12:e1005361. doi:10.1371/journal.ppat.1005361
 13. Cannarsa E, Zampicini G, Friard O, Santovito A, Cervella P. *De novo* transcriptome sequencing of a non-model polychaete species. *Mar Genomics* (2016) 29:31–4. doi:10.1016/j.margen.2016.06.001
 14. Clark KF, Greenwood SJ. Next-generation sequencing and the crustacean immune system: the need for alternatives in immune gene annotation. *Integr Comp Biol* (2016) 56:1113–30. doi:10.1093/icb/icw023
 15. Schultz JH, Adema CM. Comparative immunogenomics of molluscs. *Dev Comp Immunol* (2017) 75:3–15. doi:10.1016/j.dci.2017.03.013
 16. Coustau C, Gourbal B, Duval D, Yoshino TP, Adema CM, Mitta G. Advances in gastropod immunity from the study of the interaction between the snail *Biomphalaria glabrata* and its parasites: a review of research progress over the last decade. *Fish Shellfish Immunol* (2015) 46:5–16. doi:10.1016/j.fsi.2015.01.036
 17. Hussain A, Rizwan-Ul-Haq M, Al-Ayed H, Aljabr AM. Susceptibility and immune defence mechanisms of *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae) against entomopathogenic fungal infections. *Int J Mol Sci* (2016) 17(9):1518. doi:10.3390/ijms17091518
 18. Zhu J, Fu Q, Ao Q, Tan Y, Luo Y, Jiang H, et al. Transcriptomic profiling analysis of tilapia (*Oreochromis niloticus*) following *Streptococcus agalactiae* challenge. *Fish Shellfish Immunol* (2017) 62:202–12. doi:10.1016/j.fsi.2017.01.023
 19. Theron A, Rognon A, Gourbal B, Mitta G. Multi-parasite host susceptibility and multi-host parasite infectivity: a new approach of the *Biomphalaria glabrata*/*Schistosoma mansoni* compatibility polymorphism. *Infect Genet Evol* (2014) 26:80–8. doi:10.1016/j.meegid.2014.04.025
 20. Sminia T, Barendsen L. A comparative morphological and enzyme histochemical study on blood cells of the fresh water snails *Lymnaea stagnalis*, *Biomphalaria glabrata*, and *Bulinus truncatus*. *J Morphol* (1980) 165:31–9. doi:10.1002/jmor.1051650104
 21. Bouchut A, Roger E, Coustau C, Gourbal B, Mitta G. Compatibility in the *Biomphalaria glabrata*/*Echinostoma caproni* model: potential involvement of adhesion genes. *Int J Parasitol* (2006) 36:175–84. doi:10.1016/j.ijpara.2005.09.009
 22. Hanelt B, Lun CM, Adema CM. Comparative ORESTES-sampling of transcriptomes of immune-challenged *Biomphalaria glabrata* snails. *J Invertebr Pathol* (2008) 99:192–203. doi:10.1016/j.jip.2008.06.002
 23. Adema CM, Hanington PC, Lun CM, Rosenberg GH, Aragon AD, Stout BA, et al. Differential transcriptomic responses of *Biomphalaria glabrata* (Gastropoda, Mollusca) to bacteria and metazoan parasites, *Schistosoma mansoni* and *Echinostoma paraensei* (Digenea, Platyhelminthes). *Mol Immunol* (2010) 47:849–60. doi:10.1016/j.molimm.2009.10.019
 24. Eveland LK, Haseeb MA. Laboratory rearing of *Biomphalaria glabrata* snails and maintenance of larval schistosomes *in vivo* and *in vitro*. In: Toledo R, Fried B, editors. *Biomphalaria Snails and Larval Trematodes*. New York: Springer (2011). p. 33–55.
 25. Dheilly NM, Duval D, Mouahid G, Emans R, Allienne JF, Galinier R, et al. A family of variable immunoglobulin and lectin domain containing molecules in the snail *Biomphalaria glabrata*. *Dev Comp Immunol* (2015) 48:234–43. doi:10.1016/j.dci.2014.10.009
 26. Marchler-Bauer A, Lu S, Anderson JB, Chitsaz F, Derbyshire MK, Deweese-Scott C, et al. CDD: a conserved domain database for the functional annotation of proteins. *Nucleic Acids Res* (2011) 39:D225–9. doi:10.1093/nar/gkq1189
 27. Mone Y, Gourbal B, Duval D, Du Pasquier L, Kieffer-Jaquinod S, Mitta G. A large repertoire of parasite epitopes matched by a large repertoire of host immune receptors in an invertebrate host/parasite model. *PLoS Negl Trop Dis* (2010) 4:e813. doi:10.1371/journal.pntd.0000813
 28. Liu X, She XT, Zhu QF, Li H, Peng XX. Heterogeneous interactome between *Litopenaeus vannamei* plasma proteins and *Vibrio parahaemolyticus* outer membrane proteins. *Fish Shellfish Immunol* (2013) 34:192–8. doi:10.1016/j.fsi.2012.10.023
 29. Wu XJ, Dinguirard N, Sabat G, Lui HD, Gonzalez L, Gehring M, et al. Proteomic analysis of *Biomphalaria glabrata* plasma proteins with binding affinity to those expressed by early developing larval *Schistosoma mansoni*. *PLoS Pathog* (2017) 13:e1006081. doi:10.1371/journal.ppat.1006081
 30. Smit CH, Van Diepen A, Nguyen DL, Wührer M, Hoffman KF, Deelder AM, et al. Glycomic analysis of life stages of the human parasite *Schistosoma mansoni* reveals developmental expression profiles of functional and antigenic glycan motifs. *Mol Cell Proteomics* (2015) 14:1750–69. doi:10.1074/mcp.M115.048280
 31. Pees B, Yang W, Zarate-Potes A, Schulenburg H, Dierking K. High innate immune specificity through diversified C-type lectin-like domain proteins in invertebrates. *J Innate Immun* (2016) 8:129–42. doi:10.1159/000441475
 32. Yoshida S, Shimada T, Kondoh D, Kozuma Y, Ghosh AK, Jacobs-Lorena M, et al. Hemolytic C-type lectin CEL-III from sea cucumber expressed in transgenic mosquitoes impairs malaria parasite development. *PLoS Pathog* (2007) 3:e192. doi:10.1371/journal.ppat.0030192
 33. Wongpanya R, Sengprasert P, Amparyup P, Tassanakajon A. A novel C-type lectin in the black tiger shrimp *Penaeus monodon* functions as a pattern recognition receptor by binding and causing bacterial agglutination. *Fish Shellfish Immunol* (2017) 60:103–13. doi:10.1016/j.fsi.2016.11.042
 34. Gordy MA, Pila EA, Hanington PC. The role of fibrinogen-related proteins in the gastropod immune response. *Fish Shellfish Immunol* (2015) 46:39–49. doi:10.1016/j.fsi.2015.03.005
 35. Portet A, Pinaud S, Tetreau G, Galinier R, Cosseau C, Duval D, et al. Integrated multi-omic analyses in *Biomphalaria-Schistosoma* dialogue reveal the immunobiological significance of FREP-SmPoMuc interaction. *Dev Comp Immunol* (2017) 75:16–27. doi:10.1016/j.dci.2017.02.025
 36. Hokke CH, Fitzpatrick JM, Hoffmann KF. Integrating transcriptome, proteome and glycome analyses of *Schistosoma* biology. *Trends Parasitol* (2007) 23:165–74. doi:10.1016/j.pt.2007.02.007
 37. Peterson NA, Hokke CH, Deelder AM, Yoshino TP. Glycotope analysis in miracidia and primary sporocysts of *Schistosoma mansoni*: differential expression during the miracidium-to-sporocyst transformation. *Int J Parasitol* (2009) 39:1331–44. doi:10.1016/j.ijpara.2009.06.002
 38. Adema CM. Fibrinogen-related proteins (FREPs) in mollusks. *Results Probl Cell Differ* (2015) 57:111–29. doi:10.1007/978-3-319-20819-0_5

Q13

- 1241 39. Galinier R, Portela J, Mone Y, Allienne JF, Henri H, Delbecq S, et al. 1298
 1242 Biomphalysin, a new beta pore-forming toxin involved in *Biomphalaria* 1299
 1243 *glabrata* immune defense against *Schistosoma mansoni*. *PLoS Pathog* (2013) 1300
 9:e1003216. doi:10.1371/journal.ppat.1003216
- 1244 40. Adema CM, Hillier LW, Jones CS, Loker ES, Knight M, Minx P, et al. Whole 1301
 1245 genome analysis of a schistosomiasis-transmitting freshwater snail. *Nat* 1302
 1246 *Commun* (2017) 8:15451. doi:10.1038/ncomms15451
- 1247 41. Moran Y, Fredman D, Szczesny P, Grynberg M, Technau U. Recurrent hori- 1303
 1248 zontal transfer of bacterial toxin genes to eukaryotes. *Mol Biol Evol* (2012) 1304
 29:2223–30. doi:10.1093/molbev/mss089
- 1249 42. Schnitger AK, Yassine H, Kafatos FC, Osta MA. Two C-type lectins cooperate 1305
 1250 to defend *Anopheles gambiae* against Gram-negative bacteria. *J Biol Chem* 1306
 1251 (2009) 284:17616–24. doi:10.1074/jbc.M808298200
- 1252 43. Oliveira-Nascimento L, Massari P, Wetzler LM. The role of TLR2 in infection 1307
 1253 and immunity. *Front Immunol* (2012) 3:79. doi:10.3389/fimmu.2012.00079
- 1254 44. Williams SJ, Sohn KH, Wan L, Bernoux M, Sarris PF, Segonzac C, et al. 1308
 1255 Structural basis for assembly and function of a heterodimeric plant immune 1309
 1256 receptor. *Science* (2014) 344:299–303. doi:10.1126/science.1247357
- 1257 45. Mitta G, Galinier R, Tisseyre P, Allienne JF, Girerd-Chambaz Y, Guillou F, 1310
 1258 et al. Gene discovery and expression analysis of immune-relevant genes from 1311
 1259 *Biomphalaria glabrata* hemocytes. *Dev Comp Immunol* (2005) 29:393–407. 1312
 doi:10.1016/j.dci.2004.10.002
- 1260 46. Deleury E, Dubreuil G, Elangovan N, Wajnberg E, Reichhart JM, Gourbal B, 1313
 1261 et al. Specific versus non-specific immune responses in an invertebrate species 1314
 1262 evidenced by a comparative de novo sequencing study. *PLoS One* (2012) 1315
 1263 7:e32512. doi:10.1371/journal.pone.0032512
- 1264 47. Sandiford SL, Dong Y, Pike A, Blumberg BJ, Bahia AC, Dimopoulos G. 1316
 1265 Cytoplasmic actin is an extracellular insect immune factor which is secreted 1317
 1266 upon immune challenge and mediates phagocytosis and direct killing of 1318
 1267 bacteria, and is a *Plasmodium* antagonist. *PLoS Pathog* (2015) 11:e1004631. 1319
 1268 doi:10.1371/journal.ppat.1004631
- 1269 48. Bernimoulin M, Waters EK, Foy M, Steele BM, Sullivan M, Falet H, et al. 1320
 1270 Differential stimulation of monocytic cells results in distinct populations 1321
 1271 of microparticles. *J Thromb Haemost* (2009) 7:1019–28. doi:10.1111/j. 1322
 1272 1538-7836.2009.03434.x
- 1273 49. Pradeu T, Cooper EL. The danger theory: 20 years later. *Front Immunol* (2012) 1323
 1274 3:287. doi:10.3389/fimmu.2012.00287
- 1275 50. Coates CJ, Decker H. Immunological properties of oxygen-transport pro- 1324
 1276 teins: hemoglobin, hemocyanin and hemerythrin. *Cell Mol Life Sci* (2017) 1325
 1277 74:293–317. doi:10.1007/s00018-016-2326-7
- 1278 51. Li D, Dong H, Li S, Munir M, Chen J, Luo Y, et al. Hemoglobin subunit beta 1326
 1279 interacts with the capsid protein and antagonizes the growth of classical swine 1327
 1280 fever virus. *J Virol* (2013) 87:5707–17. doi:10.1128/JVI.03130-12
- 1281 52. Yan S, Rodrigues RG, Cahn-Hidalgo D, Walsh TJ, Roberts DD. Hemoglobin 1328
 1282 induces binding of several extracellular matrix proteins to *Candida albicans*. 1329
 1283 Identification of a common receptor for fibronectin, fibrinogen, and laminin. 1330
 1284 *J Biol Chem* (1998) 273:5638–44. doi:10.1074/jbc.273.10.5638
- 1285 53. Garg R, Qadri A. Hemoglobin transforms anti-inflammatory *Salmonella typhi* 1331
 1286 virulence polysaccharide into a TLR-2 agonist. *J Immunol* (2010) 184:5980–7. 1332
 1287 doi:10.4049/jimmunol.0903512
- 1288 54. Henry E, Fung N, Liu J, Drakakaki G, Coaker G. Beyond glycolysis: GAPDHs 1333
 1289 are multi-functional enzymes involved in regulation of ROS, autophagy, 1334
 1290 and plant immune responses. *PLoS Genet* (2015) 11:e1005199. doi:10.1371/ 1335
 1291 journal.pgen.1005199
- 1292 55. Apte SS, Parks WC. Metalloproteinases: a parade of functions in matrix biol- 1336
 1293 ogy and an outlook for the future. *Matrix Biol* (2015) 44-46:1–6. doi:10.1016/j. 1337
 1294 matbio.2015.04.005
- 1295 56. Yuan S, Tao X, Huang S, Chen S, Xu A. Comparative immune systems in ani- 1338
 1296 mals. *Annu Rev Anim Biosci* (2014) 2:235–58. doi:10.1146/annurev-animal- 1339
 1297 031412-103634
- 1298 57. Brubaker SW, Bonham KS, Zanon I, Kagan JC. Innate immune pattern rec- 1340
 1299 ognition: a cell biological perspective. *Annu Rev Immunol* (2015) 33:257–90. 1341
 1300 doi:10.1146/annurev-immunol-032414-112240
- 1301 58. Armitage SAO, Kurtz J, Brites D, Dong Y, Pasquier L, Wang HC. Dscam1 1342
 1302 in pancrustacean immunity: current status and a look to the future. *Front* 1343
 1303 *Immunol* (2017) 8:662. doi:10.3389/fimmu.2017.00662
- 1304 59. Nish S, Medzhitov R. Host defense pathways: role of redundancy and com- 1344
 1305 pensation in infectious disease phenotypes. *Immunity* (2011) 34:629–36. 1345
 1306 doi:10.1016/j.immuni.2011.05.009
- 1307 60. Pradeu T, Jaeger S, Vivier E. The speed of change: towards a discontinuity 1346
 1308 theory of immunity? *Nat Rev Immunol* (2013) 13:764–9. doi:10.1038/nri3521
- 1309 61. Korpos E, Wu C, Sorokin L. Multiple roles of the extracellular matrix in inflam- 1347
 1310 mation. *Curr Pharm Des* (2009) 15:1349–57. doi:10.2174/138161209787846685

Q14

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Copyright © 2017 Tetreau, Pinaud, Portet, Galinier, Gourbal and Duval. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

3. Mise au point d'une technique d'inactivation fonctionnelle sur les cellules immunitaires de *Biomphalaria glabrata*

Publication : An improved method for siRNA gene silencing in Biomphalaria glabrata hemocytes

PORTET Anaïs, GOURBAL Benjamin and DUVAL David

*Article en préparation

1 **An improved method for siRNA Gene Silencing in *Biomphalaria glabrata* hemocytes**

2
3 PORTET Anaïs, GOURBAL Benjamin and DUVAL David

4 5 **Abstract**

6 *B. glabrata* is the intermediate host of *Schistosoma mansoni*, the parasite responsible for
7 Schistosomiasis disease. Understanding the molecular interaction between the snail and its parasite
8 would be determinant to find new targets for disease control. To achieve this goal it is necessary to
9 develop efficient invalidation approaches to validate these potential targets. Herein we described an
10 improvement of gene silencing method by RNA interference (RNAi) in the snail *Biomphalaria*
11 *glabrata* to target specifically the hemocyte, the snail immune cells. To improve this technic, a
12 comparison of siRNA with and without Cholesterol-TEG mediated delivery of siRNA were used.
13 Cellular delivery was evaluated and optimized by flow cytometry using a “mock” fluorescent siRNA.
14 Then we used the optimized method to silencing the BgTEP expression in snail hemocytes.
15 The develop method offers a first silencing approach optimized to target the hemocyte tissue and
16 study hemocyte gene functions during the snail immune response.

17 18 **Introduction**

19 *Biomphalaria glabrata* is the vector snail host that transmit the platyhelminth parasite,
20 *Schistosoma mansoni*, responsible for the Schistosomiasis. Schistosomiasis is the second
21 most widespread human parasitic disease after the malaria. As gastropod contributes
22 importantly to transmission of human schistosomiasis, one opportunity would be to
23 emphasizes on snail control for developing new strategies to reduce schistosomiasis
24 transmission in the field (Fallon and Doenhoff 1994; Tennessen, et al. 2015). Understanding
25 the snail-parasite immunological interaction and molecular mechanisms by which snail and
26 parasite interact appears as a good way to find new targets for manipulating the snail
27 intermediate host (Tennessen, et al. 2015).

28 The compatibility between the pathogen and its host is determined by two main factors, the
29 susceptibility of the host and the infectivity of the parasite. In *Biomphalaria*, this
30 host/pathogen compatibility is based on genetic factors, a complex matching phenotype
31 model (Theron, et al. 2014) but can also be modulated by non-genetic factors, such as
32 temperature (Knight, et al. 2015), pollution (Ibrahim 2006) or UV exposure (Ruelas, et al.
33 2009).

34 To investigate and characterize the function of candidate genes or molecular pathways that
35 are involved in the snail-parasite interactions, the technology of RNA interference (RNAi)
36 widely used in other invertebrate models, appeared as a promising technology.

37 Indeed, gene silencing has been conducted with double strand RNA (dsRNA), in *Anopheles*
38 *gambiae* (Levashina, et al. 2001)(Blandin, et al. 2002) ; this method was also developed for
39 *Drosophila* adult flies (Goto, et al. 2003) ; for shrimp (Robalino, et al. 2004), for copepods
40 (Campbell, et al. 2009; Barreto, et al. 2015) or for the red flour beetle, in which dsRNA and
41 siRNA (short interference RNA) were tested (Wang, et al. 2013).

42 In *Biomphalaria glabrata*, RNA silencing has been also established. The first approach was
43 conducted to invalidate Fibrinogen Related Protein 2 (FREP2) expression with dsRNA and
44 results in a 20-30% reduction of expression (Jiang, et al. 2006). FREP3 was also silenced using
45 siRNA and a reduction of 30% of expression was observed (Hanington, et al. 2010;
46 Hanington, et al. 2012). FREP 2, 3 and 4 were also invalidated at the same time to
47 demonstrate their role in innate immune memory and a reduction by 15% of the acquired
48 protection was observed (Pinaud, et al. 2016). Finally the cytokine MIF (Macrophage
49 Migration Inhibitory Factor) was also invalidated with dsRNA (Garcia, et al. 2010).

50 Few years ago, the RNAi technic was improved by using siRNA not injected into the snail
51 tissue but transferred using a soaking method realised with siRNA or dsRNA associated with
52 Polyethyleneimine (PEI), a canonic polymer, used to optimized the penetration into the snail
53 tissues (Knight, et al. 2011). This silencing technics was used to investigate the role of a
54 lipopolysaccharide binding protein/bactericidal permeability increasing protein family
55 member from *Biomphalaria glabrata* (BgLBP/BPI1) (Baron, et al. 2013). So, a lot of studies
56 were already realised based on the RNAi silencing technics, but all these investigations were
57 realised on whole snail.

58 In this present study, we want to improve the technics efficiency on hemocytes. Given that
59 the hemocytes were the support of *B. glabrata* immune response, the specific hemocyte
60 gene silencing may contribute to explore the role of these circulating cells in the snail
61 immune response.

62 For this, according to *in vitro* and *in vivo* conditions the incorporation into hemocytes was
63 evaluated and optimized by using a 'mock' fluorescent siRNA. After that, the RNAi silencing
64 of *Biomphalaria glabrata* Thioester-containing protein (BgTEP) was realised along a kinetic
65 of infection to explore its role in the snail immune response.

66

67 **Materials and Methods**

68 ***Biological Materials***

69 Adults (10 mm in diameter) of Guadeloupian strain of *Biomphalaria glabrata* were used in
70 this study. During experimentations, the hemolymph is extracted from the head-foot
71 according to standard procedures (Sminia and Barendsen 1980) for the *in vitro* and *in vivo*
72 study.

73

74 ***Interfering RNA***

75 All of small inhibitory RNAs (siRNAs) were customised and synthesized by Eurogentec. The
76 siRNAs were purified with SePoP technic.

77 *- Test of incorporation - 'mock' fluorescent siRNA*

78 One siRNA for the GFP transcripts mRNA (GenBank accession No. EU430082) 'block it' CY3
79 was designed. siRNA_CY3 forward: CAA-GCU-GAC-CCU-GAA-GUU-C55 / siRNA_CY3 reverse:
80 GAA-CUU-CAG-GGU-CAG-CUU-G55. A second couple of siRNA, 'block it' CY3, was realised
81 with the same primer sequences but a Cholesteryl-TEG was added in 5' (siRNA_Chol_CY3).

82 *- Knockdown of a candidate gene*

83 Two small inhibitory RNA (siRNA) for the BgTEP transcripts mRNA (GenBank accession No.
84 FJ480411) was designed with a Cholesteryl-TEG added in 5'. TEP_1_forward: GAC-AGA-UUC-
85 UCA-UCA-AAC-A55 / TEP_1_reverse: UGU-UUG-AUG-AGA-AUC-UGU-C55 and
86 TEP_2_forward: GAG-UAU-GAU-UUA-CCA-AGA-U55 / TEP_2_reverse: AUC-UUG-GUA-AAU-
87 CAU-ACU-C55.

88

89 ***Procedures for siRNA Injections***

90 *- In vitro Injection*

91 Incubation of snail hemolymph with 2µg of siRNA (1µg/µL).

92 *In vitro injection*: The observation of siRNA incorporation *in vitro* during a kinetic of
93 incubation from 30 minutes, to 1, 2, 4, 6 and 8 hours of incubation was realised. For each
94 point of the kinetic, 4 replicates with the hemolymph of 3 molluscs were performed. After
95 recovered of the hemolymph, 2µg of siRNA were incorporated in each sample. The same
96 kinetic was realised with siRNA_CY3 and siRNA_Chol_CY3. The hemolymph of seventy-two
97 molluscs was recovered and incubated following the kinetic of incubation described above.

98 The hemolymph of twelve molluscs was recovered and treated with the same procedure
99 than the other samples and used as the naive condition for the normalization, with 6
100 replicates of 3 molluscs.

101 *- In vivo Injection*

102 In this case, two experimentations were realised, only with the siRNA_Chol_CY3. In the first
103 experiment we injected the same siRNA quantity than the one use in *in vitro* injection (2µg)
104 and we observed the incorporation along the kinetic. In the second experiment, 3 siRNA
105 quantities were used (2µg, 5µg and 10µg) during solely one time point of incubation.

106 The injection is realised in the pericardial cavity; a hole was realised with a 26G needle in the
107 shell and then, 2µL of siRNA were injected with a HPLC syringe.

108 *In vivo injection along a kinetic of incubation:* Observation of the siRNA incorporation *in vivo*
109 was realised following different incubation times: 2, 4, 6, 12 and 24 hours after injection. For
110 each point of the kinetic 4 snail individuals were injected and each was used as replicates.
111 Two microliters of siRNA were injected in each mollusc. Twenty-five molluscs were injected
112 with the siRNA_Chol_CY3. Four molluscs without injection used like control, were treated
113 with the same procedure than the injected samples. These samples are used as the naive
114 condition for the normalization.

115 *In vivo injection with different quantity:* Observation of the siRNA incorporation *in vivo*
116 depending on the concentration used (3 siRNA quantities: 2µg, 5µg and 10µg), 2 hours after
117 injection. For each point of kinetic 4 snail individuals were injected and used as replicates.
118 Two microliters of siRNA were injected in each mollusc. Four molluscs were injected with
119 each siRNA_Chol_CY3 concentration. Four molluscs without injection used like control were
120 treated with the same procedure that the injected samples. These samples are used as the
121 naive condition for the normalization.

122

123 ***Flow cytometry analysis***

124 After injection of siRNA (*in vitro* or *in vivo*), 100µL of hemolymph are fixed with 100µL of
125 paraformaldehyde (4%). Then, the hemocytes are centrifuged (5,000 rpm during 10 minutes)
126 and the supernatant are discard. The hemocytes are suspended in 400µL of PBS-snail. The
127 samples are stored in fridge and analysed one day after by flow cytometry.

128 The Flow Cytometry is realised on FACSCanto BD Biosciences (RIO Imaging Platform,
129 Montpellier, France) for each samples a counting of 10,000 events is made. Flow cytometry

130 results were analysed using FlowJo V 10.0.8 and significant differences in the percentage of
131 proliferation were tested using a Mann-Whitney U test with a p-value < 0.05.

132

133 **Quantitative PCR analysis**

134 *- Biological procedure*

135 Injection of 27 molluscs with 10µg of siRNA_Chol_CY3 (5µg/µL). The injection is realised in
136 the pericardial cavity; a hole was realised with a 26G needle in the shell and then, 2µL of
137 siRNA are injected with a HPLC syringe. The hemolymph is recovered along a kinetic of 2, 3
138 and 4 days after injection. For each point of the kinetic, 3 pools of 3 molluscs were realised.

139 *- RNA extraction and cDNA production*

140 The hemolymph is recovery by pool of 3 molluscs and the RNA is extracted using a
141 commercial kit, Total RNA Purification Micro Kit (Norgen Biotek) according to the
142 manufacturer's instructions. For cDNA synthesis a commercial kit was used, the Maxima H
143 Minus First Strand cDNA synthesis Kit with dsDNases (Thermo Scientific) and retro-
144 transcription was done according to the manufacturer's instructions.

145 *- Q-PCR procedure*

146 Quantitative PCR (Q-PCR) was performed using NO ROX SYBR[®] Master Mix blue dTTP
147 (Takyon) according to the manufacturer's instructions and run by using the LightCycler 480
148 (Roche). Ten microliters of Q-PCR mixture contained 30 ng cDNA, 5 µL Master Mix and 1µL of
149 each specific primers were used for the Q-PCR. For BgTEP, primers were: TEP-F CGT-ACT-
150 TAC-CCT-CGC-TC, TEP-R ACC-ATT-AGA-TCC-ACT-GGA-AGA-TA. The S19 was used as the
151 endogenous standard for normalization (Galinier, et al. 2013). Three technical replicated of
152 Q-PCR were performed with three internal controls to assess both potential genomic DNA
153 contamination (no reverse transcriptase added) and purity of the reagents used (no DNA
154 sample added). The knockdown experiment was realised with individual snail and each was
155 used as independent biological replicates.

156 *- Statistical analysis*

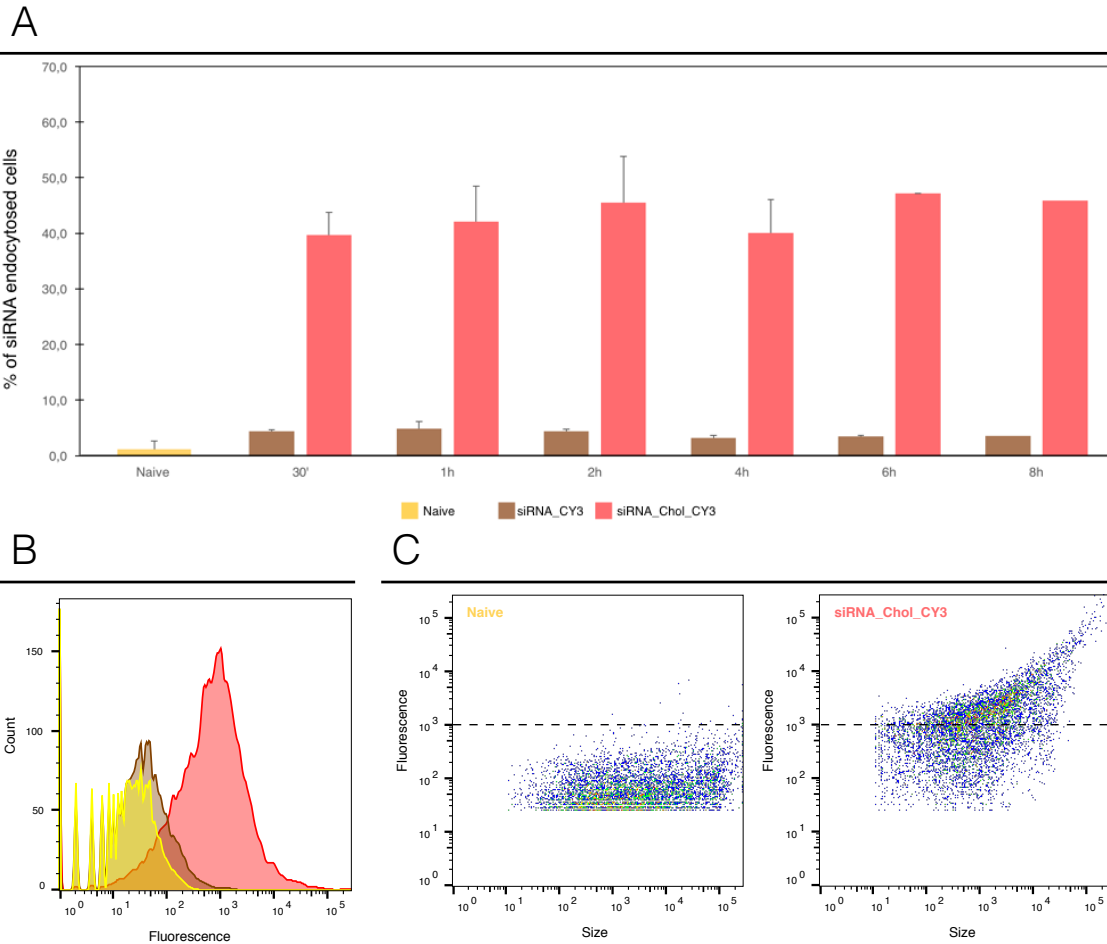
157 Significant differences in the percentage of silencing were tested using a Mann-Whitney U
158 test with a p-value < 0.05.

159

160 **Results & Discussion**

161 ***Test of in vitro incorporation***

162 To determine whether Cholesteryl-TEG can increase the uptake of siRNA into the snail
163 hemocytes, we tested separately siRNA with and without Cholesteryl-TEG couple with a
164 fluorescent tag CY3. In this approach the siRNA was injected *in vitro* on hemocyte after being
165 recovered on naive snails, the fluorescence quantification was observed by flow cytometry.
166 The siRNA was uptake by the cells along a time point kinetic, for each time point, we
167 compare the siRNA with and without Cholesteryl-TEG (fig. 1). The uptake of siRNA without
168 Cholesteryl-TEG (siRNA_CY3) is the same along the kinetic with around 5% of hemocytes
169 that were fluorescent, indicating that they have incorporated the siRNA (fig. 1A). The siRNA
170 with Cholesteryl-TEG (siRNA_Chol_CY3) is more uptake by hemocytes with around 47% of
171 fluorescent cells (fig. 1B). Here also, the incorporation of siRNA_Chol_CY3 is not significantly
172 different along the kinetic (fig. 1A). In flow cytometry to confirm the real fluorescent cell
173 uptake we compare the fluorescent profile of siRNA_CY3 and siRNA_Chol_CY3 (fig. 2B). This
174 result shows a number of cells with a high fluorescent uptake, greater in siRNA_Chol_CY3
175 condition compared to siRNA_CY3. Moreover, the fluorescent profile of siRNA_CY3 is really
176 similar to the naive profile (fig. 1B). Finally, we observe that the uptake of siRNA is realised
177 by all hemocytes. Indeed, if we look at the fluorescence uptake along the different cell size
178 in flow cytometry analyses, we observed fluorescent cells for all the gradient of potential
179 size scale (fig. 1C). It should be noted that the larger cells uptake more than the smaller cells
180 (fig. 1C). In naive condition the fluorescence is not observed (fig. 3C).
181



182

183 ***Figure 1: Comparison of siRNA with and without Cholesteryl-TEG in vitro incorporation***

184 *The naive condition is yellow, siRNA_CY3 uptake test is brown and siRNA_Chol_CY3 test is red.*

185 *A. Flow cytometry analysis of siRNA uptake. Representation of % of siRNA endocytosed cells along a*
 186 *kinetic of time during which one the 2 μ g in vitro hemocytes are incubated with siRNA. The naive*
 187 *point corresponds to cell auto-fluorescence. B. Fluorescence profile in the 3 conditions, count*
 188 *corresponds to the cells numbers and the fluorescence scale corresponds to the quantity uptake. The*
 189 *point of 10^3 corresponds to the limit of positive and negative fluorescence. C. Profile of hemocytes*
 190 *according to their fluorescence uptake and their size. Comparison to naive and siRNA_Chol_CY3. The*
 191 *dotted lines correspond to the limit of positive and negative fluorescence.*

192

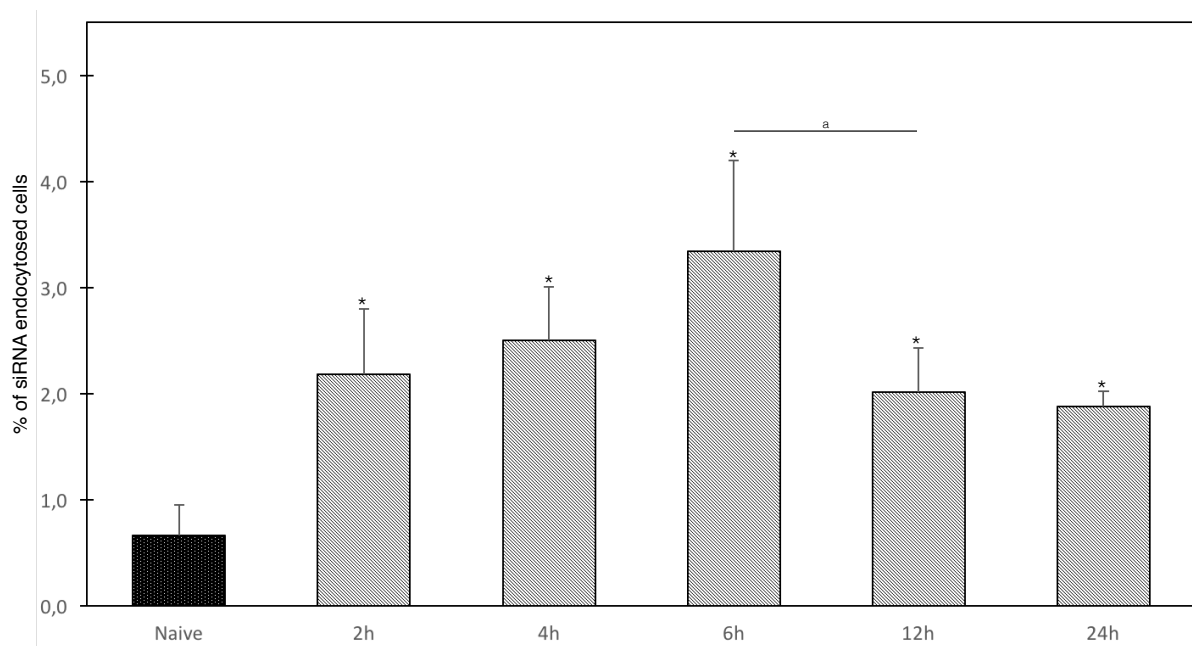
193 To conclude, the siRNA_Chol_CY3 show a best uptake capacity. Indeed, the half of cells have
 194 incorporated the fluorescence and so the siRNA. For further analysis, we used solely siRNA
 195 couple with Cholesteryl_TEG. Moreover, the time of incorporation seems not to affect the
 196 percentage of hemocytes siRNA uptake.

197

198 ***Test of in vivo incorporation***

199 To determined if the siRNA with Cholesteryl_TEG is a good tool to target real gene on
 200 hemocytes in whole snail, we injected the siRNA *in vivo* in pericardial cavity and quantified

201 the fluorescence uptake by flow cytometry. The siRNA was uptake by the cells along a time
202 point kinetic, for each time point we recovered the hemolymph and quantified the
203 fluorescence into the hemocytes (fig. 2). The percentage of siRNA internalised by the
204 hemocytes is lower than in the *in vitro* tests. Indeed, between 3 and 4 % of cells have
205 incorporated siRNA at 2, 4 and 6 hours post-injection compared to around 40% in 30 min for
206 *in vitro* tests . After 6 hours the cell incorporation decrease significantly to 2% of the cells
207 that were fluorescents (fig. 2). For all kinetic time points the percentage of fluorescent cells
208 was more important than in naive condition.
209



210

211 Figure 2: Injection of siRNA with Cholesteryl-TEG in vivo incorporation

212 The naive condition is black and siRNA_Chol_CY3 test is grey.

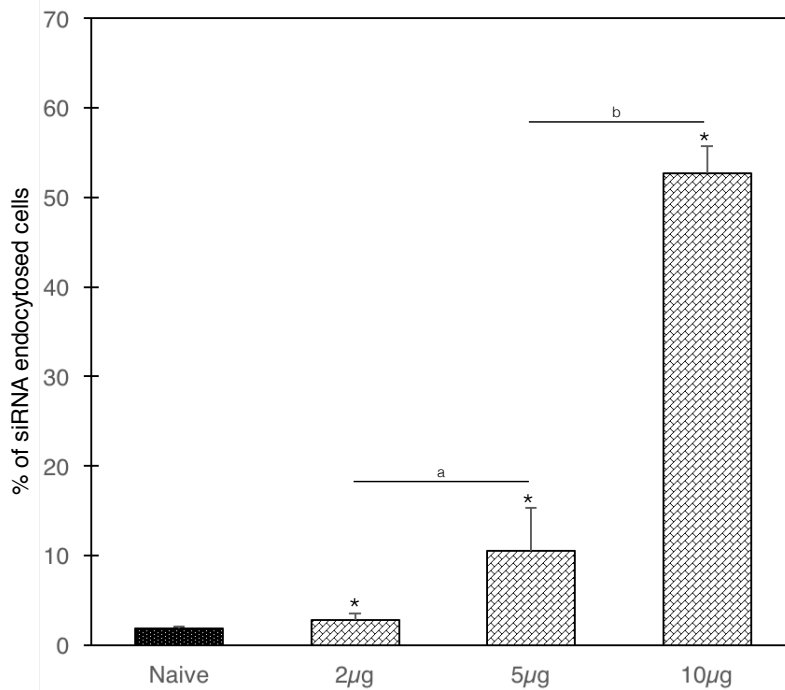
213 Flow cytometry analysis of siRNA with Cholesteryl-TEG uptake. Representation of % of siRNA
214 incorporated in cells along a time point kinetic following injection of 2 µg of siRNA in vivo in whole
215 snail. The naive point corresponds to cell auto-fluorescence. The "*" corresponds to significant
216 differences between the different time point kinetic and naive condition (tested with Mann-Whitney U
217 test). The "a" corresponds to significant differences between 6 and 12 hours post-injection (Mann-
218 Whitney U test).

219

220 To conclude, siRNA with Cholesteryl_TEG is incorporated in hemocyte after an *in vivo*
221 injection. But the cell proportion which has uptake the siRNA is less important than in *in*
222 *vitro* experiment using the same siRNA. Moreover, the percentage of cells, which has
223 incorporated the siRNA, increased between 2 and 6 hours after injection. We decide to
224 conserve 2 hours as a unique point for further analyses to avoid the point of maximum
225 incorporation and the uptake decrease observed at the end of the kinetic.

226
227
228
229
230
231
232
233
234
235
236

To improve the percentage of siRNA incorporated in cells we have to increase the siRNA dose injected into the pericardial cavity. To determine the best siRNA concentration, we recovered the hemocytes after siRNA *in vivo* injection of 3 different concentrations in whole snail pericardial cavity. For that we injected 2, 5 and 10 μg of siRNA-Chol_CY3 and recovered the hemocytes 2 hours after injection. The percentage of siRNA incorporated by hemocyte increases significantly according to the dose injected (fig. 3). Indeed, with 2 μg of siRNA injected around 4% of cells are fluorescents and with 5 and 10 μg injected, 11 and 55% of cells are fluorescents, respectively (fig. 3). So, the uptake of siRNA seems to be correlated and proportional to the injected quantity of siRNA.



237
238
239
240
241
242
243
244
245
246

Figure 3: Comparison of different doses of siRNA with Cholesteryl-TEG in vivo incorporation

The naive condition is black and siRNA_Chol_CY3 test is grey.

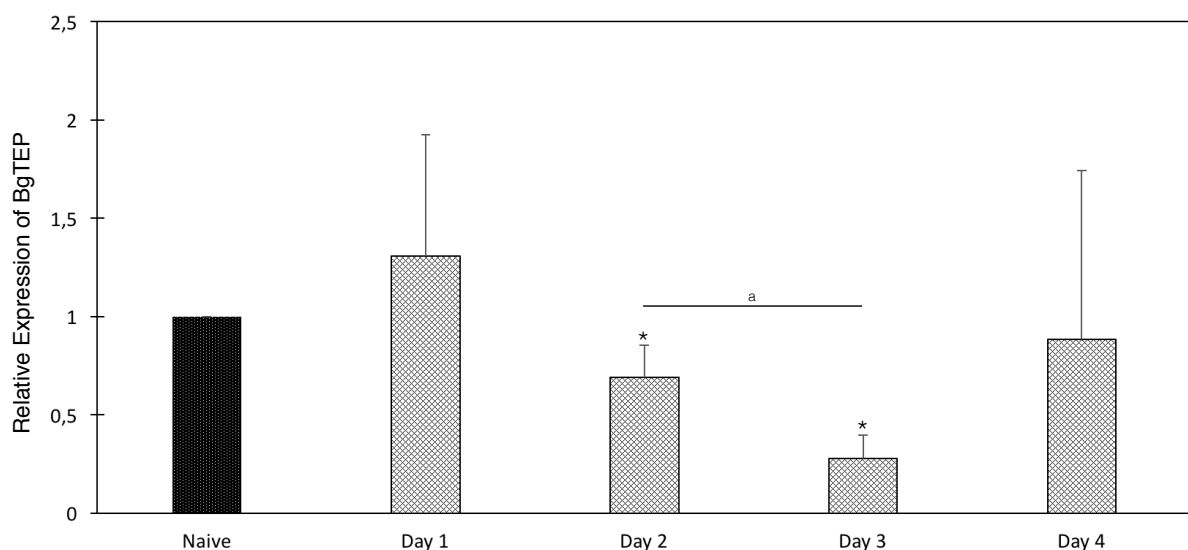
Flow cytometry analysis of siRNA with Cholesteryl-TEG uptake. Representation of % of siRNA incorporated cells after different injected dose 2 hours post-injection. The naive point corresponds to cell auto-fluorescence. The "*" corresponding to significant differences between the different kinetic point and naive condition (Mann-Whitney U test). The "a" corresponds to significant differences between 2 and 5 μg of siRNA injected and "b" to significant differences between 5 and 10 μg of siRNA injected (Mann-Whitney U test).

247 To conclude, the siRNA_Chol_CY3 is uptake by hemocytes after an *in vivo* injection (fig. 2 and
248 3). Moreover, the percentage of cell which has incorporated the siRNA is correlated to the

249 injected doses (fig. 3). Indeed, among the 3 doses tested the greatest concentration seems
250 the better and has the biggest percentage of cell incorporation. For further analysis we thus
251 propose to use an injection of 10 µg of siRNA coupled with Cholesteryl-TEG.

252
253 To determine the efficiency of this new technique of siRNA silencing we have realised siRNA
254 against a hemocyte target the BgTEP. BgTEP is a thioester-containing protein identified in *B.*
255 *glabrata* within an immune complex constituted by snail receptors and parasite antigens
256 (Moné, et al. 2010). This BgTEP seems to have an opsonin role (Portet, et al. *in prep*) like
257 observed in *A. gambiae* with AgTEP1 (Levashina, et al. 2001). Moreover, in addition to
258 having an opsonin role the BgTEP is also found mainly in hemocytes. So, this protein is the
259 best candidate to explore hemocytes gene silencing. To observe the siRNA silencing effect
260 on BgTEP expression we have realised an injection of 10 µg in snail pericardial cavity and we
261 recovered the hemolymph at 1, 2, 3 and 4 days after injection. We compared the expression
262 of BgTEP with the expression of S19 housekeeping gene (fig. 4). The expression of BgTEP
263 decreases significantly 2 and 3 days after siRNA injection compared to naive BgTEP
264 expression (fig. 4). At 4 days the BgTEP expression increase and return to basal expression
265 level similar to naive snails. We can thus propose that the siRNA is consumed (fig. 4). One
266 day after injection, the expression of BgTEP seems to be more important than naive
267 condition but the difference is not significant, a transitory compensation of the silencing can
268 explain such observation.

269



270

271 Figure 4: Time course for optimum gene knockdown after injection of siRNA with Cholesteryl-TEG

272 *Injection of 10 µg of siRNA-Chol against BgTEP in pericardial cavity. Analysis of BgTEP expression*
273 *during 4 days after siRNA injection. The expression of BgTEP is normalized with the S19 housekeeping*
274 *gene expression. The “*” corresponding to significantly difference between the different kinetic point*
275 *and naive condition (Mann-Whitney U test). The “a” corresponding to significantly difference*
276 *between 2 and 3 days after injection (Mann-Whitney U test).*
277

278 To conclude, this new siRNA silencing technic seems to work on snail cells during 3 days.
279 Three days after injection appeared as the best choice to explore the phenotype associated
280 with the silenced target gene.

281
282 The Cholesteryl-TEG method improved significantly the siRNA silencing into snail hemocytes.
283 The BgTEP, expressed mainly by hemocytes have been successfully silenced and show a
284 decrease of the expression into cells after silencing. Our results showed the possibility of
285 silencing into hemocyte tissue compartment and observing a decrease of target gene
286 expression. For obtaining such results, the uptake technic has been optimized to succeed in
287 having near half of the hemocytes that are silenced.

288 To complete this analysis a confocal microscopy approach would be necessary to confirm the
289 incorporation of the siRNA into the hemocytes. Moreover, cryosection analysis would be
290 undertaken to observe the specific incorporation of siRNA into hemocytes.

291

292 **References**

- 293 Baron OL, van West P, Industri B, Ponchet M, Dubreuil G, Gourbal B, Reichhart J-M, Coustau C. 2013.
294 Parental Transfer of the Antimicrobial Protein LBP/BPI Protects Biomphalaria glabrata Eggs
295 against Oomycete Infections. PLoS Pathogens 9:e1003792-e1003792.
296 Barreto FS, Schoville SD, Burton RS. 2015. Reverse genetics in the tide pool: Knock-down of target
297 gene expression via RNA interference in the copepod Tigriopus californicus. Molecular Ecology
298 Resources 15:868-879.
299 Blandin S, Moita LF, Köcher T, Wilm M, Kafatos FC, Levashina EA. 2002. Reverse genetics in the
300 mosquito Anopheles gambiae: Targeted disruption of the Defensin gene. EMBO Reports 3:852-
301 856.
302 Campbell EM, Pert CC, Bowman aS. 2009. RNA-interference methods for gene-knockdown in the sea
303 louse, Lepeophtheirus salmonis: studies on a putative prostaglandin E synthase. Parasitology
304 136:867-874.
305 Fallon PG, Doenhoff MJ. 1994. Drug-Resistant Schistosomiasis: Resistance to Praziquantel and
306 Oxamniquine Induced in Schistosoma Mansoni in Mice is Drug Specific. The American Journal of
307 Tropical Medicine and Hygiene 51:83-88.
308 Galinier R, Portela J, Moné Y, Allienne JF, Henri H, Delbecq S, Mitta G, Gourbal B, Duval D. 2013.
309 Biomphalysin, a New β Pore-forming Toxin Involved in Biomphalaria glabrata Immune Defense
310 against Schistosoma mansoni. PLoS Pathogens 9:e1003216-e1003216.

311 Garcia AB, Pierce RJ, Gourbal B, Werkmeister E, Colinet D, Reichhart JM, Dissous C, Coustau C. 2010.
312 Involvement of the cytokine MIF in the snail host immune response to the parasite *Schistosoma*
313 *mansoni*. *PLoS Pathogens* 6.

314 Goto A, Blandin S, Royet J, Reichhart JM, Levashina EA. 2003. Silencing of Toll pathway components
315 by direct injection of double-stranded RNA into *Drosophila* adult flies. *Nucleic Acids Research*
316 31:6619-6623.

317 Hanington PC, Forys Ma, Dragoo JW, Zhang S-M, Adema CM, Loker ES. 2010. Role for a somatically
318 diversified lectin in resistance of an invertebrate to parasite infection. *Proceedings of the*
319 *National Academy of Sciences of the United States of America* 107:21087-21092.

320 Hanington PC, Forys Ma, Loker ES. 2012. A somatically diversified defense factor, FREP3, is a
321 determinant of snail resistance to schistosome infection. *PLoS Neglected Tropical Diseases* 6.

322 Ibrahim MM. 2006. Energy allocation patterns in *Biomphalaria alexandrina* snails in response to
323 cadmium exposure and *Schistosoma mansoni* infection. *Experimental Parasitology* 112:31-36.

324 Jiang Y, Loker ES, Zhang S-M. 2006. In vivo and in vitro knockdown of FREP2 gene expression in the
325 snail *Biomphalaria glabrata* using RNA interference. *Dev Comp Immunol* 30:855-866.

326 Knight M, Elhelu O, Smith M, Haugen B, Miller A, Raghavan N, Wellman C, Cousin C, Dixon F, Mann V,
327 et al. 2015. Susceptibility of Snails to Infection with Schistosomes is influenced by Temperature
328 and Expression of Heat Shock Proteins. *Epidemiology (Sunnyvale, Calif.)* 5:189.

329 Knight M, Miller A, Liu Y, Scaria P, Woodle M, Ittiprasert W. 2011. Polyethyleneimine (PEI) mediated
330 siRNA gene silencing in the *Schistosoma mansoni* snail host, *Biomphalaria glabrata*. *PLoS*
331 *neglected tropical diseases* 5:e1212-e1212.

332 Levashina EA, Moita LF, Blandin S, Vriend G, Lagueux M, Kafatos FC. 2001. Conserved Role of a
333 Complement-like Protein in Phagocytosis Revealed by dsRNA Knockout in Cultured Cells of the
334 Mosquito, *Anopheles gambiae*. *Cell* 104:709-718.

335 Moné Y, Gourbal B, Duval D, du Pasquier L, Kieffer-Jaquinod S, Mitta G. 2010. A large repertoire of
336 parasite epitopes matched by a large repertoire of host immune receptors in an invertebrate
337 host/parasite model. *PLoS Neglected Tropical Diseases* 4.

338 Osta MA, Christophides GK, Vlachou D, Kafatos FC. 2004. Innate immunity in the malaria vector
339 *Anopheles gambiae*: comparative and functional genomics. *The Journal of experimental biology*
340 207:2551-2563.

341 Pinaud S, Portela J, Duval D, Nowacki FC, Olive M-A, Allienne J-F, Galinier R, Dheilily NM, Kieffer-
342 Jaquinod S, Mitta G, et al. 2016. A Shift from Cellular to Humoral Responses Contributes to
343 Innate Immune Memory in the Vector Snail *Biomphalaria glabrata*. *PLOS Pathogens*
344 12:e1005361-e1005361.

345 Robalino J, Browdy CLCL, Prior S, Metz A, Parnell P, Gross P, Warr G. 2004. Induction of antiviral
346 immunity by double-stranded RNA in a marine invertebrate. *Journal of ...* 78:10442-10448.

347 Ruelas DS, Karentz D, Sullivan JT. 2009. Effects of UVB on interactions between *Schistosoma mansoni*
348 and *Biomphalaria glabrata*. *Journal of Invertebrate Pathology* 101:140-142.

349 Sminia T, Barendsen L. 1980. A comparative morphological and enzyme histochemical study on blood
350 cells of the freshwater snails *Lymnaea stagnalis*, *Biomphalaria glabrata*, and *Bulinus truncatus*.
351 *Journal of Morphology* 165:31-39.

352 Tennesen JA, Théron A, Marine M, Yeh J-Y, Rognon A, Blouin MS. 2015. Hyperdiverse Gene Cluster
353 in Snail Host Conveys Resistance to Human Schistosome Parasites. *PLoS Genetics* 11:e1005067.

354 Theron A, Rognon A, Gourbal B, Mitta G. 2014. Multi-parasite host susceptibility and multi-host
355 parasite infectivity: A new approach of the *Biomphalaria glabrata*/*Schistosoma mansoni*
356 compatibility polymorphism. *Infection, Genetics and Evolution* 26:80-88.

357 Wang J, Wu M, Wang B, Han Z. 2013. Comparison of the RNA interference effects triggered by dsRNA
358 and siRNA in *Tribolium castaneum*. *Pest Management Science* 69:781-786.

359

Discussion

L'immunité des invertébrés présente un haut niveau de complexité, malgré cela nous observons de manière générale un manque de connaissance des molécules de reconnaissance impliquées dans la réponse immunitaire contre les pathogènes ainsi que leur spécificité. En effet, chez *Biomphalaria glabrata*, la mise en évidence de molécules très diversifiées et polymorphes, les FREP, est particulièrement intéressante mais leur fonction et en particulier leur rôle de reconnaissance reste encore à réellement démontrer. Des approches d'inactivations fonctionnelles de 2 variants des FREP, les FREP 2 et 3 présentent une augmentation de la sensibilité du mollusque sans pour autant réverter complètement le phénotype (Hanington, et al. 2012; Pinaud, et al. 2016). Ces résultats montrent que la réponse immunitaire et plus précisément la reconnaissance ne se limite pas à une famille de molécule. Dans le but de contribuer à la découverte d'autres molécules participant à la réponse immunitaire, nous avons réalisé une étude visant à identifier les molécules de reconnaissance potentielle chez *Biomphalaria glabrata* en réponse à une large gamme de pathogènes, ainsi que l'étude d'une molécule immunitaire cible, la BgTEP, dont l'orthologue AgTEP1 a déjà été mise en évidence dans la réponse contre les pathogènes chez *Anopheles gambiae*.

Les protéines contenant des sites ThioEster (TEP), homologue des protéines du complément C3 des vertébrés, sont présentes dans une large gamme d'espèces allant des deuterostomiens aux protostomiens, et ont été caractérisées pour leur rôle dans l'immunité innée. Chez les vertébrés, le système du complément de l'hôte effectue une fonction défensive au cours de la phase initiale de l'infection, jouant un rôle d'opsonine, en déclenchant des cascades protéolytiques contre les agents pathogènes et entraînant ensuite une série de réponses inflammatoires qui permettent de lutter contre les infections microbiennes (Sarma and Ward 2011). Le système du complément est activé par trois voies initiales distinctes qui convergent à l'activation du troisième composant du complément (C3) par clivage protéolytique et conduisent à la génération d'une série de molécules effectrices impliquées dans la phagocytose ou la lyse des agents pathogènes (Sarma and Ward 2011). L'identification récente de protéines homologues au système du complément chez les arthropodes, suggère que ce mécanisme de défense immunitaire pourrait être un système ancestral partagé entre les vertébrés et les invertébrés (Levashina, et al. 2001; Sekiguchi, et al. 2012; Wu, et al. 2012).

Les TEP d'invertébrés sont intégrées dans un plus grand groupe que l'on nomme la super-famille des TEP. Cette super-famille comprend les alpha-2-macroglobulines des

Chapitre 2

vertébrés et invertébrés, les facteurs du complément des vertébrés et bien entendu les TEP d'invertébrés (Moné, et al. 2010).

Chez les invertébrés, les TEP ont été principalement étudiés chez les moustiques et la drosophile. Le génome d'*Anopheles gambiae* code pour 19 protéines homologues de la iTEP (TEP d'insecte) (AgTEP1 à 19), dans lequel AgTEP1 a été décrit plus en détail (Blandin, et al. 2008). AgTEP1 joue un rôle très important d'opsonine dans la phagocytose des bactéries gram-positifs et gram-négatifs, ainsi que des champignons, par des phagocytes (Levashina, et al. 2001; Yassine, et al. 2012). En outre, AgTEP1 est également capable de se lier à la surface des parasites (*Plasmodium sp.*) et de promouvoir leur lyse et mélanisation (Blandin, et al. 2008). Par ailleurs, l'AgTEP1 joue aussi un rôle actif et essentiel dans la lyse des ookinetes et par conséquent dans la diminution du nombre d'oocyste (Blandin, et al. 2008; Smith, et al. 2015). Dans le génome de *Drosophila melanogaster*, 6 gènes codant pour des protéines homologues de la iTEP ont été identifiés (DmTEP1 à 6) (Lagueux, et al. 2000; Blandin and Levashina 2004). La plupart des TEP sont exprimées par les hémocytes, et peuvent être induites dans le corps gras (Bou Aoun, et al. 2010). L'étude plus en détail du rôle des différentes TEP dans la réponse immunitaire de la drosophile, montre une augmentation de l'expression des DmTEP1, 2, 3 et 4 suite à un challenge bactérien ou fongique (Wertheim, et al. 2005; Bou Aoun, et al. 2010; Arefin, et al. 2017). De plus, ces études suggèrent que les DmTEP2 et 3 sont requises pour phagocyter des bactéries gram-positifs et gram-négatifs alors que la phagocytose de levure nécessitera la DmTEP6 (Bou Aoun, et al. 2010). En outre, les DmTEP2 et 6 joueront aussi un rôle dans la mélanisation des pathogènes (Shokal, et al. 2017). Par ailleurs, la DmTEP4 est également impliquée dans la défense ou la régulation contre des bactéries pathogènes ou non (Shokal, et al. 2017). Chez les crustacés, un gène homologue de la iTEP est mis en évidence chez la crevette, *Litopenaeus vannamei*, nommé LvTEP1 (Li, et al. 2017). LvTEP1 est exprimé dans la plupart des tissus liés à l'immunité, tels que l'intestin, l'épithélium et les hémocytes ; son niveau d'expression augmente dans les hémocytes suite à un challenge bactérien (avec des bactéries gram-négatifs ou gram-positifs) ou viral, ce qui indique son implication dans la réponse immunitaire innée des crevettes (Li, et al. 2017).

Chez *Biomphalaria glabrata*, c'est au sein de ce complexe immun entre les FREP et les SmPoMuc, que ce troisième acteur a été identifié, une Thioester-containing Protein de mollusque, la BgTEP (Moné, et al. 2010). La séquence de cette protéine a été caractérisée et présente une structure proche de celle des TEP d'invertébrés. Dans la partie C-terminal de la BgTEP, nous retrouvons un domaine liaison aux récepteurs α -2-

Chapitre 2

macroglobuline, qui, chez les vertébrés, est impliqué dans l'interaction avec les récepteurs spécifiques des macrophages ou des phagocytes et participe ainsi au recrutement des hémocytes et donc à l'encapsulation des parasites (van Lookeren Campagne, et al. 2007).

Depuis son identification en 2010, nous avons cherché à aller plus loin dans sa caractérisation structurale ainsi qu'à mettre en évidence son rôle dans la réponse immunitaire du mollusque (fig. 9). Pour cela nous avons pu reconstruire sa structure intron-exon, grâce à la publication récente du génome de *B. glabrata* (Adema, et al. 2017). Sa structure tridimensionnelle prédite par modélisation présente une très forte similitude avec celle de l'AgTEP1. Une analyse phylogénétique complète des différentes TEP d'invertébrés ainsi que des facteurs du complément C3, C4, C5 et des α -2-macroglobuline des vertébrés a permis de mettre en évidence une ségrégation des TEP de mollusques dans un sous-groupe de la super famille des TEP. La TEP étant identifiée chez d'autres invertébrés comme étant impliquée dans la réponse immunitaire et étant présente dans le complexe immun FREP/SmPoMuc chez *B. glabrata*, nous avons cherché à mettre en évidence son implication dans la réponse immunitaire du mollusque. Pour cela une analyse de l'expression tissulaire de la BgTEP nous révèle, qu'elle est une protéine sentinelle présente chez le mollusque à l'état naïf. De plus, son expression varie fortement d'un tissu à l'autre. La BgTEP présente la plus forte expression dans les hémocytes, les cellules circulantes du mollusque spécialisées dans l'immunité. Cependant une forte expression est aussi observée dans le pied et l'ovotestis. Le pied étant le premier tissu exposé aux pathogènes extérieurs et l'ovotestis, le lieu de formation de production des oeufs et spermatozoïdes pour la reproduction, il n'est pas aberrant d'identifier une plus forte expression de molécules immunitaires dans ces tissus. En effet, une implication antagoniste entre reproduction et immunité a été suggéré grâce à l'expression de l'AgTEP1 chez *A. gambiae* (Pompon and Levashina 2015).

De plus, l'expression tissulaire révélant la présence de la BgTEP dans les hémocytes, une analyse d'immunolocalisation sur hémocytes étalés sur lame révèle qu'elle n'est exprimée que par un seul type de cellules circulantes, les blast-like cells. En effet, 3 types d'hémocytes ont pu être identifiés chez *B. glabrata* selon leur caractéristiques morphologiques, parmi eux les blast-like cells, qui sont les hémocytes les plus petits possédant un rapport taille/noyau très important (Cavalcanti, et al. 2012). De plus, grâce à des analyses de cytométrie en flux, nous avons pu mettre en évidence que seulement un tiers de ces blast-like cells expriment la BgTEP, ce résultat met peut-être en lumière une sous catégorisation des différents types hémocytaires déjà connus.

Chapitre 2

Nous avons ensuite cherché à mettre en évidence son rôle dans la réponse immunitaire du mollusque, pour cela l'hôte a été stimulé par différents types de pathogènes, des bactéries (*E. coli* et *M. luteus*), levures (*S. cerevisiae*) et un parasite métazoaire (*S. mansoni*). La BgTEP montre une modulation de son expression quelque soit le parasite qui est présenté au mollusque. Suite à une infection par *E. coli* et *S. cerevisiae* on observe une sous-expression de la BgTEP alors que suite à une infection par *M. luteus* et *S. mansoni* elle est sur-exprimée. Non seulement, elle semble impliquée dans la réponse aux différents pathogènes mais en plus, de manière différentielle. Des expériences d'interactions entre l'hémolymphe déplétée de cellules et les différents pathogènes vivants, à température physiologique du mollusque montrent que la BgTEP possède une capacité de fixation sur tout les pathogènes ainsi qu'une capacité de maturation différentielle selon le type d'interaction. En effet, 3 heures après l'interaction avec *E. coli*, *M. luteus* et *S. cerevisiae* la BgTEP présente un nouveau fragment, issu de protéolyse et révélé par western-blot. Il semblerait donc que la BgTEP soit impliquée dans la reconnaissance ou tout du moins dans la réponse contre différents pathogènes. Cependant, à ce jour nous ne sommes pas capable d'affirmer si la BgTEP se fixe directement à la surface des pathogènes, comme il semblerait que ce soit le cas chez le moustique ou bien si elle est comprise dans un complexe moléculaire de reconnaissance par exemple. Pour cela des expériences de co-immunoprecipitation, grâce à des anticorps anti-BgTEP, entre l'hémolymphe du mollusque et les pathogènes seront nécessaires. A ce jour, nous pouvons tout de même affirmer que les cellules exprimant la BgTEP ne sont impliquées directement ni dans la phagocytose ni dans l'encapsulation. Ces résultats vont dans le sens du rôle d'opsonine de la BgTEP comme c'est le cas chez le moustique.

Cependant, une protéine α -2-macroglobuline présentant une activité anti-protase contre le schistosome a été mise en évidence chez *B. glabrata* dans les années 90 (Bender and Bayne 1996). Nous pourrions imaginer qu'il s'agit de la même molécule. Des études complémentaires doivent aujourd'hui être réalisées dans le but de mieux comprendre la le rôle de la BgTEP et confirmer ou non qu'il s'agit bien de la même molécule que celle identifiée en 1989. Cette découverte pourrait amener une bi-fonctionnalité à la BgTEP et par conséquent une nouvelle fonction encore inconnue chez les invertébrés.

Dans ce contexte, une analyse fonctionnelle de la BgTEP est aujourd'hui essentielle, dans le but de confirmer son rôle d'opsonine et peut-être mettre en évidence une bi-fonctionnalité avec une lyse des cellules des pathogènes.

Chapitre 2

En parallèle, une caractérisation plus fine de la reconnaissance immunitaire chez le mollusque, nous permettrait de mieux comprendre l'interaction avec les pathogènes (fig. 9). De plus, cela nous permettrait peut-être de mettre en évidence un complexe de reconnaissance dans lequel la BgTEP serait impliquée. Pour cela, l'étude des mécanismes de reconnaissances chez *B. glabrata* à différentes stimulations a été réalisée par une approche protéomique d'interactome et révélé par électrophorèse bidimensionnelle. L'hémolymphe déplétée de cellules est mise en contact avec différents pathogènes (*E. coli*, *M. luteus*, *S. cerevisiae*, *E. caproni* et *S. mansoni*) vivants pendant un temps court (20 minutes) à température physiologique du mollusque.

Les premiers résultats montrent la présence de molécules dont la fonction première est de participer à la réponse immunitaire, que nous avons appelé PriPaR (Primary Pathogens Recognition proteins) (fig. 9). Cette catégorie contient les PRR (Pathogens Recognition Receptors). Parmi ces récepteurs nous retrouvons, des lectines, grande famille impliquée dans la reconnaissance immunitaire (Pasquier 2005; Pees, et al. 2016). Plus précisément des C-types lectines impliquées dans la reconnaissance de *M. luteus* et *S. cerevisiae* ; et des H-types lectines impliquées dans la reconnaissance de *E. coli*. Toujours parmi les PriPaR, nous retrouvons un autre type de molécule les biomphalysines, toxines mises en évidence dans la réponse immunitaire contre *S. mansoni* (Galinier, et al. 2013). Des spots identifiés comme étant de la biomphalysine sont retrouvés en réponse au 5 pathogènes, suggérant la présence d'une famille de biomphalysines, avec un certain niveau de spécificité, avec des isoformes impliquées dans la reconnaissance de *S. mansoni* et d'autres impliquées dans celle des autres pathogènes.

Nous retrouvons aussi des molécules dont la première fonction n'est pas la reconnaissance de pathogène mais qui sont plutôt impliquée dans une fonction physiologique et seront appelées SePaR (Secondary involved in Pathogens Recognition protein) (fig. 9). Nous retrouvons parmi ces molécules l'actine, déjà mise en évidence dans le rôle de clotting des pathogènes pour améliorer la phagocytose chez *A. gambiae* (Sandiford, et al. 2015), ou encore la dermatopontine, spécifique de l'interaction avec *S. mansoni* (Mitta, et al. 2005).

Finalement, nous retrouvons une troisième catégorie de molécules, il s'agit des enzymes impliquées dans le métabolisme d'un grand nombre de molécules, que l'on nommera EnPaR (Enzymes involved in Pathogens Recognition) (fig. 9). Parmi elles, nous retrouvons l' α -amylase, déjà mise en évidence dans les expériences de co-immunoprécipitation

Chapitre 2

réalisée entre des protéines de *S. mansoni* et de *B. glabrata* (Moné, et al. 2010). Ici, nous la retrouvons aussi liée à *M. luteus*. Finalement, nous retrouvons aussi des ADAMTS et des GAPDH, respectivement mises en évidence dans la réponse immunitaire des vertébrés et des plantes (Apte and Parks 2015; Henry, et al. 2015).

En conclusion, les résultats obtenus démontrent que la liaison avec la surface des agents pathogènes ne peut plus être considérée comme accessoire. En effet, parmi chacune de ces catégories, nous observons des molécules spécifiques à un type de pathogènes et d'autres communes à plusieurs voir à toutes. D'autres expériences sont maintenant nécessaires pour comprendre si les EnPaR peuvent se lier directement à la surface des pathogènes de surface ou si leur implication est liée à leurs activités enzymatiques pour contribuer à la maturation des complexes immuns après association avec d'autres PriPaR et/ou SePaR. De plus, les enzymes pourraient modifier directement les propriétés physico-chimiques de la membrane externe du pathogène et favoriser ainsi une réponse immunitaire humorale et/ou cellulaire efficace.

Lors de ces expériences d'interactome, nous nous attentions à mettre en évidence des molécules telles que les FREP ou la BgTEP révélées dans le complexe immunitaire entre le mollusque et le parasite grâce à des expériences de co-immunoprécipitation réalisées au laboratoire (Moné, et al. 2010). Cependant, ces expériences ne révèlent ni la présence de FREP ni de BgTEP dans les interactions avec les différents pathogènes. Les études menées sur les approches de co-immunoprécipitation ont été réalisées sur le premier stade parasitaire intramolluscal, c'est à dire le sporocyste primaire, les approches d'interactomes, elles, ont été réalisées sur le stade parasitaire infestant le mollusque, le miracidium. Cette différence avec les résultats antérieurs pourrait provenir des différents stades de développement des parasites utilisés. Cela suggère une capacité subtile de reconnaissance dans la machinerie immunitaire du mollusque à distinguer les différents stades de développement intra-molluscaux du parasite : les miracidium, sporocystes primaires et secondaires voir même les cercaires. Nous pourrions aussi imaginer peut-être que les SmPoMucs ne sont pas accessibles. En effet, mises en évidence dans la glande apicale du miracidium, elles ne sont peut-être excrétées qu'au moment de la pénétration du parasite dans les tissus de l'hôte et donc du passage en sporocyste primaire. De plus, étant donné que la FREP est retrouvée dans le complexe immunitaire en étant associée à la BgTEP, leur liaison est peut-être obligatoire pour la reconnaissance par les FREP des agents pathogènes. Le temps d'exposition est peut-être trop court pour que le complexe ait le temps de se former. Par ailleurs, lorsque nous laissons les pathogènes

Chapitre 2

et l'hémolymphe en contact 30 minutes, la BgTEP est retrouvée fixée à la surface de ces derniers. En conclusion, nous pouvons imaginer que le temps minimum requis pour la formation du complexe est d'au moins 30 minutes.

En parallèle, toujours chez *B. glabrata*, une analyse protéomique réalisée dans le but de mettre en évidence des molécules de reconnaissances impliquées dans l'interaction avec le sporocyste primaire suite à l'infection d'un mollusque sensible et résistant a été réalisée cette année par une autre équipe de recherche (Wu, et al. 2017). Dans cette étude, comme dans celle que nous avons réalisée, des molécules immunitaires et non immunitaires sont retrouvées impliquées dans la reconnaissance. Par ailleurs, dans ces approches, ils identifient non seulement des FREP mais aussi la BgTEP, ce qui signifie que ces deux molécules sont bien impliquées dans la réponse et plus précisément la reconnaissance immunitaire du mollusque (Wu, et al. 2017). Les expériences ont été réalisées à partir d'extrait parasitaire purifié et d'une matrice d'affinité enrichie en protéine plasmatique de mollusque. Cette différence de plan expérimental peut aussi amener à une différence dans le résultat. En effet, nous pouvons imaginer que la BgTEP soit très faiblement présente dans l'hémolymphe en comparaison avec d'autre molécule, ce qui accroît la possibilité que l'on ne la détecte pas du tout sur nos gels alors que qu'elle est bien présente dans le complexe immun et à participer à la reconnaissance de différents pathogènes.

De plus nous pouvons imaginer que les SmPoMucs ne soit pas réellement des molécules de surface du parasite mais plutôt des molécules internes. Dans ce cas là cela peut expliquer pourquoi les FREP sont retrouvées liées aux molécules du parasite dans l'expériences de l'équipe de Yoshino et non dans celle que nous avons réalisé sur des pathogènes entiers. De plus toujours dans les expériences de l'équipe de Yoshino de nombreuses molécules identifiées sont communes à celles mises en évidence lors des expériences de CoIP réalisées au laboratoire (Moné, et al. 2010) mais pas retrouvées dans les expériences d'interactomes. Nous pouvons donc imaginer qu'en utilisant des extraits de parasites on libère des molécules de parasite qui ne sont parfois jamais exposées à l'hémolymphe, et par conséquent on met en évidence des interactions artificielles qui n'arrivent jamais *in vivo*.

En revanche quand on utilise des pathogènes entier, avec seulement les déterminants de surface accessibles, dans ce cas on ne met en évidence des interactions possibles en condition *in vivo*.

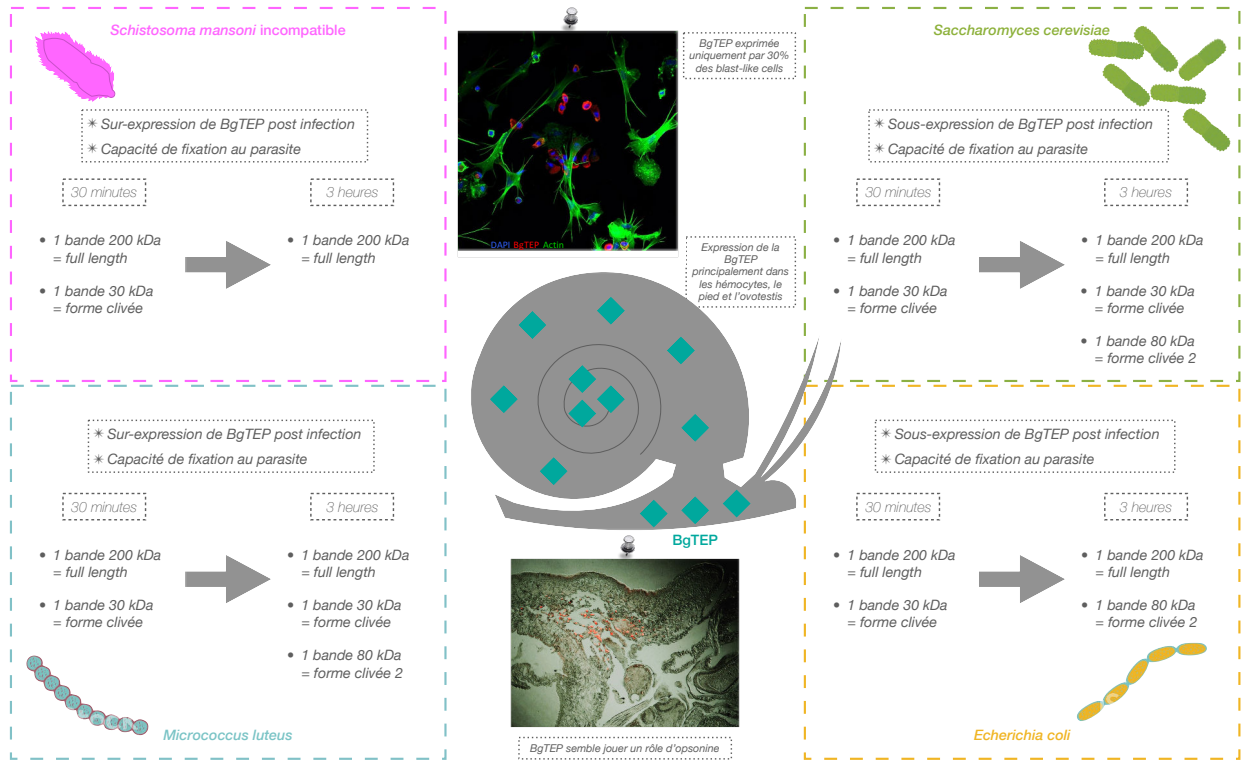
Chapitre 2

L'interaction entre *B. glabrata* et *S. mansoni* est régit par le polymorphisme de compatibilité qui entraîne un véritable dialogue moléculaire entre les deux acteurs, suite à une reconnaissance obligatoire. Le premier complexe immun caractérisé entre des FREP de mollusque et des SmPoMuc de parasite présente un rôle important dans ce polymorphisme de compatibilité mais ne peut à lui seul expliquer le phénotype. A la suite des analyses de protéomique globale, réalisées suite à l'infection par différents pathogènes, des molécules immunitaires ou non ont été mises en évidence dans la reconnaissance de ces derniers. Une invalidation par RNAi de ces dernières permettrait de confirmer ou non leur rôle dans la reconnaissance. De plus une invalidation multiple, en y associant l'invalidation des FREP par exemple, permettrait peut-être de réverter complètement les phénotypes d'incompatibilité et/ou de susceptibilité. Il en est de même pour la BgTEP, en effet, une invalidation fonctionnelle de cette dernière nous permettrait de confirmer son implication dans la réponse immunitaire du mollusque.

Dans le but de réaliser une invalidation fonctionnelle performante et ciblée, nous avons réalisé un grand nombre de mise au point sur la technique de RNAi chez *Biomphalaria glabrata*. Ceci nous a permis d'améliorer grandement la technique de RNAi déjà existante chez ce dernier. En effet, l'utilisation de siRNA pour une cible précise, couplé à un bras cholestérol permettra une meilleure internalisation du siRNA par les hémocytes et par conséquence l'invalidation sera plus performante. Cette nouvelle technique permettra une invalidation plus performante de molécules ciblées dans les hémocytes, comme par exemple la BgTEP.

La caractérisation des bases moléculaires du polymorphisme de compatibilité régissant l'interaction entre *B. glabrata* et *S. mansoni*, permettra une meilleure compréhension de la réponse immunitaire globale et plus précisément de la reconnaissance immunitaire du mollusque. Cependant, de nouveaux axes de recherches émergents dans les interactions hôtes / parasites, nous poussent aujourd'hui à considérer l'hôte comme un ensemble d'organismes vivants comprenant l'hôte lui-même, et son microbiote associé. La compréhension profonde des interactions hôtes / parasite ne peut plus se faire sans l'étude de ce 3^{ème} protagoniste, ce qui complexifie encore le schéma en le faisant évoluer vers une interaction pluripartite.

Chapitre 2



	<i>Micrococcus luteus</i>	<i>Escherichia coli</i>	<i>Saccharomyces cerevisiae</i>	<i>Schistosoma mansoni</i>
PriPaR ?		BgTEP		
PriPaR		Biomphalysine		
	C-type lectine		C-type lectine	
		H-type lectine		
SePaR	CREP		CREP4	
	Actine		Actine	Dermatopontine
	Collagène α		Collagène α	Collagène α
EnPaR		Hémoglobine 1 et 2		Hémoglobine 1
	α-amylase			α-amylase
	ADAMTS			ADAMTS
			GAPDH	GAPDH
				CECR1

Figure 9 : Du Polymorphisme de Compatibilité vers la complexité de la réponse immunitaire

D'après PORTET et al. en préparation et (TETREAU et al. 2017). Mise en évidence du rôle de la BgTEP dans la réponse immunitaire de *B. glabrata*. Caractérisation de molécules immunes et non immunes impliquées dans la reconnaissance de divers pathogènes chez *B. glabrata*.

Chapitre 3

Chapitre 3 : L'influence du microbiote sur l'interaction *Biomphalaria* / *Schistosoma*.

Introduction

"Le concept d'organisme montre aujourd'hui ses limites : il faut désormais prendre en compte le fait qu'un animal ou une plante ne peut vivre sans les multiples micro-organismes qui l'habitent." Marc-André Selosse, professeur au Muséum National d'Histoire Naturelle, vice-président de l'International Society for Symbiosis

Microbiote vient du grec ancien "*mikros-biotos*", qui désigne ce qui est petit et vivant, en d'autres termes il s'agit de l'ensemble des micro-organismes (bactéries, archées, champignons, virus et protozoaires eucaryotes) vivants dans un environnement spécifique appelé le microbiome.

De plus en plus de scientifiques, aujourd'hui, ne considèrent plus un organisme (animal ou plante) seul, mais considèrent un holobionte (Zilber-Rosenberg and Rosenberg 2008). Holobionte vient du grec "*holo-bios*", qui désigne tout ce qui est vivant, autrement dit nous prenons en compte l'hôte (animal ou plante) ainsi que son microbiote. Dans ce contexte-là, nous ne travaillons plus à l'échelle du génome de l'hôte, mais à celle de l'hologénome, comprenant le génome de l'hôte ainsi que ceux de tous les micro-organismes symbiotiques composant le microbiote.

Devant l'importance croissante de l'holobionte dans la compréhension des organismes vivants, de nombreuses études ont été réalisées pour caractériser le microbiote associé à différents modèles invertébrés, la majorité étant réalisées sur le microbiote bactérien.

Comme chez les vertébrés, le rôle du microbiote dans l'homéostasie de l'hôte invertébré a aussi été mis en évidence. En effet, chez la drosophile, une modification du microbiote bactérien intestinal entraîne une altération globale de la physiologie de l'animal (Brummel, et al. 2004; Buchon, et al. 2009a; Broderick, et al. 2014; Clark, et al. 2015).

Les premières études du microbiote bactérien de mollusques ont été réalisées dès les années 70. Une caractérisation de la flore bactérienne totale de *Biomphalaria glabrata* (Ducklow, et al. 1979) et de la fraction intestinale de *Helix aspersa* et *Helix pomatia* (Charrier, et al. 2006) a été réalisée par culture en milieu solide. La fraction totale du microbiote bactérien cultivable de *Biomphalaria glabrata* a également été analysée par

Chapitre 3

séquençage ARNr 16S (Silva, et al. 2013) ce qui permet de compléter les résultats obtenus précédemment. Toujours avec la même technique de séquençage, la fraction intestinale du microbiote de nombreux mollusques a été abordée, parmi eux *Helix aspersa* (Švec, et al. 2002), *Biomphalaria pfeifferi*, *Bulinus africanus* et *Heliosoma duryi* (Van Horn, et al. 2012).

Toutes ces études ont pour but une caractérisation brute de fraction bactérienne du microbiote, avec l'identification des genres bactériens majoritaires, présents chez les mollusques à savoir les *Gammaproteobacteries*, *Bacteroidetes* ou encore les *Acidobacteries*.

Plus récemment, le rôle clé du microbiote bactérien dans l'interaction entre l'hôte et divers pathogènes a pu être décrit. En effet, chez le moustique, *Anopheles gambiae*, la flore microbienne peut limiter l'infection par *Plasmodium falciparum*, en inhibant le développement du parasite (Dong, et al. 2009), et favoriser l'infection par l'arbovirus *o'nyong-nyong virus* (Carissimo, et al. 2015). Chez *Aedes sp.*, le microbiote permet le maintien d'une activité basale du système immunitaire, plus particulièrement de la voie de signalisation Toll, permettant une meilleure réponse lors d'une infection par le virus de la dengue (Xi, et al. 2008; Ramirez, et al. 2012).

Plus spécifiquement, les virus souvent considérés comme pathogènes ou nuisibles, sont aussi une partie intégrante du microbiote et peuvent jouer un rôle dans l'interaction hôtes / parasites (Martinez, et al. 2015; Pradeu 2016). En effet, des virus à ADN sont capables de manipuler le système immunitaire des guêpes parasitoïdes (Strand and Burke 2012). Chez la coccinelle, des virus ARN simple-brins, sont capables de modifier le comportement de cette dernière lorsqu'elle est parasitée par des guêpes parasitoïdes pour protéger physiquement les larves de leur parasitoïde (Dheilly, Maure, et al. 2015). De manière plus générale, les virus peuvent jouer un rôle dans le contrôle du système immunitaire de l'hôte et son développement (Christiaansen, et al. 2015) ou bien dans la défense de ce dernier contre les pathogènes (Bettarel, et al. 2015).

De manière générale, le microbiote influence grandement son hôte lors d'interactions avec des pathogènes, que ce soit positivement ou négativement. Dans ce contexte, nous nous proposons de réaliser une étude de caractérisation du microbiote de *Biomphalaria sp.*

Pour cela, nous avons réalisé une caractérisation de cinq virus résidents chez quatre souches de *B. glabrata* et une de *B. pfeifferi*. L'identification de ces virus fut réalisée par une approche de séquençage massif avant une reconstruction génomique complète ainsi

Chapitre 3

qu'une analyse taxonomique. Finalement, basé sur une analyse d'abondance, il apparaît une grande spécificité virale au travers des différentes souches de mollusques étudiées.

Nous avons aussi réalisé une caractérisation du microbiote bactérien de *B. glabrata* provenant du Brésil ainsi que l'étude des modifications de la communauté microbienne au cours d'infections par *Schistosoma mansoni*. Deux isolats géographiques du parasite ont été utilisés, *S. mansoni* provenant du Brésil, parasite sympatrique, et *S. mansoni* provenant du Vénézuéla, parasite allopatrique, affichant tous deux les mêmes prévalences et intensités parasitaires. En parallèle, la réponse anti-microbienne du mollusque a été étudiée pour établir des liens potentiels entre la modification des communautés bactériennes au cours de l'infection et l'immunité de l'hôte. Pour cela une approche de séquençage métabarcoding 16S (MiSeq) et une approche de transcriptomique massive (RNAseq) ont été utilisées.

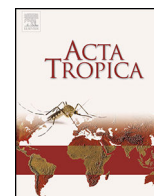
Les résultats montrent non seulement une affectation de la diversité et la richesse de la communauté bactérienne du mollusque mais aussi de sa composition au cours d'une réponse immunitaire complexe, faisant intervenir des conditions sympatrique et allopatrique et de primo-infections et challenges qui sont associées à des réponses immunitaires fondamentalement différentes.

1. Caractérisation de virus résidents chez *Biomphalaria*

Publication: First characterization of viruses from freshwater snails of the genus *Biomphalaria*, the intermediate host of the parasite *Schistosoma mansoni*.

GALINIER Richard, TETREAU Guillaume, PORTET Anaïs, PINAUD Silvain, DUVAL David and GOURBAL Benjamin

*Article publié en 2017 dans Acta Tropica



First characterization of viruses from freshwater snails of the genus *Biomphalaria*, the intermediate host of the parasite *Schistosoma mansoni*



Richard Galinier*, Guillaume Tetreau, Anaïs Portet, Silvain Pinaud, David Duval, Benjamin Gourbal

CNRS, IHPE UMR 5244, Univ. Perpignan Via Domitia, IFREMER, Univ. Montpellier, F-66860 Perpignan, France

ARTICLE INFO

Article history:

Received 16 September 2016

Received in revised form

16 November 2016

Accepted 18 December 2016

Available online 22 December 2016

Keywords:

Schistosomiasis vector

Biomphalaria

Viral microbiota

Picorna-like virus

Totiviridae,

ABSTRACT

We report the genome sequence and organization of five viruses infecting snails of both *Biomphalaria glabrata* and *Biomphalaria pfeifferi*, which are vectors of the intestinal schistosomiasis. Four viruses presented a polyadenylated positive single strand RNA genome encoding one or two large open reading frames (ORFs) flanked by untranslated region. Conserved protein motifs typical of the picorna-like virus superfamily were identified in these viruses but they all presented different genome organization. Phylogenetic analysis confirmed their assignment to this superfamily. The partially characterized fifth virus presented sequence similarity for *Totiviridae*, a family of non-polyadenylated double-strand RNA viruses. Virus distribution and relative abundance between the five strains of *Biomphalaria* originating from different geographical areas was determined. Our results provide valuable information of new viruses from *Biomphalaria* and pave the way for future studies dedicated to their impact on snail fitness and *Biomphalaria*/*Schistosoma* interactions.

© 2016 Elsevier B.V. All rights reserved.

1. Introduction

Schistosomiasis is the second most widespread human parasitic disease after malaria. It is reported to be endemic in more than 78 countries in the tropical and subtropical areas, in Africa, Asia and Americas. Around 200 million people are estimated to be infected all over the world, with 200 000 death per year (Chitsulo et al., 2000). Infection is caused by a trematode parasite of the genus *Schistosoma*, which comprises 22 different species. Three of them, *Schistosoma mansoni*, *S. haematobium* and *S. japonicum* are mainly responsible for human infections. *S. mansoni* is the causative agent of intestinal schistosomiasis and it is vectored by a freshwater snail from the genus *Biomphalaria* (Basch, 1976). The compatibility between the pathogen and its host is determined by two main factors, the susceptibility of the host and the infectivity of the parasite. In *Biomphalaria*, this host/pathogen compatibility is determined by genetic factors but can also be modulated by non-genetic factors, such as temperature (Knight et al., 2015), pollution (Ibrahim, 2006)

or UV exposure (Ruelas et al., 2009), and could also be influenced by its microbiota composition.

The microbiota of an organism is constituted of all microbial organisms, such as bacteria, fungi and viruses that live within the host in a mutualistic interaction. The microbiota is known to impact host evolution and adaptation by affecting several fitness parameters (Bosch and McFall-Ngai, 2011; Zilber-Rosenberg and Rosenberg, 2008). Notably, it has been shown to play a key role in the interaction between a host and its pathogens. In the mosquito *Anopheles gambiae*, the microbial flora can limit the infection with *Plasmodium falciparum* by inhibiting the development of the parasite within the host (Dong et al., 2009). More specifically, although viruses have often been characterized by their pathogenic and harmful effects, they are also an integral part of the microbiota and they can modulate host-parasite interaction in invertebrates (Martinez et al., 2015; Pradeu, 2016). For example, PolyDNA viruses are able to manipulate the immune system of parasitoid wasps (Burke and Strand, 2012), while SSRNA DcPv can modify the behavior of a ladybird parasitized by a parasitoid wasp to protect the parasitoid larvae (Dheilly et al., 2015b). More generally, it is widely accepted that viruses can control the immune system of their host for their own development (Christiaansen et al., 2015), but also for

* Corresponding author.

E-mail address: richard.galinier@univ-perp.fr (R. Galinier).

the defense of their host against other pathogens (Bettarel et al., 2015).

In this context, RNAseq data were used to perform the first identification of sequences of resident viruses from *Planorbidae* of the genus *Biomphalaria*. Next-generation sequencing (NGS) has already been successfully used as a powerful tool for the discovery of new viruses (Liu et al., 2011; Marston et al., 2013), even when they are present at a low dose in a latency phase or at higher dose but without any associated symptoms. In this work, we report the identification and characterization of 5 viruses from four strains of *B. glabrata* and one strain of *B. pfeifferi*. The taxonomic position of these *Biomphalaria* viruses was assessed by a phylogenetic analysis using the most conserved domains from protein sequences. Finally, based on virus abundance analysis, we evidenced a high level of virus specificity toward the different snail strains investigated herein.

2. Material and methods

2.1. Biological material

The experiments were conducted using four strains of *Biomphalaria glabrata* (Bg) and one of *Biomphalaria pfeifferi* (Bp). The four South American *B. glabrata* strains were named as follow: BgBAR (origin Brazil, Belo Horizonte); BgVEN (origin Venezuela, Guaraca); BgBRE (origin Brazil, Recife) and BgGUA (origin Guadeloupe, Le Lamentin). The *B. pfeifferi* strain came from Arabica peninsula and was named BpOMAN (origin Oman, Dhofar). These five *Biomphalaria* strains exhibit differential susceptibility to each of the five *Schistosoma* strains, from a generally high compatibility with their sympatric strains (i.e., originating from the same location), to a generally moderate/low compatibility with other allopatric strains (i.e., originating from different locations) (Theron et al., 2014).

2.2. RNAseq data

RNA extraction, cDNA library construction and Illumina SOLEXA sequencing were performed as previously described (Dheilly et al., 2015a). Each paired-end cDNA library (72 bp) was constructed from a pool of 30 snails, ranging from juveniles to old adults. *De novo* transcriptome assemblies were performed as previously described (Dheilly et al., 2015a). Finally, five high quality reference transcriptomes were obtained. They comprised 70 533, 79 664, 117 269, 82 500 and 99 507 transcripts for the strains BgBAR, BgVEN, BgBRE, BgGUA and BpOMAN, respectively. Automatic annotation of transcripts was performed using Blast2GO version 2.4.2. Putative virus sequences were identified by focusing on key viral features that are RNA-dependent RNA polymerase, capsid protein and polyprotein domains. Sequence analysis was performed using either the GeneCodes software package (Sequencher version 4.5, Ann Arbor MI) or the BioEdit Sequence Alignment Editor version 7.1.3.0. (Hall, 1999).

2.3. PCR and RACE-PCR

RACE-PCR was performed to expand the virus genome sequences at the 5' and/or 3' ends when they were missing. First strand cDNA synthesis and RACE-PCR were performed using the SMARTer RACE cDNA Amplification Kit (Clontech) from the RNA extracted in section 2.2 by following manufacturer's instructions. RACE-PCR products were analyzed by agarose gel electrophoresis and cloned into the pCR4-TOPO vector according to the manufacturer's instructions (ThermoFisher Scientific). Clones were then sequenced using GenoScreen facilities (GenoScreen, France).

PCR were performed to validate the sequences obtained by RNAseq, as well as to complete virus sequence gaps. One microliter

of a ten times diluted cDNA (see Section 2.2) was used as template to amplify desired fragments using the GoTaq G2 Hot Start polymerase (Promega) using the manufacturer's instructions. PCR products were cloned and sequenced following the same procedure as for RACE-PCR products.

2.4. Sequence analysis and phylogenetic trees

Homologous sequences were searched using BLAST searches against GenBank non redundant database on the National Center for Biotechnology Information web server (Bethesda, USA). A search for the presence of conserved motifs in the sequences obtained by RACE-PCR and PCR was done using the Conserved Domain Search Service (CD-Search) from the NCBI web server (Marchler-Bauer et al., 2015).

For phylogenetic analyses, multiple protein sequence alignments were performed with ClustalW using the BLOSUM62 substitution matrix model. The neighbor-joining method (Poisson substitution model; uniform substitution rate; gaps/missing data treatment: pairwise deletion) was used to generate the phylogenetic trees. A bootstrap analysis of 2000 replications was carried out on the trees inferred from the neighbor-joining method to assess the robustness of the tree branches. Two phylogenetic trees were generated: one using the RdRP protein sequences, the other one using the helicase protein sequences. In addition to the 5 RdRP and 4 helicase sequences from *Biomphalaria* viruses, 76 RdRP and 59 helicase sequences from 9 different families of *Picornavirales* were obtained from NCBI database (Supplementary Table S1). These different phylogenetic groups of viruses were chosen based on the closest domain organization of the virus genome to the *Biomphalaria* viruses and on the best BLAST matches against the NCBI database. All phylogenetic analyses were performed using MEGA 7.0.14 software (Kumar et al., 2016).

2.5. Analysis of viral abundance

A viral transcriptome containing the nucleotide sequences of the five *Biomphalaria* viruses previously characterized was created in order to analyze viral abundance in the five *Biomphalaria* strains. For each RNAseq data of each *Biomphalaria* strain, high quality reads (phred score >26) were aligned to viral transcriptome using Bowtie2 (V2.2.6.2), which was run locally on the Galaxy project server (<http://bioinfo.univ-perp.fr>) (Giardine et al., 2005). The mapping was realized with paired library and sensitive end-to-end mode. Differential abundance of each identified virus was estimated and normalized by the upper quartile (UQ) within each snail strain (Dillies et al., 2012).

3. Results and discussion

3.1. Viral sequence analysis and genome organization

3.1.1. Discovery of five new viral sequences from *Biomphalaria* RNAseq data

The presence of virus-derived transcripts was investigated in four *de novo* assembled transcriptomes from *B. glabrata* coming from different geographical areas and one from *B. pfeifferi*. A total of five viruses were identified from *B. glabrata* and *B. pfeifferi* species, and named BV1 to BV5 for *Biomphalaria* virus 1–5, respectively (Fig. 1). Sequence of BV1 virus was almost completely obtained from RNAseq data while only partial contig coverage was observed for the four other viral sequences. Joining PCR and RACE-PCR followed by Sanger sequencing allowed fulfilling some gaps and extending viral sequences to generate respectively full or near-full length sequences for BV1 and BV3 while only partial sequences were obtained for BV2, BV4 and BV5 (Fig. 1). The obtained viral

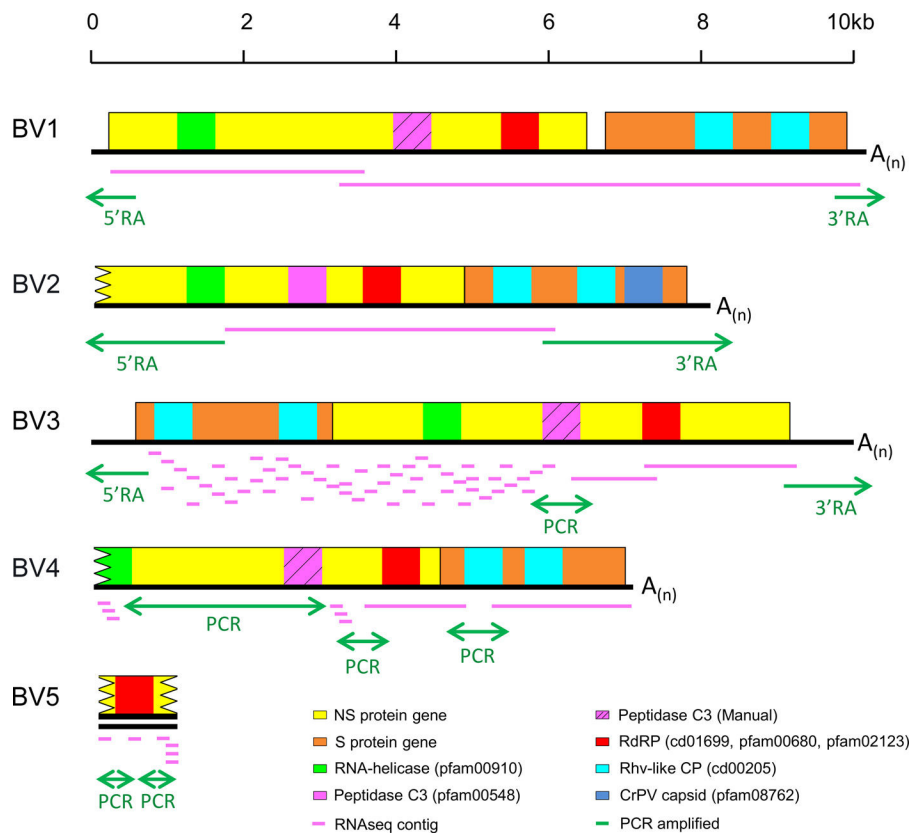


Fig. 1. Genome structures of *Biomphalaria* RNA-viruses. Black lanes represent the RNA strands (one for BV1, BV2, BV3 and BV4 or two for BV5). Yellow and orange boxes correspond to the region coding for non-structural proteins and structural proteins, respectively. Colored squares in these boxes relate the presence of conserved protein domains identified either by CD-search analysis or manually (hatched). Transcripts from RNAseq data are materialized by pink lines, while gaps and cDNA ends obtained only by PCR and RACE-PCR are illustrated by green arrows. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sequences were deposited in GenBank under the following accession numbers: KY024317-KY024321 for BV1, KY024322 for BV2, KY024323 for BV3, KY024324 for BV4 and KY024325 for BV5. Rearring facilities (water, food, and material used) were screened by PCR for the presence of these viruses in order to avoid misinterpretation. None of the *Biomphalaria* viruses was detected.

3.1.2. *Biomphalaria glabrata* Virus 1 (BV1)

The BV1 genome was found to be 10 116 nucleotides long, excluding the poly(A) tail present at the 3' end (Fig. 1). No sequence match was found in GenBank based on the complete nucleotide sequence. The genome was enriched in Adenine/Uracile (27% A, 37% U, 19% C and 17% G), which is typical of Picorna-like viruses. Two large ORFs located on the same positive RNA strand but on different reading frames account for 88% of the total genome length. The other 12% of the genome consisted in a 253 nucleotides long 5' UTR, a 160 nt intergenic region (IGR), and a longer 756 nt 3' UTR ended by a poly(A) tail (Fig. 1). No large ORF was found in reverse orientation, suggesting that BV1 is a positive strand RNA virus.

The 5' proximal ORF (ORF1) started with an AUG codon at position 254 and ended at a UAA stop codon at nucleotide 6403. The predicted product was 2049 amino acid long with a theoretic molecular mass of 227 kDa. It encoded a picornavirus-like H-P-Rep cassette, which included in this order a RNA helicase (pfam00910), a 3C cysteine protease (pfam00548) and RNA-dependent RNA polymerase (RdRP) (cd1699) as described for *Picornaviridae* (Koonin and Dolja, 1993). A search for conserved domains in NCBI database revealed the presence of both helicase and polymerase domains but not of protease domain. However, manual analysis of the sequence allowed the identification of a protease domain spanning

from amino acid position 1227–1427, with the conserved cysteine protease motif ¹³⁹⁹GxCG¹⁴⁰² and the putative substrate binding residues ¹⁴¹⁸GxHxxG¹⁴²³ (Gorbalenya et al., 1989). In addition, we also found the amino acids that could constitute the protease catalytic triad previously described by (Ryan and Flint, 1997) at position H¹²⁷⁹, D¹³³¹ and C¹⁴⁰¹, confirming the presence of the protease domain. The best hit after protein blast analysis was for a *Nora virus* replication polyprotein (GenBank accession no. KP970079), with 26% identity over 653 residues (e-value: 8⁻⁴²). *Nora virus* is a recently characterized positive single strand RNA virus, which is still unclassified among the picorna-like viruses (Habayeb et al., 2006).

The 3' proximal ORF (ORF2) started at nucleotide position 6564 with an AUG codon and ended at position 9362 with a UAA stop codon. It encoded a predicted product of 932 amino acids with a theoretical molecular mass of 103 kDa. BLAST analysis against NCBI database didn't retrieve any homologous sequences. Nevertheless, CD search analysis revealed the presence of two picornavirus capsid protein domain-like (cd 00205), typical of picorna-like non-structural virus proteins.

The genome organization of BV1 suggests that it could be a new species in the picorna-like virus superfamily, considering that it possesses all the typical domain of structural and non-structural proteins of these viruses. More precisely, its di-cistronic genome organization suggests that it might be a candidate species for the *Dicistroviridae* family.

The BV1 genome has been sequenced in all *Biomphalaria* strains and compared at the nucleic acid level (Table 1). BV1 nucleotide sequences were highly similar between most snail strains, with 99–100% identity for BV1 sequences from BgVEN, BgGUA and

Table 1
Identity percentages between BV1 nucleic sequences.

	GUA	OMAN	VEN	BRE	BAR
GUA	100				
OMAN	99.79	100			
VEN	99.25	99.39	100		
BRE	97.12	97.32	97.32	100	
BAR	86.7	86.75	86.75	86.56	100

Pairwise sequence alignment between nucleic sequences of BV1 from the four *B. glabrata* strain and the *B. pfeifferi* one was performed using EMBOSS Needle tools from the EBI server (<https://www.ebi.ac.uk/>).

BpOMAN, and more than 97% identity with the one from BgBRE. Interestingly, the BV1 sequence from BgBAR strain was the most different, as it only shared 87% identity with the BV1 genome sequences from the four other *Biomphalaria* strains. In addition, BV1 sequence from BgBAR strain presented a longer 5'UTR (261 nt instead of 167 nt in the other strains), a single codon insertion in the ORF1 sequence, and an insertion of 8 codons in the ORF2, which thus encoded a 940 residues product. The polyprotein from ORF1 and ORF2 presented respectively 89% and 93% identity and 95% and 96% similarity with the ones of BV1 from the four other *Biomphalaria* strains, respectively (Table 1). Additional studies based on morphological and biochemical data, as well as host range and tropism specificities will be necessary to determine if BV1 from BgBAR is a distinct species or simply a variant of BV1 identified in the four other *Biomphalaria* species.

3.1.3. *Biomphalaria glabrata* Virus 2 (BV2)

BV2 virus was identified thanks to a long contig located in the middle of the genome sequence of the virus (Fig. 1). RACE-PCRs allowed to fully sequence the 3' end but only partially the 5' part of the virus genome. The partial genome sequence obtained for BV2 virus was 7 958 nt long. Only one ORF was predicted by *in silico* nucleotide sequence translation with a partial length of 7 749 nt lacking the initial start codon. The 3' UTR was 209 nt long and ended by a poly(A) tail.

The partial ORF encoded a 2 582 amino acid product and included several conserved motifs; at the 5' end of the ORF, typical domains from picorna-like viruses were identified including non-structural proteins, such as the RNA helicase domain (pfam00910), two mingled protease domains, the 3C cystein protease (pfam00548) and the peptidase C3G (pfam12381) domains, and a RdRP (cd1699) domain. At the 3' end, the unique ORF of BV2 encoded two *Picornavirus* capsid protein-like domains (cd00205) followed by a CRPV capsid protein-like domain (pfam08762), which are all typical domains from picorna-like non-structural virus proteins. It is noteworthy that such genome organization comprising one ORF coding for non-structural proteins at the 5' end and structural proteins at the 3' end was only characterized for the *Marnaviridae* family inside the picorna-like virus supergroup, and it comprises only one species so far. Nevertheless, protein blast analysis provided the highest similarity for the non-structural protein of *Solenopsis invicta* virus-1, a *Dicistrovirus*, with 26% identity over 797 residues (e-value: 7^{-56}).

3.1.4. *Biomphalaria glabrata* Virus 3 (BV3)

The RNAseq contig coverage of BV3 genome sequence was composed of multiple small overlapping fragments from the 5' side to the two-thirds of the sequence, separated by a gap from two overlapping fragments at the 3' side (Fig. 1). The sequence of the gap was completed by PCR and the two ends were obtained by RACE-PCR. The near full-length viral genome sequence generated was constituted of 9 982 nt comprising one ORF of 8 556 nt, a 5' UTR and a 3' UTR of 619 and 807 nt, respectively. A poly(A) tail was also recovered at the 3' end of the genome.

The unique BV3 ORF encoded a 2 852 amino acids product. Two picornavirus capsid protein domain-like (cd00205) were found at the 5' side of BV3 and non-structural protein domains for RNA helicase (pfam00910) and RdRP (cd1699) at the 3' side, which is the reverse organization as compared to the one of BV2. Cysteine protease domain was not detected as it probably differed from already known sequences; however, the cysteine protease motif $^{1910}\text{GxCG}^{1913}$ was retrieved as well as the potential catalytic triad constituted by H¹⁸⁰⁴, E¹⁸⁴⁶ and C¹⁹¹². Inside the *Picornavirales* order, only *Secoviridae*, *Picornaviridae* and *Iflaviridae* families have the replication block in the C-terminal region of the polyprotein, while the structural proteins are found in the N-terminal region. Protein BLAST analysis provided the best hit for mouse *Encephalomyocarditis virus*, a member of the mammalian *Picornaviridae* family, with 28% identity over 297 residues (e-value: $1e^{-17}$).

3.1.5. *Biomphalaria pfeifferi* Virus 4 (BV4)

The contig coverage of BV4 was the weakest of the five viruses identified in *Biomphalaria* species (Fig. 1). The many gaps within the sequence were successfully filled by PCR but RACE-PCR failed to amplify both genome ends. The partial genome sequence of BV4 that we obtained was 7 264 nt long, which is constituted of a unique ORF, incomplete at its 5' side, and of a fragment of the 3'UTR (103 nt).

Partial sequence of BV4 ORF coded for a 2 386 residues product. CD search analysis revealed the same domain organization as BV2, with non-structural proteins at the 5' end and structural proteins at the 3' end (Fig. 1). The 5' end of the ORF started inside the RNA helicase domain (pfam00910) and was followed by the RdRP domain (cd1699). No protease domain was detected and its manual identification was less obvious than for other viruses. The cysteine protease motif $^{1003}\text{GxCG}^{1006}$ was clearly identifiable but the putative substrate binding site $^{1022}\text{GxMxxG}^{1027}$ was divergent from the conserved one (GxHxxG). In addition, the first H⁸⁹⁸ and last C¹⁰⁰⁵ residues of the catalytic triad were found, but several candidate residues were found for the second one (E⁹³⁰, D⁹⁴¹, E⁹⁴⁹, E⁹⁵¹ and D⁹⁵⁴). In the structural protein part at the 3' side of the ORF, two picornavirus capsid protein-like domains (cd00205) were identified. Protein BLAST analysis provided the best hit for the polyprotein of *Fisavirus 1* (GenBank accession NC.025836), with 32% identity over 1 350 residues (e-value: $3e^{-179}$). *Fisavirus 1* has been recently described as an unclassified *Picornavirales* isolated from fresh water carp feces (Reuter et al., 2015), with a 8 712 nt long genome bearing the same genome organization as BV4.

3.1.6. *Biomphalaria pfeifferi* Virus 5 (BV5)

The last virus BV5 was identified by the presence of a few non-overlapping contigs corresponding to the RdRP gene. Sequence gaps were fulfilled by PCR, but only a partial 1013 nt long sequence of the unique ORF of its genome was obtained (Fig. 1).

The ORF encoded a 337 residues product. This comprised the RdRP domain (cd1699) and a RdRP 4 domain (pfam02123), which is a RdRP protein found in *Luteovirus*, *Totivirus* and *Rotavirus*. The best BLAST hit was for the RdRP of *Golden shiner totivirus*, with 54% identity over 319 amino acids (e-value: $3e^{-111}$). This virus was recently characterized from a fresh water fish from a North American lake and it presented a double-stranded RNA genome (Mor and Phelps, 2016). Moreover, all the other BLAST hits also corresponded to RdRP from other *Totivirus*, suggesting that BV5 could be a new candidate species for this family.

3.2. Taxonomic position of viruses

Both phylogenetic analyses performed using the RdRP domain (Fig. 2) and helicase domain sequences (Supp. Fig. Helicase) clus-

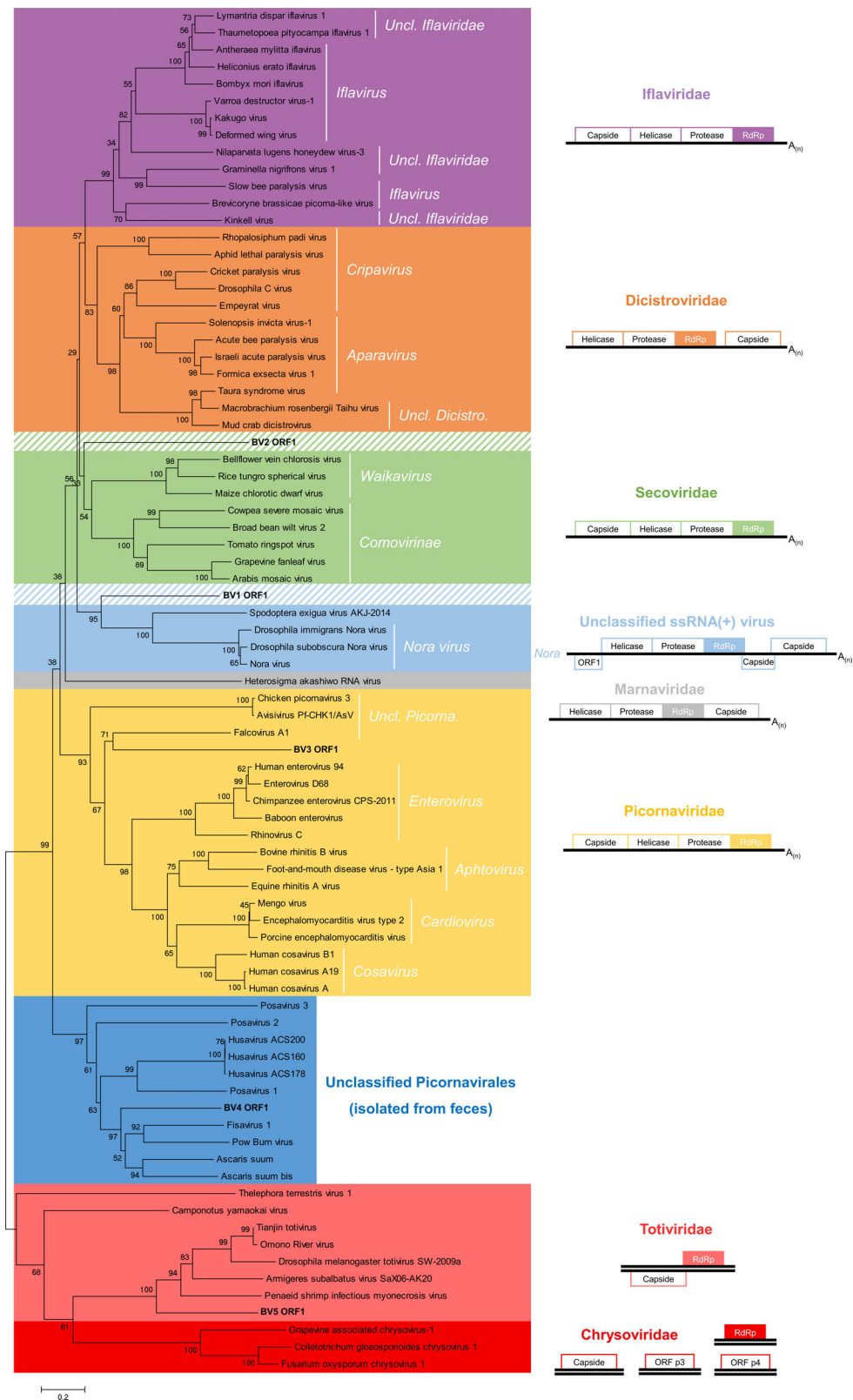


Fig. 2. Phylogenetic analysis of the RdRp protein sequence domains from 81 different viruses belonging to the *Iflaviridae* (13; purple), *Dicistroviridae* (12; orange), *Secoviridae* (8; green), *Unclassified ssRNA(+)* virus (4; light blue), *Marnaviridae* (1; grey), *Picornaviridae* (17; yellow), *Unclassified Picornavirales* isolated from feces (10; dark blue), *Totiviridae* (8; light red) and *Chrysoviridae* (3; dark red) families in addition to the five viruses isolated from *Biomphalaria*. Viruses from *Biomphalaria* (BV1 to BV5) are indicated in bold and are highlighted in the color of the family if they were clearly included in or they are hatched with the color of the closest family. A bootstrap analysis of 2000 replications was carried out on the trees inferred from the neighbor joining method. Bootstrap values are shown at each branch of the trees. The accession number of each protein sequence used is listed in Supplementary Table S1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

tered BV1 with a group of unclassified *Picornaviridae* supported with high bootstrap values for RdRP sequences.

This group of unclassified *Picornaviridae* comprised an isolate from *Spodoptera exigua* and several *Nora* viruses from different *Drosophila* species. In addition to structural and non-structural proteins, each encoded by a specific ORF, *Nora* viruses possess 2 additional ORFs; one is coding for a RNAi suppressor protein, and the other one encodes a protein of unknown function. The *S. exigua* virus possesses 5 ORFs, one that encodes non-structural proteins and the four others that encode proteins without any conserved motif sequence. Therefore, the clustering of BV1 on the basis of RdRP and helicase protein sequence similarity is not consistent with the genome organization of viruses from this cluster. BV1 presents the typical dicistrovirus organization with its dicistronic genome structure comprising the 2 ORFs coding from 5' to 3' end for non-structural and structural proteins, respectively. This phylogenetic relationship of BV1 with *Nora* viruses is surprising as it did not have the same genomic structure. As *Nora* viruses have been previously described to be enteric viruses transmitted by feces (Habayeb et al., 2009); it could be interesting to investigate in the future the tropism of BV1 for snail tissues.

Analysis of RdRP sequences led to the clustering of BV2 with the *Secoviridae*, which is a family of viruses known to infect plants. But on one hand, this taxonomic position is weakly supported by the bootstrap analysis, and on the other hand, its genomic organization is not that of *Secoviridae*, but rather the one of *Marnaviridae*. A complementary phylogenetic analysis based on the helicase domain (Fig. S1) positioned BV2 between BV1 and the *Marnaviridae*, but again supported by a low bootstrap value. To be noted, the helicase sequence used for this analysis was truncated, which could have biased the phylogenetic analysis due to the treatment of the missing amino-acids in the neighbor-joining model used (gap treatment: pairwise deletion). It is obvious that BV2 is a picorna-like virus as it possesses all their typical domains, but to date we were not able to clearly classify it among the already known families by phylogenetic analyses.

The two phylogenetic analyses positioned BV3 with high bootstrap values inside the *Picornaviridae* family, which groups the mammalian picornaviruses. Moreover, the genome organization of BV3 is in accordance with that of the *Picornaviridae* family. This family comprises several genus and BV3 is clustered together with several unclassified *Picornaviridae* identified from different bird species. The discovery of other viruses phylogenetically closer to BV3 may help to give more insights into its exact position within this family.

BV4 clustered with a group of unclassified *Picornavirales* in analyses performed with RdRP and helicase sequences. It also shared a similar domain organization with viruses from this group. All viruses from this group were isolated from feces and it comprises viruses identified from farmed animal's feces like swine and fish, but also from human and fly feces. It would be interesting in the future to study the tissue tropism of BV4 to determine if it also has an intestinal origin. It seems clear that a new group of picorna-like viruses, which includes BV4, is emerging and that it has to be assigned to a new family or to a new group inside an existing family.

The phylogenetic analysis of the RdRP protein sequence of BV5 clustered it with the *Totiviridae* family. It particularly positioned it with *Totiviruses* from other invertebrates, including shrimp, mosquito, and fly. *Totiviruses* are non-polyadenylated double-stranded RNA viruses and they exhibit the same dicistronic genome organization with a large first ORF encoding structural proteins, and non-structural proteins encoded by a shorter second ORF. BV5 presents a very strong bootstrap support in spite of a very short protein fragment.

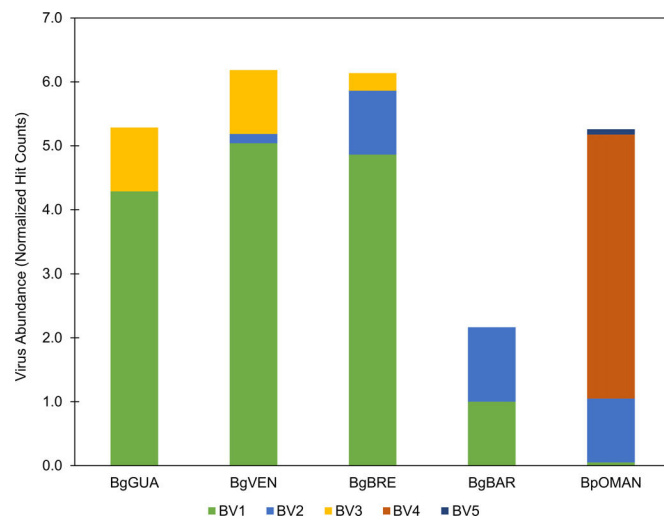


Fig. 3. Relative abundance of virus into *Biomphalaria* strains. Cumulative expression (Hit count normalized by the upper-quartile) of the *Biomphalaria* virus transcripts, in the different *Biomphalaria* strains, determined by Bowtie2 analysis. Each histogram corresponds to a *Biomphalaria* strain (BgGUA, BgVEN, BgBRE, BgBAR and BpOMAN) and each color corresponds to a virus (green for BV1, blue for BV2, yellow for BV3, orange for BV4 and dark blue for BV5). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Virus distribution and relative abundance in different *Biomphalaria* strains and species

The five viruses identified in this study were not all present in all snail strains studied (Fig. 3). This confirms that our snail rearing process and facilities properly isolate the different snail strains with their own virus community and do not allow inadvertent transmissions between all different snail strains. Previous studies have already shown a correlation between viral reads number and viral load (Valenzuela-Miranda et al., 2014; Wilk et al., 2015). Even if the exact number of viral particles cannot be estimated by this way, it gives a relative viral abundance between different biological samples. Therefore, we used our RNA-seq data to estimate the relative abundances of each virus within the five *Biomphalaria* strains. Total viral load was similar in the *Biomphalaria* strains BgVEN, BgBRE, BgGUA and BpOMAN (between 5.2 and 6.1 normalized hit counts) but it was 2.4–2.9 times lower in BgBAR. Proportion of the five viruses varied between the different *Biomphalaria* strains studied (Fig. 3).

BV1 was present in all tested snail strains, including both *B. glabrata* and *B. pfeifferi* species. It was the most abundant virus within three *B. glabrata* strains (81.0, 81.4 and 79.0% of total viral load in BgVEN, BgBAR and BgGUA, respectively) whereas it represented only 46.2% of total viral load in BgBAR strain. Interestingly, genome sequence of BV1 from BgBAR strain was slightly different from BV1 from other snail strains. In *B. pfeifferi*, BV1 was also present but its abundance was very low (0.9% of total viral load), which could explain why it was more difficult to amplify by PCR from BpOMAN and why we only managed to get 8 732 nt over 10 116 at the 3' side. Such variation in the relative abundance of this virus between the two snail species suggests that it might be differently constrained by the presence of different other viruses and potentially different other members of the microbiota but also by differences in the metabolism, immunity and physiology of the different species.

In *B. glabrata*, BV2 and/or BV3 were also recovered. Their abundance varied according to the snail strain. It is noteworthy that Bowtie2 analysis revealed the presence of BV2 reads in BgVEN transcriptome, whereas no viral transcript nor PCR amplicon corresponding to BV3 was recovered from BgVEN. We could hypothesize

that viral load might be extremely low in this snail strain that could partly explain why PCR-based approaches failed. Another possibility is that mapped reads might belong to another uncharacterized virus from BgVEN having sequence homology with BV3. In BgBRE, BV2 was much more abundant than BV3, while in BgVEN it was the opposite (Fig. 3). Interestingly, BV3 abundance within the different *B. glabrata* strains seems to decrease from populations sampled in Caribbean Island and Venezuela to Brazil (from BgGUA to BgVEN, BgBRE and BgBAR) while BV2 load is increasing. This potential trade-off in the geographical distribution of BV2 and BV3 needs to be further investigated. In *B. pfeifferi*, BV2 was as abundant as in BgBRE and BgBAR. Consequently, this virus was able to infect both *Biomphalaria* species, like BV1. The most abundant virus in *B. pfeifferi* was BV4 (78.4% of total viral load), which was only recovered in this strain. Similarly, BV5 is specific to *B. pfeifferi* but it exhibited a 48-fold lower abundance than BV4. Again, this particular viral distribution could be due to a specific tropism of BV4 and BV5 to *B. pfeifferi* species and/or could be linked to the geographical origin of the snail strain. Viral specificity should be investigated by performing viral transmission experiments.

Nevertheless, we have to keep in mind that RNAseq data were obtained from pools of 30 animals. Therefore, it provides a global overview of virus distribution and abundances within each strain of snails but it fails in reaching the inter-individual plasticity of virus abundance and diversity. Future studies are thus also needed to solve this question.

3.4. *Biomphalaria resident viruses as potential new schistosomiasis control agents?*

The control of host snail population is one of the fighting strategies proposed by the WHO for schistosomiasis eradication (Rollinson et al., 2013). Limiting snail populations in endemic areas could constrain parasite transmission. In this context, several approaches have been developed or proposed. Molluscicides have been developed (McCullough et al., 1980), but their toxicity to the abiotic and biotic environments is a limit to their massive use in the field. The management of snail habitat together with the introduction of predators or snail competitors for habitat have also been used (Giboda et al., 1997; Guimaraes et al., 2001; Hofkin et al., 1992; Pointier and Jourdan, 2000; Sokolow et al., 2014). Unfortunately, snail population dynamics are highly variable depending on environmental conditions and thus the efficacy of such strategies is therefore difficult to predict.

The use of natural snail pathogens could also be considered for controlling snail populations. However, only few studies have been conducted using natural snail microbes. Two snail pathogens have been reported to become potential control agents. *Brevibacillus lacterosporus* was shown to induce mortality in some but not all *B. glabrata* populations, and only on juvenile snails (3–12 days-old post-eclosion) (de Oliveira et al., 2004). More recently, a new bacterial strain of *Paenibacillus*, *P. glabratella*, was reported to be lethal for *B. glabrata* snails, with a high rate of mortality at all developmental stages of the snail (Duval et al., 2015). Nevertheless, its pathogenicity against other schistosome intermediate host species as well as its safety toward other local endemic species is still to be determined. Our results open a new field of research using *Biomphalaria* viruses. Nowadays, the role of holobiont in the modulation of host immune system is well known in other models (Gilbert et al., 2012). Thus, it will be important to discover the role (positive or negative) played by these viruses on the snail homeostasis, especially during *S. mansoni* parasite infection. Without evident pathogenicity, virus could affect snail life history traits potentially depending on the viral tropism. Future work will investigate viral prevalence at individual level and potential pathogenicity in reciprocal host/virus

crosses to investigate if these viruses could be used as a new strategy to control vector snail populations in the field.

4. Conclusion

In this study, we report the identification and characterization of viruses from five *Biomphalaria* snail strains from RNAseq data. We discovered the presence of at least 5 new RNA viruses in both *B. glabrata* and *B. pfeifferi* species. The analysis of their sequence and genome organization revealed that four viruses were associated with the picorna-like virus superfamily, while the fifth one is close to the *Totiviridae* family. These results are supported by a phylogenetic analysis based on the RdRP and helicase protein sequences. The distribution analysis showed that BV1 was present in all the 5 snail strains while the four others were not equally distributed. Some of them, such as BV2 and BV3, seem to have a geographic-specific origin while others, such as BV4 and BV5, might be species-specific as they were only recovered from *B. pfeifferi*. BV1 is clearly the most abundant virus in *B. glabrata*, while it is BV4 in *B. pfeifferi*.

This work is a first step towards the identification of natural biological agents able to infect several populations of *Biomphalaria*, which is the intermediate host of the parasite *S. mansoni*. Future work will investigate viral prevalence at individual level, but also in field populations. The viral tropism, as well as the mode of transmission, has to be studied to better understand the different steps of the contamination process. The role played by these viruses on snail homeostasis also has to be investigated and more particularly during *S. mansoni* parasite infection to know if the presence of these viruses would be adaptive for the host.

Acknowledgements

This work was supported by ANR JCJC INVIMORY (number ANR-13-JSV7-0009) funded to BG. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.actatropica.2016.12.021>.

References

- Basch, P.F., 1976. Intermediate host specificity in *Schistosoma mansoni*. *Exp. Parasitol.* 39, 150–169.
- Bettarel, Y., Bouvier, T., Nguyen, H.K., Thu, P.T., 2015. The versatile nature of coral-associated viruses. *Environ. Microbiol.* 17, 3433–3439.
- Bosch, T.C., McFall-Ngai, M.J., 2011. Metaorganisms as the new frontier. *Zoology (Jena, Germany)* 114, 185–190.
- Burke, G.R., Strand, M.R., 2012. Polydnviruses of parasitic wasps: domestication of viruses to act as gene delivery vectors. *Insects* 3, 91–119.
- Chitsulo, L., Engels, D., Montresor, A., Savioli, L., 2000. The global status of schistosomiasis and its control. *Acta Trop.* 77, 41–51.
- Christiaansen, A., Varga, S.M., Spencer, J.V., 2015. Viral manipulation of the host immune response. *Curr. Opin. Immunol.* 36, 54–60.
- Dheilly, N.M., Duval, D., Mouahid, G., Emans, R., Allienne, J.F., Galinier, R., Genthon, C., Dubois, E., Du Pasquier, L., Adema, C.M., Grunau, C., Mitta, G., Gourbal, B., 2015a. A family of variable immunoglobulin and lectin domain containing molecules in the snail *Biomphalaria glabrata*. *Dev. Comp. Immunol.* 48, 234–243.
- Dheilly, N.M., Maure, F., Ravallec, M., Galinier, R., Doyon, J., Duval, D., Leger, L., Volkoff, A.N., Misse, D., Nidelet, S., Demolombe, V., Brodeur, J., Gourbal, B., Thomas, F., Mitta, G., 2015b. Who is the puppet master? Replication of a parasitic wasp-associated virus correlates with host behaviour manipulation. *Proceedings* 282, 20142773.
- Dillies, M.A., Rau, A., Aubert, J., Hennequet-Antier, C., Jeanmougin, M., Servant, N., Keime, C., Marot, G., Castel, D., Estelle, J., Guernec, G., Jagla, B., Jouneau, L., Laloe, D., Le Gall, C., Schaeffer, B., Le Crom, S., Guedj, M., Jaffrezic, F., 2012. A

- comprehensive evaluation of normalization methods for Illumina high-throughput RNA sequencing data analysis. *Brief. Bioinform.* 14, 671–683.
- Dong, Y., Manfredini, F., Dimopoulos, G., 2009. Implication of the mosquito midgut microbiota in the defense against malaria parasites. *PLoS Pathog.* 5, e1000423.
- Duval, D., Galinier, R., Mouahid, G., Toulza, E., Allienne, J.F., Portela, J., Calvayrac, C., Rognon, A., Arancibia, N., Mitta, G., Theron, A., Gourbal, B., 2015. A novel bacterial pathogen of *Biomphalaria glabrata*: a potential weapon for schistosomiasis control? *PLoS Negl. Trop. Dis.* 9, e0003489.
- Giardine, B., Riemer, C., Hardison, R.C., Burhans, R., Elnitski, L., Shah, P., Zhang, Y., Blankenberg, D., Albert, I., Taylor, J., Miller, W., Kent, W.J., Nekrutenko, A., 2005. Galaxy: a platform for interactive large-scale genome analysis. *Genome Res.* 15, 1451–1455.
- Giboda, M., Malek, E.A., Correa, R., 1997. Human schistosomiasis in Puerto Rico: reduced prevalence rate and absence of *Biomphalaria glabrata*. *Am. J. Trop. Med. Hyg.* 57, 564–568.
- Gilbert, S.F., Sapp, J., Tauber, A.I., 2012. A symbiotic view of life: we have never been individuals. *Q. Rev. Biol.* 87, 325–341.
- Gorbalenya, A.E., Donchenko, A.P., Blinov, V.M., Koonin, E.V., 1989. Cysteine proteases of positive strand RNA viruses and chymotrypsin-like serine proteases: a distinct protein superfamily with a common structural fold. *FEBS Lett.* 243, 103–114.
- Guimaraes, C.T., Souza, C.P., Soares, D., 2001. Possible competitive displacement of planorbid by *Melanooides tuberculata* in Minas Gerais, Brazil. *Memorias do Instituto Oswaldo Cruz* 96 (Suppl), 173–176.
- Habayeb, M.S., Ekengren, S.K., Hultmark, D., 2006. Nora virus, a persistent virus in *Drosophila*, defines a new picorna-like virus family. *J. Gen. Virol.* 87, 3045–3051.
- Habayeb, M.S., Cantera, R., Casanova, G., Ekstrom, J.O., Albright, S., Hultmark, D., 2009. The *Drosophila* Nora virus is an enteric virus, transmitted via feces. *J. Invertebr. Pathol.* 101, 29–33.
- Hall, T., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* 41, 95–98.
- Hofkin, B.V., Hofinger, D.M., Koech, D.K., Loker, E.S., 1992. Predation of *Biomphalaria* and non-target molluscs by the crayfish *Procambarus clarkii*: implications for the biological control of schistosomiasis. *Ann. Trop. Med. Parasitol.* 86, 663–670.
- Ibrahim, M.M., 2006. Energy allocation patterns in *Biomphalaria alexandrina* snails in response to cadmium exposure and *Schistosoma mansoni* infection. *Exp. Parasitol.* 112, 31–36.
- Knight, M., Elhelu, O., Smith, M., Haugen, B., Miller, A., Raghavan, N., Wellman, C., Cousin, C., Dixon, F., Mann, V., Rinaldi, G., Ittiprasert, W., Brindley, P.J., 2015. Susceptibility of snails to infection with schistosomes is influenced by temperature and expression of heat shock proteins. *Epidemiology (Sunnyvale, Calif.)*, 5.
- Koonin, E.V., Dolja, V.V., 1993. Evolution and taxonomy of positive-strand RNA viruses: implications of comparative analysis of amino acid sequences. *Crit. Rev. Biochem. Mol. Biol.* 28, 375–430.
- Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33, 1870–1874.
- Liu, S., Vijayendran, D., Bonning, B.C., 2011. Next generation sequencing technologies for insect virus discovery. *Viruses* 3, 1849–1869.
- Marchler-Bauer, A., Derbyshire, M.K., Gonzales, N.R., Lu, S., Chitsaz, F., Geer, L.Y., Geer, R.C., He, J., Gwadz, M., Hurwitz, D.I., Lanczycki, C.J., Lu, F., Marchler, G.H., Song, J.S., Thanki, N., Wang, Z., Yamashita, R.A., Zhang, D., Zheng, C., Bryant, S.H., 2015. CDD: NCBi's conserved domain database. *Nucleic Acids Res.* 43, D222–226.
- Marston, D.A., McElhinney, L.M., Ellis, R.J., Horton, D.L., Wise, E.L., Leech, S.L., David, D., de Lamballerie, X., Fooks, A.R., 2013. Next generation sequencing of viral RNA genomes. *BMC Genomics* 14, 444.
- Martinez, J., Fleury, F., Varaldi, J., 2015. Competitive outcome of multiple infections in a behavior-manipulating virus/wasp interaction. *Ecol. Evol.* 5, 5934–5945.
- McCullough, F.S., Gayral, P., Duncan, J., Christie, J.D., 1980. Molluscicides in schistosomiasis control. *Bull. World Health Organ.* 58, 681–689.
- Mor, S.K., Phelps, N.B., 2016. Molecular detection of a novel totivirus from golden shiner (*Notemigonus crysoleucas*) baitfish in the USA. *Arch. Virol.* 161, 2227–2234.
- Pointier, J.P., Jourdan, J., 2000. Biological control of the snail hosts of schistosomiasis in areas of low transmission: the example of the Caribbean area. *Acta Trop.* 77, 53–60.
- Pradeu, T., 2016. Mutualistic viruses and the heteronomy of life. *Stud. Hist. Philos. Biol. Biomed. Sci.*
- Reuter, G., Pankovics, P., Delwart, E., Boros, A., 2015. A novel posavirus-related single-stranded RNA virus from fish (*Cyprinus carpio*). *Arch. Virol.* 160, 565–568.
- Rollinson, D., Knopp, S., Levitz, S., Stothard, J.R., Tchuente, L.A., Garba, A., Mohammed, K.A., Schur, N., Person, B., Colley, D.G., Utzinger, J., 2013. Time to set the agenda for schistosomiasis elimination. *Acta Trop.* 128, 423–440.
- Ruelas, D.S., Karentz, D., Sullivan, J.T., 2009. Effects of UVB on interactions between *Schistosoma mansoni* and *Biomphalaria glabrata*. *J. Invertebr. Pathol.* 101, 140–142.
- Ryan, M.D., Flint, M., 1997. Virus-encoded proteinases of the picornavirus super-group. *J. Gen. Virol.* 78 (Pt 4), 699–723.
- Sokolow, S.H., Lafferty, K.D., Kuris, A.M., 2014. Regulation of laboratory populations of snails (*Biomphalaria* and *Bulinus* spp.) by river prawns *Macrobrachium* spp. (Decapoda, Palaemonidae): implications for control of schistosomiasis. *Acta Trop.* 132, 64–74.
- Theron, A., Rognon, A., Gourbal, B., Mitta, G., 2014. Multi-parasite host susceptibility and multi-host parasite infectivity: a new approach of the *Biomphalaria glabrata*/*Schistosoma mansoni* compatibility polymorphism. *Infect. Genet. Evol.* 26, 80–88.
- Valenzuela-Miranda, D., Cabrejos, M.E., Yanez, J.M., Gallardo-Escarate, C., 2014. From the viral perspective: infectious salmon anemia virus (ISAV) transcriptome during the infective process in Atlantic salmon (*Salmo salar*). *Mar. Genomics* 20, 39–43.
- Wilk, E., Pandey, A.K., Leist, S.R., Hatesuer, B., Preusse, M., Pommerenke, C., Wang, J., Schughart, K., 2015. RNAseq expression analysis of resistant and susceptible mice after influenza A virus infection identifies novel genes associated with virus replication and important for host resistance to infection. *BMC Genomics* 16, 655.
- Zilber-Rosenberg, I., Rosenberg, E., 2008. Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution. *FEMS Microbiol. Rev.* 32, 723–735.
- de Oliveira, E.J., Rabinovitch, L., Monnerat, R.G., Passos, L.K., Zahner, V., 2004. Molecular characterization of *Brevibacillus laterosporus* and its potential use in biological control. *Appl. Environ. Microbiol.* 70, 6657–6664.

2. Influence de la réponse immunitaire sur le microbiote bactérien caractérisé de novo

Publication : Bacterial communities homeostasis a reciprocal interplay between microbiota and immune system in the lophotrochozoan snail *Biomphalaria glabrata*

PORTET Anaïs, TOULZA Eve, DUVAL David, HUOT Camille, LOKMER Ana, GALINIER Richard and GOURBAL Benjamin

*Article en préparation, soumission dans International Society for Microbial Ecology Journal, prévue pour Septembre 2017

1 Bacterial communities homeostasis a reciprocal interplay between microbiota and the
2 immune system in the lophotrochozoan snail *Biomphalaria glabrata*.

3

4 PORTET Anaïs, TOULZA Eve, DUVAL David, HUOT Camille, LOKMER Ana, GALINIER Richard
5 and GOURBAL Benjamin

6

7 **Abstract:**

8 Since few years, the importance of the microbiota in host-parasite interactions has been pointed out, and so
9 microbiota has to be taken into account as a third part in these studies. Indeed, the microbiota is constituted of
10 all microbial organisms that live within the host in a mutualistic interaction, such as bacteria, fungus or virus,
11 and can play a role in the host-parasite interaction. Here we pay a particular attention to the microbiota in the
12 interaction between the trematode *Schistosoma mansoni*, agent of human schistosomiasis and its invertebrate
13 intermediate host, the gastropod *Biomphalaria glabrata*. As a first approach to decipher the potential role of
14 the microbiota in this interaction, we analyse how the snail bacterial microbiota is affected or modified by
15 schistosome infections.

16 A massive 16s DNA sequencing approach (MiSeq) was carried out to characterize the changes of the
17 *Biomphalaria* bacterial microbiota. Moreover, sympatric and allopatric infections were realised between *B.*
18 *glabrata* and *S. mansoni* to observe differences in the dynamic of microbiota modifications according to this co-
19 evolutionary context. In parallel, using RNAseq data, we were able to establish a relation between the bacterial
20 microbiota modifications and the snail anti-microbial immune response.

21

22 **Introduction:**

23 Nowadays, rather than a single individual, an organism is considered by the individual
24 himself and all the potential symbiotic (mutualistic or pathogen) bacteria, archea, fungi,
25 virus and protozoan eukaryotes, organisms living in association that were named microbiota.
26 Consequently, the association of the host genome and microbiota genome should be
27 considered as the real unit of selection. Based on this concept, authors suggest that
28 symbiotic changes under environmental stress would have important role of the microbiota
29 both in adaptation and in the evolution of the host (Zilber-Rosenberg and Rosenberg 2008;
30 Theis, et al. 2016).

31 In the past decade, several studies have been conducted on the microbiota
32 characterization in invertebrate models, among them a lot of was realised on drosophila and
33 depict the influence of the microbiota on the fly phenotypes. The exogenous bacteria
34 improve the life expediency of drosophila (Brummel, et al. 2004), a strong diet cause a
35 microbiota resignation which leads to modification of mating preferences (Sharon, et al.
36 2011) ; finally microbiota modification results in the epithelial barrier dysfunction, which
37 play a role of protection against external attack (Buchon, et al. 2009; Clark, et al. 2015). The

38 microbiota establishes also a relationship between the energy storage and the body mass,
39 indicating a coordination of general metabolism; and so its involvement in diet and other
40 vital functions (Shin, et al. 2011; Broderick, et al. 2014; Wong, et al. 2014). In field
41 populations, it seems that the diet of an organism plays a major role on the bacterial
42 community acquisition and structuring (Chandler, et al. 2011), however the abundance of
43 each bacterial species seems to be influenced by host genotype and/or physiological status
44 (Chaston, et al. 2016; Martinson, et al. 2017).

45 So, the microbiota can affect some physiology compartment but also the invertebrate
46 immunity. Indeed, in *Aedes sp.*, the midgut microbiota elicits a basal immune activity of
47 mosquito immunity (Ramirez, et al. 2012), by activating mostly, the Toll pathways (Xi, et al.
48 2008). In *Anopheles gambiae*, the bacteria *Enterobacter* which is part of the microbiota was
49 shown to produce reactive oxygen species (ROS) against pathogens (Cirimotich, et al. 2011).
50 Always in *Anopheles sp.*, the endosymbiotic bacteria of microbiota *Wolbachia* leads to an
51 induction of immune genes, like TEP1, LRIM1 or defensin 1 (Joshi, et al. 2017). In drosophila,
52 the bacterial microbiota is necessary to secreted the Pvf2 factor, during stimulations
53 (Sansone, et al. 2015). The microbiota may also play a role of immune-shaping in its host.
54 Indeed, immune priming by gut microbiota have been demonstrated in insect models
55 following nutrition processes. In *Anopheles gambiae*, an unspecific priming based on
56 hemocyte differentiation, naturally induced by the gut microbiota is observed when
57 *Plasmodium* disrupts the barriers that normally prevent the gut microbiota from coming in
58 direct contact with epithelial cells (Rodrigues, et al. 2010). Similarly, the gut microbiota is
59 necessary for priming in *Tribolium castaneum* against *Bacillus thuringiensis* *bv. tenebrionis*
60 (Futo, et al. 2015). Finally, the host immunity may also play a role of tolerance or control on
61 microbiota. In drosophila, the intestinal homeobox gene Caudal regulates the commensal-
62 gut mutualism by repressing nuclear factor kappa B-dependent antimicrobial peptide genes
63 (Ryu, et al. 2008). Always in invertebrate, the host immunity tolerates the intestinal
64 commensal communities by regulation of the pro-inflammatory and anti-inflammatory
65 activities (Vitetta, et al. 2013).

66 Schistosomiasis is the second most widespread human parasitic disease after malaria
67 and affects over 200 million people worldwide (WHO 2010). *Schistosoma mansoni*
68 (Platyhelminthes, Lophotrochozoa) causes intestinal schistosomiasis. The *Schistosoma*
69 parasite needs a freshwater snail as a first intermediate host, where it undergoes part of its

70 life cycle before infecting humans. Patently infected snails support the continuous
71 production of hundreds of generations of cercariae, the free-living human-infective stage of
72 the parasite. Because of their medical and epidemiological importance as intermediary host
73 for *Schistosoma mansoni* parasites, the freshwater snails, *Biomphalaria glabrata*, have
74 attached significant research attention.

75 Numerous studies have been conducted on immunobiological interactions between
76 *Biomphalaria glabrata* et *Schistosoma mansoni* (Mitta, et al. 2005; Bouchut, et al. 2008;
77 Moné, et al. 2011; Coustau, et al. 2015; Galinier, et al. 2017; Mitta, et al. 2017), but to date
78 non studies have been carried out on the possible tripartite interaction between
79 *Biomphalaria glabrata*, its microbiota and its trematode parasite *Schistosoma mansoni*. Few
80 studies have been conducted on *Biomphalaria glabrata* snails to characterize their bacterial
81 microbiota (Ducklow, et al. 1979; Silva, et al. 2013). Indeed, in the 70s, the aerobic
82 heterotrophic flora of 200 snails has been investigated by analysing the cultivable bacteria
83 on Agar-Petri-dishes (Ducklow, et al. 1979). Five predominant bacteria genera including
84 *Pseudomonas*, *Acinetobacter*, *Aeromonas*, *Vibrios* and *Enterobacteria* were identified. In
85 2013, a first characterization of the *Biomphalaria glabrata* bacterial microbiota has been
86 conducted by 16S rRNA sequencing (Silva, et al. 2013) but exclusively on predominant
87 colonies grown in culture. The 5 genera previously identified were found with 6 additional
88 bacteria genera including *Citrobacter*, *Cupriavidus*, *Rhizobium*, *Stenotrophomonas*, *Klebsiella*
89 and *Sphingomonas* (Silva, et al. 2013).

90 In the present paper we investigate how an immune stress may influence the host
91 microbiota. In our study the immune stress of *B. glabrata* is caused by *S. mansoni* infections.
92 The immune system of *B. glabrata* is affect in different way along infections. Indeed, in
93 sympatric interaction, we observed a strong immunosuppression of the host immune
94 response, rather than in allopatric interaction an activation of the immune response is
95 observed (unpublished data). Moreover, when a snail was primo-infection by a snail strain
96 and challenged by the same snail strain, we observed a strong difference in the immune
97 response (fig.1). Indeed, following primo-infection a cellular immune response is observed
98 while following challenged a humoral immune response is activated (Pinaud, et al. 2016). So,
99 in this context we investigate the influence of this several immune responses on the host
100 microbiota dynamic.

101 In the present study, we realised a global characterization of the uncultivable bacterial

102 microbiota in naive South American *Biomphalaria glabrata* using 16S metabarcoding
103 sequencing approach (MiSeq); until now studies were exclusively conducted on a limited
104 portion of bacterial community (i.e. the cultivable bacteria fraction). We also investigated
105 microbiota community modifications following infection with two different parasite isolates
106 (*S. mansoni* Brazil (sympatric) and *S. mansoni* Venezuela (allopatric)) displaying the same
107 infectivity (i.e., same prevalence and intensity) toward the Brazilian snail host.
108 Concomitantly, the antimicrobial immune response of the snail was investigated using a
109 massive sequencing transcriptomic approach (RNAseq). The aim of the present study was to
110 depict the inter-individual structure of microbiota communities in naive snail population and
111 its dynamic according to infection immune stress. Did parasite infection disrupt the bacterial
112 microbiota communities, would this be reversible, which role for the snail immune system?

113

114 **Materials and Methods:**

115 ***Biological Materials***

116 One snail strain is used in this study, the albino Brazilian strain of *Biomphalaria glabrata*
117 (BgBRE). They are exposed to sympatric or allopatric infection by a Brazilian or a Venezuelan
118 strain of *Schistosoma mansoni* (named SmBRE, SmVEN respectively).

119

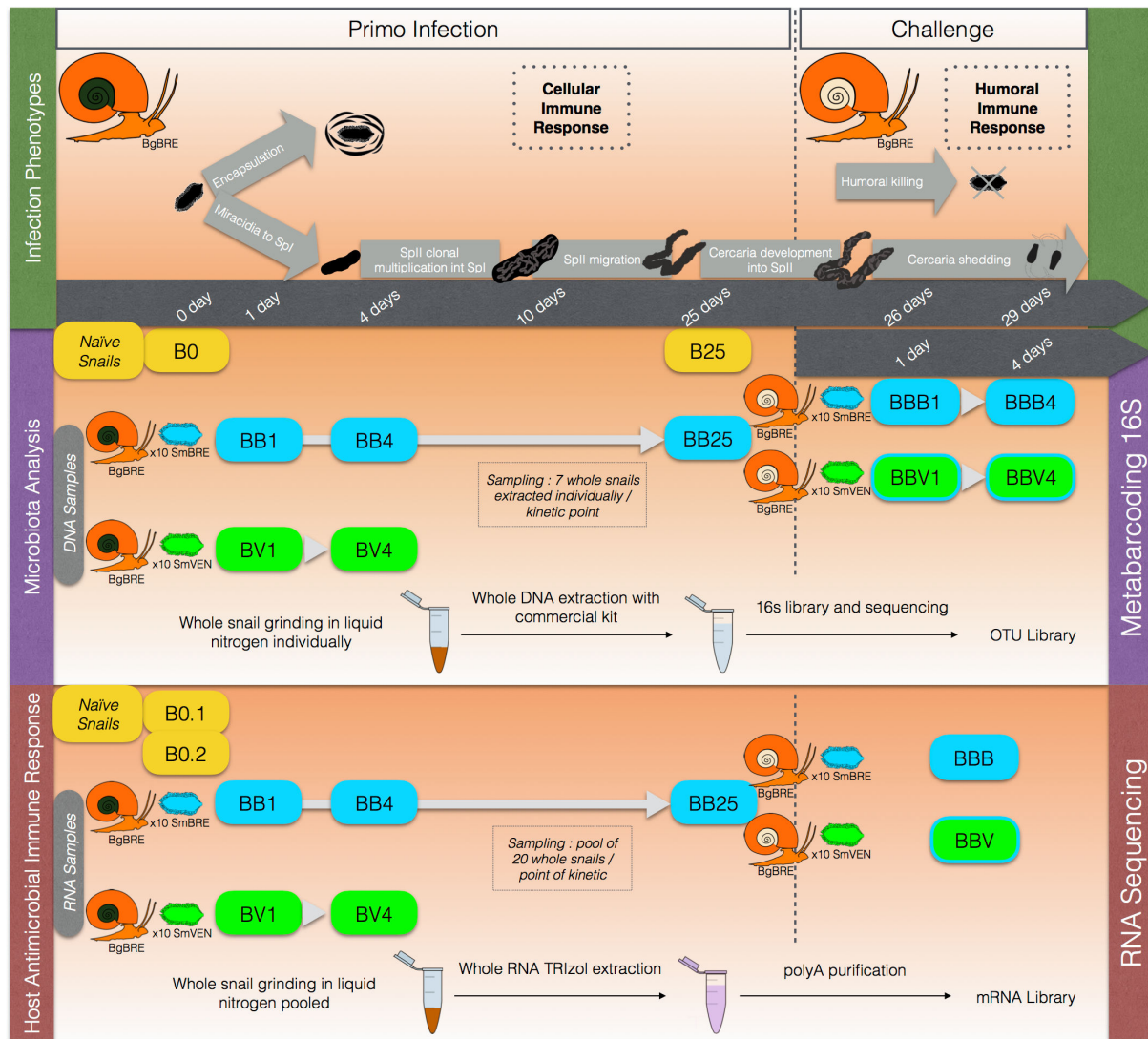
120 ***Experimental Protocol (fig. 1)***

121 In order to investigate and characterize changes in microbial communities following BgBRE
122 infection by SmBRE or SmVEN parasites, we conducted a complex experimental infection
123 protocol (fig. 1). Snails were either primo-infected or challenged with the two parasite
124 strains and 7 individual snails were recovered at 1, 4 and 25 days after primo-infection and
125 at 1, 4 days after challenge. Whole DNA was extracted for each individual snail and send to
126 MiSeq sequencing for 16S meta-barcoding. Briefly, 63 BgBRE snails are primo-infected with
127 SmBRE (49 snails) or SmVEN (14 snails). Then, twenty-five days after primo-infection, 28
128 snails primo-infected by SmBRE were challenged with SmBRE (14 snails) or SmVEN (14
129 snails). For all experimental infections, the snails were individually exposed for 12h to 10
130 miracidia in 5mL of pond water.

131 Samples of BgBRE primo-infected with SmBRE and recovered at 1, 4 and 25 days after the
132 primo-infection were named: BB1, BB4 and BB25. Samples of BgBRE primo-infected with
133 SmVEN and recovered at 1, 4 days after the primo-infection were named: BV1 and BV4.

134 Samples of BgBRE primo-infected with SmBRE and challenge with SmBRE and recovered at 1,
 135 4 days after the challenge were named: BBB1, BBB4 and named BBV1 and BBV4 for the one
 136 challenged with SmVEN.

137 As controls, 6 naïve snails were individually recovered at the starting of the experiment and
 138 6 other naïve snails were recovered 25 days later. Those samples were named: B0 and B25.
 139 These samples were used to demonstrate the stability of the bacterial communities of naïve
 140 BgBRE snails in breeding and rearing laboratory conditions.



141
 142 Fig. 1: Overview of experimental procedures

143 A parallel between the bacterial community evolution and immune response were carry out according to a
 144 *Biomphalaria* infection. For primo-infection Brazilian *Biomphalaria glabrata* (BgBRE) snails were individually
 145 exposed to 10 miracidia of either their sympatric Brazilian *Schistosoma mansoni* trematode parasite (SmBRE –
 146 BB - blue) or Venezuelian *Schistosoma mansoni* (SmVEN – BV - green). Following the infection some of
 147 miracidia were encapsulated by the hemocytes or developed into primary sporocysts (first intra-molluscan
 148 stage). Intra-molluscan parasite stages include two generations of sporocysts (primary sporocyst (SPI) and

149 secondary sporocyst (SpII)) and the production of cercariae. Twenty-five days after primo infection, the snails
150 primo infected with SmBRE were challenged with either 10 SmBRE (BBB) or 10 SmVEN (BBV). In this case all
151 miracidia degenerated into snail tissues, demonstrating the activation of a humoral immune response (Pinaud
152 et al. 2016). The bacterial community in naïve snails (see Fig. 2) and its evolution along the infection (see Fig.
153 3,4,5) were analysed by metabarcoding 16S sequencing. The antimicrobial immune response was analysed
154 along the bacterial community evolution (see Fig. 6).

155

156 ***Whole-body DNA extraction and sequencing***

157 Total snails are individually grind in nitrogen and the total DNA was extracted according to
158 the DNeasy Blood and Tissue Kit (Qiagen). The DNA quantification is realised by
159 spectrometry approach with a Qubit 2.0 Fluorometer. For each sample, 16S rDNA amplicon
160 libraries were generated using the 341F (CCTACGGGNGGCWGCAG) and 805R
161 (GACTACHVGGGTATCTAATCC) primers targeting the variable V3V4 loops (Klindworth et al.,
162 2012). Paired-end sequencing with 250 bp read length was performed on the Illumina MiSeq
163 PE (GenomeQuébec, Montréal, Québec, Canada) using the v2 chemistry according to the
164 manufacturer's protocol.

165

166 ***Data analysis of 16S sequences***

167 The FROGS pipeline ((Find Rapidly OTU with Galaxy Solution) implemented on a
168 galaxy instance (<http://sigenae-workbench.toulouse.inra.fr/galaxy/>) was used for data
169 processing (Escudié et al., 2015). In brief, paired reads were merged using FLASH (Magoč &
170 Salzberg, 2011). After denoising and primer/adapters removal with cutadapt (Martin, 2011),
171 de novo clustering was done using SWARM that uses a local clustering threshold, with
172 aggregation distance $d=3$ after denoising. Chimera were removed using VSEARCH (Rognes,
173 Flouri, Nichols, Quince, & Mahé, 2016) . We filtered the dataset for singletons and we
174 performed affiliation using Blast+ against the Silva database (release 128, sept 2016) for 16S
175 amplicons. Finally, an OTU table in standard BIOM format was produced for subsequent
176 analysis.

177 All statistical analyses were done using R v3.3.1 (R: a language and environment for
178 statistical computing, 2008; R Development Core Team, R Foundation for Statistical
179 Computing, Vienna, Austria [<http://www.R-project.org/>]). We used the phyloseq R package
180 for community composition analysis (McMurdie & Holmes, 2013) to infer alpha diversity
181 metrics at the OTU level, as well as beta diversity (between sample distance) from the OTU

182 table. Community similarity was assessed by Principal Coordinate Analysis (PCoA) using the
183 bray-curtis distance matrices.

184 We performed a Mann Withney U test ($p < 0.05$) to compare taxonomic diversity and one-
185 way ANOVA to compare functional diversity metrics between groups of samples. For all
186 analyses, the threshold significance level was set at 0.05.

187

188 **Analyse of core-microbiota:**

189 Core-microbiota is defined as the part of microbial communities that are present in
190 100% of the naive snails. To determine which families are part of core-microbiota, we made
191 a pivot table from the OTUs and the taxa ones, and we noted those which were present in all
192 the naive individuals. We obtained the abundance of each family in naive conditions and
193 then checked the abundance of each of these families. It appears that the family composing
194 the core-microbiota were also part of the most abundant ones in snails.

195 We then wanted to see if this core-microbiota is affected by infectious conditions. For that,
196 we compared the abundance of the families, part of core-microbiota, in infected conditions
197 to those for naive conditions with a Student test. Families that were significantly varying
198 ($p < 0,05$) were noted. The amount of significant differentially represented families for each
199 infected condition (1, 4 and 25 days primo infected and 1, 4 days challenged) were
200 calculated to see if there is a difference between it.

201

202 **Transcriptomic analysis of antimicrobial response**

203 *- Snail infections and biological samples recovery*

204 One hundred and eighty BgBRE snails were infected with SmBRE, SmVEN, 80 were
205 secondarily challenged 25 days later with SmBRE or SmVEN. Each snail was individually
206 exposed for 12 h to 10 miracidia in 5mL of pond water. For each experimental infection, 20
207 snails were recovered at 1, 4 and 25 days after primo-infection. The samples were named as
208 follows: BB1, BB4 and BB25 for BgBRE infected with SmBRE; BV1, BV4 for BgBRE infected
209 with SmVEN. At 1 and 4 days after challenge, 20 snails were recovered in each conditions;
210 equimolar amounts of each of these experimental groups were joined together into a single
211 sample named BBB for BgBRE infected by SmBRE and challenged by SmBRE; BBV for BgBRE
212 infected by SmBRE and challenged by SmVEN. Two pools of 30 uninfected BgBRE snails (B0.1
213 and B0.2) were used as controls.

214 - *Whole-snail RNA extraction and sequencing*

215 Total RNA was extracted using TRIZOL (Sigma Life Science, USA) according to the
216 manufacturer's instructions. Sequencing was realized in paired-end 72-bp read lengths,
217 using an Illumina Genome Analyzer II (MGX-Montpellier GenomiX, Montpellier, France).

218 - *De novo transcriptome assembly*

219 An antimicrobial transcriptome was realised with transcripts known to be involved in
220 *Biomphalaria* immune response against bacteria (Table 1). This small transcriptome was
221 concatenated with a transcriptome of *Biomphalaria* already available in the laboratory after
222 subtraction of transcripts corresponding to the small antimicrobial transcriptome to avoid
223 redundancy during the mapping of the reads.

224

225 Table 1: Antimicrobial transcriptome construction

226 Sequences used to perform a *Biomphalaria* antimicrobial transcriptome.

227

228 - *Differential expression analysis*

229 High-quality reads (Phred score >29) were aligned to the concatenate transcriptome using
230 Bowtie2 (v.2.0.2), which was run locally on a Galaxy server. The DESeq2 (v2.12) software was
231 used to identify differential transcript representation between uninfected (B0.1 and B0.2)
232 and infected conditions (p value < 0.05).

233

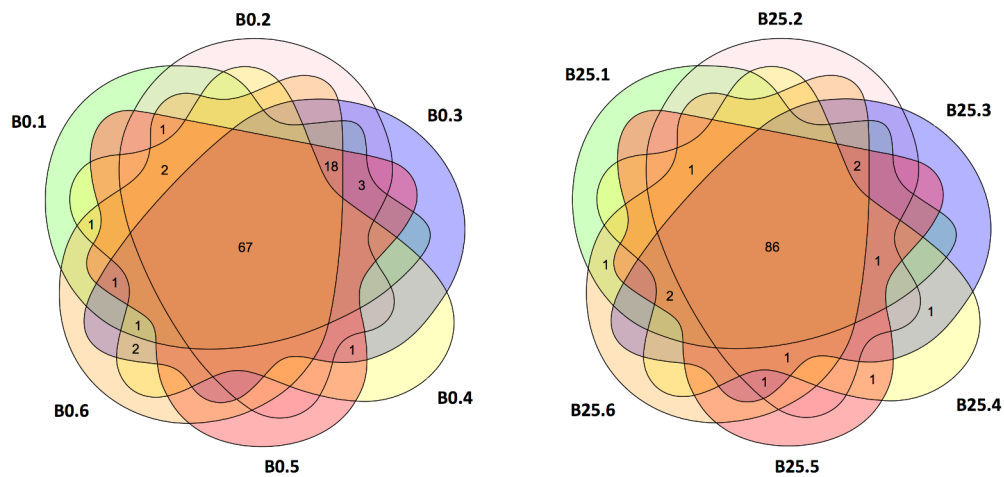
234 **Results:**

235 *Biomphalaria* microbiota characterization

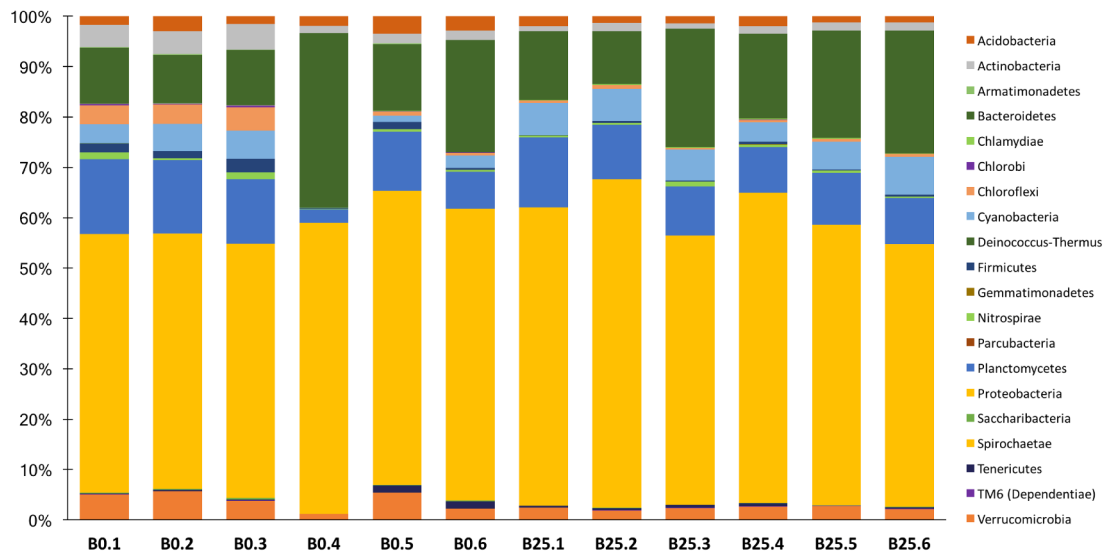
236 Microbiota communities would be known to be associated mainly with tegument, gut and
237 even, in some specific cases, with hemolymphatic compartment. In *Biomphalaria glabrata*
238 tegumental microbiota is difficult to isolate specifically and an installed hemolymphatic
239 microbiota community seems not to be present (personal observation). Thus. we used a
240 meta-barcoding 16S sequencing to characterize the whole snail *Biomphalaria glabrata*
241 bacterial microbiota that is expected to be associated mainly to gut. To characterize this
242 microbiota, we identify microorganisms from naive BgBRE individuals at day 0 and 25 (B0
243 and B25) of the experiment. The taxonomic diversity of the naive conditions is quantified
244 (table 2); the OTU numbers (observed richness), their expected richness (Chao index),
245 diversity (Shannon index) and evenness (Pielou index) are not significantly different into the

246 two naive condition time points. Moreover, Venn diagrams were used to highlight the inter-
247 individual similarity between microbiotas of naive snails. We observed that 67 (69%) and 89
248 (92%) of the 97 OTUs (family scale) are shared by B0 and B25 naive snails respectively (fig.
249 2A). Sixty-two on ninety-seven families present are common in all naive individuals and
250 correspond to what we described as the stable core microbiota (Table 1). We observed
251 92.5% of naive B0 snails OTUs family corresponding to the core microbiota and in naive B25
252 snails the core microbiota corresponding to 69.6% of OTUs family. Finally, the phylum
253 composition of individual naive snails at B0 and B25 time point shows a few inter-individual
254 variability and a constant proportion along the time (fig. 2B). In naive snails the
255 proteobacteria, bacteroidetes, cyanobacteria and planctomycetes phyla were the more
256 represented (fig. 2B). To conclude the bacterial microbiota of BgBRE seems have a strong
257 temporal stability and very few inter-individual variabilities in our laboratory rearing
258 conditions and also between individuals. Moreover, we identified the core microbiota of *B.*
259 *glabrata* witch corresponds to microbiota part of similarity between all individuals.
260

A



B



261

262 Fig. 2: *Biomphalaria* microbiota characterization

263 Characterisation of the *Biomphalaria* bacterial microbiota with six naive snails recovered at the start of
 264 experimentation (B0.1; B0.2; B0.3; B0.4; B0.5 and B0.6) and 6 naive snails recovered 25 days after the start of
 265 experimentation (B25.1; B25.2; B25.3; B25.4; B25.5 and B25.6). A. Venn diagram demonstrating the
 266 distribution of the OTUs (Family scale) shared by the 6 naive snails at B0, T=0 day (left Venn diagram), and
 267 shared by the 6 naive snails at B25, T=25 days (right Venn diagram). The number corresponding to the OTUs
 268 number. In our analysis 97 OTUs family was recovered. B. Phylum level composition of the 20 most abundant
 269 OTUs among the 12 naive snails.

270

271 Microbiota dynamics and *Biomphalaria* infection

272 As the bacterial microbiota of naive *Biomphalaria* seems to be stable in time, we next
 273 investigated whether some differences in microbiota composition and structure would
 274 change following *Schistosoma mansoni* infections.

275 Thus to investigate the microbiota dynamics, we used one mollusc strain, a Brazilian
 276 *Biomphalaria glabrata* (BgBRE) and two parasite strains, its sympatric parasite, the Brazilian
 277 *Schistosoma mansoni* (SmBRE) and an allopatric parasite, *Schistosoma mansoni* from
 278 Venezuela (SmVEN). *Biomphalaria glabrata* immune response was different against
 279 sympatric or allopatric parasite infections, and comparing primo-infection to challenge
 280 infection. We thus analysed microbiota dynamic during sympatric and allopatric primo-
 281 infection and challenge infection to investigate the influence of trematode infection on the
 282 microbiota communities (fig. 1).

283

Experimental Conditions	OTUs Observed	Estimate Richness (Chao 1)	Shannon Diversity Index	Simpson Index	Pielou Evenness Index
Naive snails	1,400 +/- 321.3	2,162.9 +/- 571.6	4.56 +/- 0.44	0.96 +/- 0.03	0.63 +/- 0.05
Primo infected snails	1,255.4 +/- 313.8	1,920.7 +/- 504.9	4.45 +/- 0.48	0.95 +/- 0.03	0.63 +/- 0.05
Primo SmBRE	1,266.4 +/- 278.3	1,975.6 +/- 443.2	4.30 +/- 0.38 ^b	0.94 +/- 0.04	0.61 +/- 0.06
Primo SmVEN	1,244.4 +/- 345.2	1,865.8 +/- 554.3	4.59 +/- 0.53 ^b	0.97 +/- 0.02	0.65 +/- 0.05
Primo infected snails 25 days	1,223.9 +/- 207.3	1,863.1 +/- 375.8	4.21 +/- 0.59	0.93 +/- 0.08	0.59 +/- 0.08
Challenge snails	1,063.3 +/- 252.5 ^a	1,656.2 +/- 452.8 ^a	4.01 +/- 0.39 ^a	0.94 +/- 0.03 ^a	0.58 +/- 0.06 ^a
Challenge SmBRE	1,027.2 +/- 191.1 ^a	1,568.9 +/- 348.8 ^a	4.01 +/- 0.43 ^a	0.94 +/- 0.03 ^a	0.58 +/- 0.07 ^a
Challenge SmVEN	1,099.3 +/- 297.4 ^a	1,743.5 +/- 522.6	3.99 +/- 0.36 ^a	0.93 +/- 0.03 ^a	0.57 +/- 0.05 ^a

284

285 Table 2: Taxonomic diversity

286 Operational Taxonomic Units (OTUs), Richness and Diversity/Evenness indices (mean +/- CI 95%) from control,
 287 primo-infected and challenged snails were recovered. For infected experimental conditions the values were
 288 calculated by pooling 1 and 4 days.

289 Different superscript letters indicate significant differences (Mann Whitney U test p<0.05); "a" corresponding
 290 to significant differences with naive snails; "b" corresponding to significant difference between parasite strains.

291

292 In a first time, we investigate the taxonomic diversity into each samples (table 2). The OTU
 293 numbers (observed richness), their richness (Chao index), diversity (Shannon index) and
 294 evenness (Pielou index) are quantified. After the primo-infection the taxonomic diversity of
 295 inection and time points are not significantly different. Conversely, it appears that all indices
 296 seem to be affected following the immune challenge. Indeed, the number of OTUs (Mann-
 297 Whitney U test, p=0.002), their Chao index richness (Mann-Whitney U test, p=0.006),
 298 Shannon index diversity (p=0.0004) and Pielou index evenness (Mann-Whitney U test,
 299 p=0.009) are significantly reduced in challenge compared to naive and primo-infected snails
 300 (table 2). Among the infected conditions (primo-infection and challenge), if we look

301 separately at the different time of kinetics post-infection (1 and 4 days) the richness and
 302 diversity are always less important after the challenge than after the primo-infection (fig. 3).
 303 So, to conclude the taxonomic diversity level does not seem to change following primo-
 304 infection whatever was the parasite strains used or the time point of infection. A difference
 305 on the taxonomic diversity is observed exclusively following challenge infection.

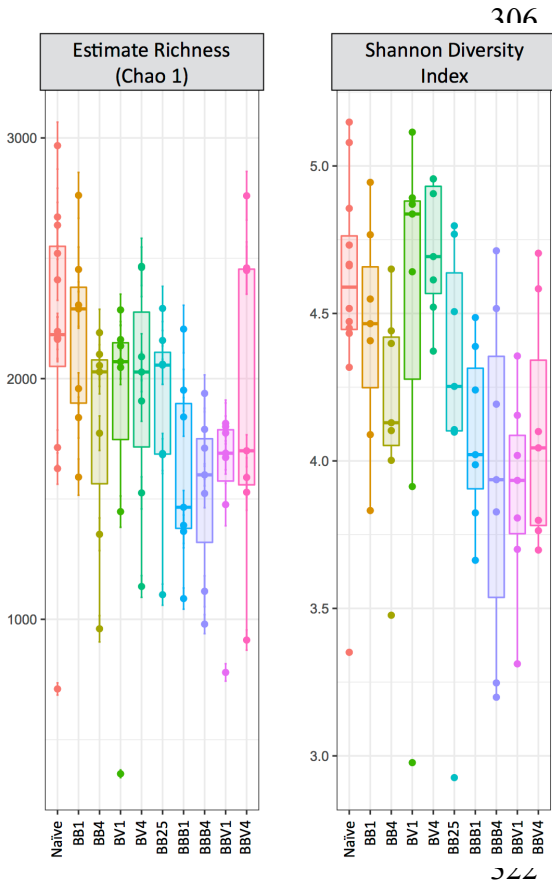


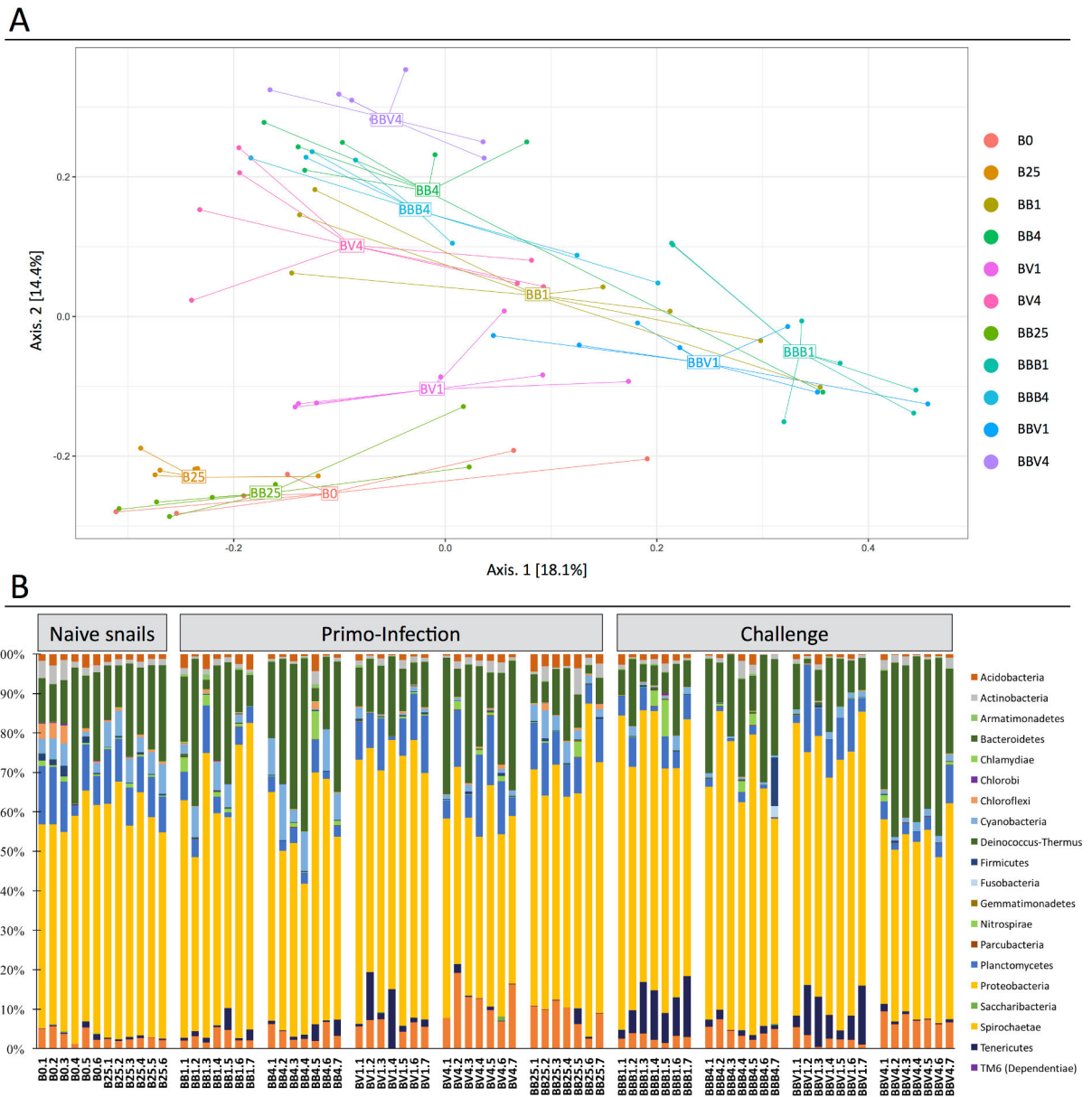
Fig. 3: Taxonomic Diversity

Focus on Richness and Diversity indices of OTUs. Boxplots of Chao1 and Shannon indices for all experimental points. Naive corresponding to naive snails, B0 and B25, are pooled; BB primo infection of BgBRE by SmbRE; BV primo infection of BgBRE by SmVEN; BBB primo infection of BgBRE by SmbRE and challenge by SmbRE; BBV primo infection of BgBRE by SmbRE and challenge by SmVEN. The time of kinetic point is mentioned with 1,4 or 25 corresponding of the days after primo-infection or challenge.

323 In a second time, we investigate the diversity of all samples using with a Bray-Curtis distance
 324 (fig. 4A). We observed the quantity and quality of specific OTUs in each samples. Through an
 325 ordination by Principal Coordinate Analysis (PCoA), we observed that naïve snail samples (B0
 326 and B25) grouped with BB25 compare to the other infection samples (fig. 4A). Even if BB25
 327 was significantly different from naive snails the fact that they are grouped in the PCoA
 328 (fig.3A) seems to demonstrate that the microbiota communities tend to return to a structure
 329 and a composition similar to naïve snails, at 25 days after the primo-infection.

330
 331 Moreover, always with the PCoA ordination, we observe also that samples of time point of
 332 infection (1 and 4 days) segregated according to the first axis (fig. 4A); as well as the type of
 333 infection (primo-infection and challenge) are also separated according to the first axis (fig.

334 4A). These results seem to show an impact of the infection dynamic and infection type on
 335 the microbiota structure rather than an impact of a specific parasite strains. Another, Bray-
 336 Curtis distance shows a significant difference between naive conditions and primo-infected
 337 samples (Mann-Whitney U test, $p=0.001$) and also between naive conditions and challenged
 338 samples ($p=0.001$) (supplementary table 1).
 339



340
 341 **Fig. 4: Functional diversity and bacterial community along *Biomphalaria* infection**
 342 Evolution of the bacterial microbiota of *Biomphalaria glabrata* along the *Schistosoma* infection and challenge.
 343 Sample abbreviations: B0, naive snails at T=0 day; B25, naive snails at T=25 days; BB primo infection of BgBRE
 344 by SmBRE; BV primo infection of BgBRE by SmVEN; BBB primo infection of BgBRE by SmBRE and challenge by
 345 SmBRE; BBV primo infection of BgBRE by SmBRE and challenge by SmVEN. The time of kinetic point is

346 mentioned with 1,4 or 25 corresponding of the days after primo-infection or challenge. Finally, “.number”
347 corresponding to the biological replicates. A. Functional diversity comparisons of *Biomphalaria* microbiota
348 along infection. Principal coordinate analysis of pairwise Bray-Curtis distances between all infection type and
349 time samples. Axes represent the two synthetic variables explaining the greatest proportion of variation in the
350 data set. The name of the infection point corresponding to the barycenter of biological replicates. B. Phylum
351 level composition of the 20 most abundant OTUs among all point of kinetic. In this representation the replicate
352 naive snails were pooled for more readability.

353
354 Finally, we observed the bacterial community composition of all samples to highlight the
355 most affected phyla (fig. 4B). In global analysis, we observed an impact of infections on
356 bacterial phyla composition while the parasite strains do not appear to affect differently
357 their composition. Most interestingly some phyla decrease or increase after primo-infeccion
358 or challenge. Among them, clusters are less abundant after primo-infection, at BB25 their
359 abundance seems to return to a rate similar to naïve snails and finally, they are again less
360 abundant after the challenge. This is the case for the Planctomycetes (fig.3B,
361 Planctomycetes, blue), the most abundant phylum within this profile; the Actinobacteria
362 (fig.3B, Actinobacteria, grey), the gram positive bacteria main abundant phylum presents in
363 our analysis; the Acidobacteria (fig.3B, Acidobacteria, red) and the Chloroflexi (fig.3B,
364 Chloroflexi, orange). Other clusters are more abundant than naive condition after primo-
365 infection, at BB25 their abundance decrease and they are again more abundant after
366 challenge. This is the case for the Bacteroidetes (fig.3B, Bacteroidetes, green), its represent
367 the common bacteria phylum mostly present in environment; Tenericutes (fig.3B,
368 Tenericutes, blue). The proteobacteria is the most abundant phylum in all conditions. The
369 number of sequence is stable along the infections.

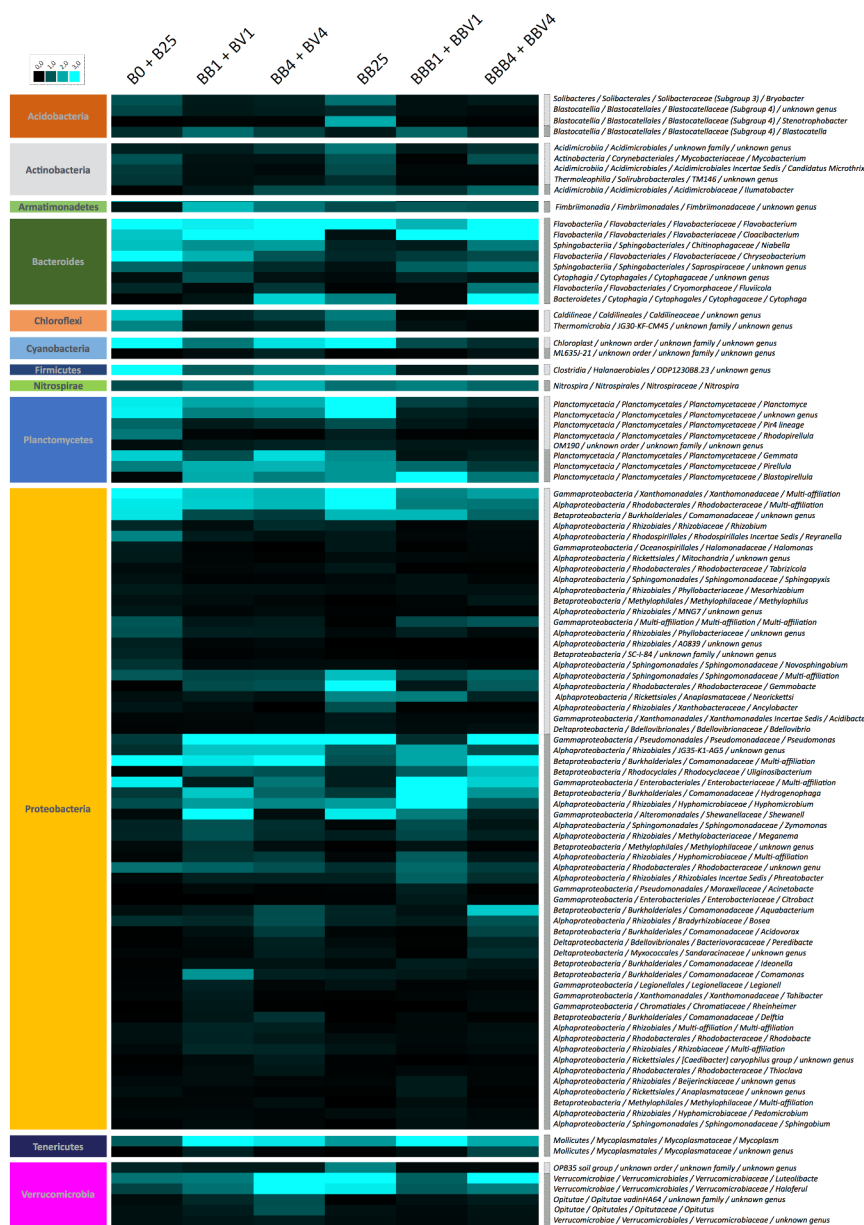
370 Moreover, the Genus level seems to be affected by the infection (fig. 5). We have recovered
371 the 100 most abundant Genus in all conditions; into a same phylum it appears a Genus
372 abundance shift after infection (fig. 5). This is the case for Acidobacteria phylum,
373 Blastocatella genus increase after infection while Byobacter and Stenotrophobacter genera
374 decrease after infection (fig. 5). Some phyla represented by few genera, increase globally
375 after infection, like Armatimonadetes, Bacteroidetes or Tenericutes. In opposition, the
376 Chloroflexi phylum decreases globally after infection (fig. 5).

377 In the present study, some phyla have few represented Genus but each highly abundant
378 (i.e., Armatimonadetes, Cyanobacteria, Firmicutes, Nitrospirae and Tenericutes); and some

379 phyla have a great number of genera highly represented (i.e., Planctomycetes or
 380 Proteobacteria) (fig. 5).

381 Finally, the microbiota composition is highly modified by the parasite infection; indeed, we
 382 have test the proportion of core microbiota which are modified by infections compared to
 383 naive condition. We observed 80.6% of the core microbiota is significantly impacted after
 384 infections. Furthermore, 9.7% the core-microbiota is specific significantly impacted only
 385 after at the primo-infection and 8.1% is specific significantly impacted only after the
 386 challenge.

387



388

389 Fig. 5: Focus on most abundant genus

390 Clustering of 100 most abundant Genus according to naive and infection conditions. Each Genus is representing
391 once, each line represents one Genus and each row corresponding to one condition on the Heatmap. Color
392 scale shows the abundance of each Genus in each condition (naive and infected). For infected experimental
393 conditions the different parasite strains were pooled for 1 and 4 days in primo-infection and challenge. Each
394 box corresponding to Phylum containing different Genus. The colors of the box corresponding to Phylum colors
395 and Genus are assembling into Phylum. The grey bars corresponding to Genus which are less present after
396 infections (primo-infection, challenge or both). The black bars corresponding to Genus which are more present
397 after infections. The name of each Genus is composed of its Class / Order / Family / Genus.

398

399 The link between the microbiota and the antimicrobial immune response of *Biomphalaria*

400 In a host-pathogen system, like between *Biomphalaria glabrata* and *Schistosoma*, the
401 microbiota can play an important role for the outcome of the interaction. Massive
402 transcriptomic approaches on *B. glabrata* immune response have been performed
403 previously in the sympatric / allopatric context (unpublished data) and in the primo-
404 infection/challenge context (Pinaud, et al. 2016). In this paper, we propose to investigate the
405 impact or link between the microbiota perturbation and the antimicrobial immune response.
406 For this purpose, we focus not only on the antimicrobial peptide response but also on the
407 antimicrobial proteins, that have been identified in the antibacterial response of
408 *Biomphalaria glabrata*. In *Biomphalaria* genome, 3 types of these molecules have been
409 identified the LBP/BPI, that belongs to antimicrobial proteins, the biomphamacin (type of
410 macin) and the achacin, belonging to antimicrobial peptides (fig. 6). We followed the
411 expression of these different proteins after primo-infections and challenges with the
412 sympatric (SmbRE) and allopatric (SmVEN) parasite strains. In a first time, these transcripts
413 are differentially expressed after infections by Schistosomes. For LBP/BPI the 3.1 and 3.2 are
414 over-expressed until 25 days after infection, while all other are under-expressed in all
415 conditions (fig. 6). Concerning the biomphamacins, the biomphamacin 1, 4, 5 and 6 are over-
416 expressed along infections, while biomphamacin 3 is under-expressed (fig. 6). Finally, the
417 achacin are under-expressed in sympatric primo-infection and following challenges and not
418 differentially expressed in allopatric primo-infection (fig. 6).

419

420

421

422

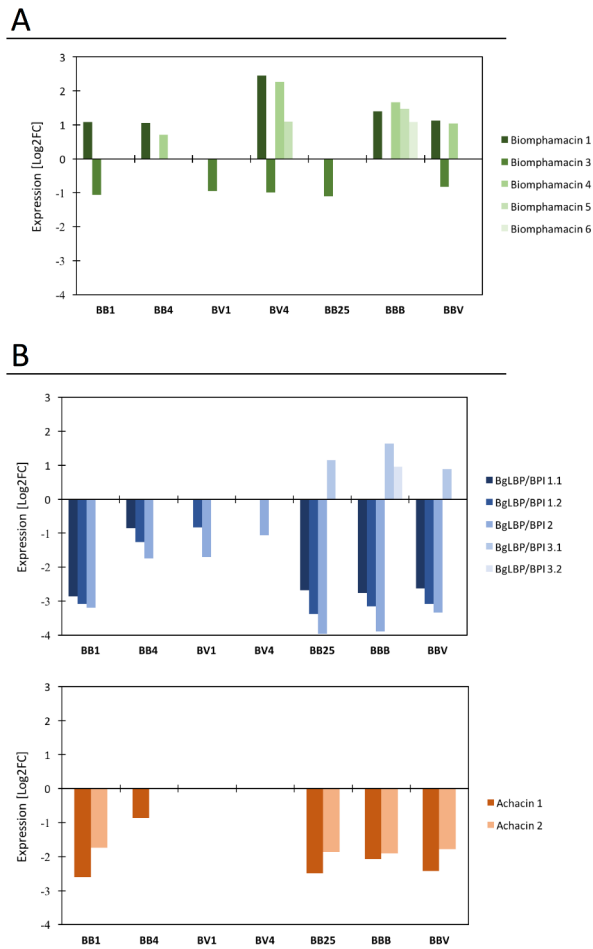


Fig. 6: The antimicrobial peptide of *Biomphalaria*

Expression [Log₂FC (fold change) from the DESeq2 analysis] of antimicrobial immune transcripts. The positive bars corresponding to the over-represented expression compared to the naive snails and the negative bars corresponding to the under-represented expression. Sample abbreviations: BB primo infection of BgBRE by SmBRE; BV primo infection of BgBRE by SmVEN; BBB primo infection of BgBRE by SmBRE and challenge by SmBRE; BBV primo infection of BgBRE by SmBRE and challenge by SmVEN. The time of kinetic point is mentioned with 1,4 or 25 corresponding of the days after primo-infection or challenge. Antimicrobial peptide categories including 6 biomphamacins (type of macin) in green, 5 LBP/BPIs (*Lipopolysaccharide-binding protein / Bactericidal Permeability-Increasing protein*) in blue and 2 achacins in orange.

443 The LBP/BPI and achacin expressions seems not to be correlated directly with the microbiota
 444 community modifications; for all the kinetic of infections all transcripts are under-expressed
 445 even when the microbiota community is changing. The biomphamacins, except the 6, are
 446 over-expressed after infection, from 24h in BB infection and 96h in BV infection and in
 447 challenge (BBB and BBV infection). Theses results suggest that the 3 *Biomphalaria*
 448 antimicrobial protein families are not involved in the same immune response; and only the
 449 biomphamacin family can potentially impacted the microbiota communities.

450

451 **Discussion:**

452 It is generally accepted that microbiota can serve beneficial functions in their hosts. Indeed,
 453 endosymbiotic bacterial communities can contributed to the host's health and fitness. One
 454 important biological trait that microbiota was demonstrated to affect is the host's immune
 455 system and consequently the protection against pathogens. An intimate interaction between
 456 the microbiota, the host immunity and pathogens has been highlighted in different

457 vertebrate and invertebrate models. In *Aedes sp.*, the midgut microbiota elicits a basal
458 immune activity (Ramirez, et al. 2012), by activating mostly, the Toll pathways (Xi, et al.
459 2008). An other example, in *Anopheles sp.*, of the role of microbiota, is the induction of
460 immune genes, like TEP1, LRIM1 or defensin 1, by the endosymbiotic bacteria *Wolbachia*
461 (Joshi, et al. 2017). These immune activations have important repercussions on pathogen
462 infections. In *Drosophila melanogaster*, the bacterial microbiota is necessary to secrete the
463 Pvf2 factor essential for the activation of an efficient anti-viral immune response (Sansone,
464 et al. 2015). In *Anopheles gambiae*, the bacteria *Enterobacter* which is part of the microbiota
465 was shown to produce reactive oxygen species (ROS) that were involved in the protection
466 against *Plasmodium falciparum* infections (Cirimotich, et al. 2011).

467 However, following an infection by a pathogen, the host mount a specific or adapted
468 immune response against the intruder. This activation of the immune response could have
469 huge repercussions on the microbiota community structure. For example, in *Aedes sp.* and
470 dengue virus interaction, the immune response against the pathogen influences the
471 microbiota population dynamic (Ramirez, et al. 2012).

472 These observations suggested that an intricate dialogue exist between the host and the
473 microbiota through the immune system. Indeed, two hypotheses have been proposed to
474 explain the role of the immune system in the microbiota acquisition and stability. Firstly, it
475 has been proposed that the host immune system ensures constant pressure on the
476 microbiota and thus constrains its implantation and its expansion permanently (Hooper, et
477 al. 2012). The microbiota has also an immune-shaping capacity, the resident bacterial
478 microbiota profoundly shape the host immunity conversely the host immune system play a
479 role to maintaining the homeostasis with resident microbial communities (Hooper, et al.
480 2012). Secondly, the antigenic discontinuity theory proposes that the immune system
481 controls and answers against a strong variation of antigenicity detected for example
482 following an infection, but would be tolerant against weak and continuous stimulations
483 experienced during their life span, which could be associated with the microbiota (Pradeu
484 and Eric 2014). In this case no immune response and no immune control of the microbiota
485 by the host would be expected.

486 All these hypothesis, theories, or observations were mainly proposed based on vertebrate
487 models and more precisely for mammals. Herein we propose to investigate this intimate and
488 intriguing interaction between the host, the microbiota and the immune system in an

489 invertebrate model. To achieve this goal, we choose the model involving the gastropod snail
490 *Biomphalaria glabrata* and its trematode parasite *Schistosoma mansoni*, the agent of human
491 schistosomiasis. The *S. mansoni* parasite was used as an external stress to modify the
492 immune system in different issues. Indeed, we demonstrate that depending on the past
493 evolutionary history between snail and schistosome different immune responses were
494 observed. In sympatric interaction, parasite was perfectly adapted to its host and exerts a
495 strong immunosuppression, whereas in allopatric interactions the immune response is
496 activated (personal communication). Moreover, in innate immune memory process a cellular
497 immune response was observed following primo-infection and a humoral immune response
498 following secondary challenge (Pinaud, et al. 2016). Thus using *S. mansoni* infections, we
499 were able to generate different immune stresses (i.e. immunosuppression, immune cellular
500 response, humoral response) and we analyse the impacts of these stresses, experienced by
501 the host, on microbial community modifications or dynamics.

502 The first step of this analysis was to characterize the microbiota community in *Biomphalaria*
503 healthy snails. The bacterial microbiota of BgBRE has a strong temporal and inter-individual
504 stability. We were thus able to identify a core microbiota shared by all individuals. Then we
505 analysed changes in the microbiota structure and core microbiota composition following
506 infections. We demonstrate that the taxonomic diversity does not seem to change
507 following primo-infection whatever was the parasite strains used or the time point of
508 infection. A difference on the taxonomic diversity is observed exclusively following challenge
509 infection. However, infections affected the bacterial OTUs composition while the parasite
510 strains do not appear to affect differently their composition. Some phyla decrease or
511 increase after primo-infection or challenge. Some OTUs increased both after primo-infection
512 and challenge, other decreased following both also. Interestingly, for all these affected
513 OTUs, at BB25 their abundances returned to a value similar to naïve snails. It seems that we
514 have a kind of resilience of the microbiota community following the first stress of infection
515 before changing in the same manner after the second stress. The immune responses
516 associated with primary or challenge infection seemed to affect the bacterial communities.
517 Thus we try to analyse if the antimicrobial immune response could explain such microbiota
518 variation. We pay a particular attention to the antimicrobial peptides or proteins and we
519 demonstrate that solely the AMPs belonging to biomphamacin family can potentially explain
520 the impact on the microbiota communities. It has been yet demonstrated that the immune

521 system could be a key determinant of host-associated bacterial communities, and more
522 precisely that species-specific antimicrobial peptides can shape species-specific bacterial
523 associations (Franzenburg, et al. 2013). Other immune pathways have been also
524 demonstrated to regulate or control the microbiota communities. In drosophila, the
525 intestinal homeobox gene Caudal regulates the commensal-gut mutualism by repressing
526 nuclear factor kappa B-dependent antimicrobial peptide genes (Ryu, et al. 2008). Always in
527 invertebrate, the host immunity tolerates the intestinal commensal communities by
528 regulation of the pro-inflammatory and anti-inflammatory activities (Vitetta, et al. 2013).

529 Thus, if a biological stress (intruders infections) modifies the expression of the antimicrobial
530 peptide or of other immune pathways, a repercussion on the microbiota community is
531 expected.

532 Our first results, seems to indicate that we have in *Biomphalaria* the same type of regulation
533 of the bacterial communities by the immune system. Based on the hypothesis proposed by
534 Hooper (Hooper and Macpherson 2010; Hooper, et al. 2012) the immune system maintain
535 permanently the endosymbiotic microbial communities in a physiological threshold
536 compatible with their role of symbionts. Thus following an infection, the immune system is
537 diverted from its function of managing the microbiota and bacterial communities change
538 profoundly. When the immune response stopped and return to a basal level the microbiota
539 is again under-control and a resilience is observed. In this context, the microbiota
540 modification would be a consequence of the infection and of the activation of the immune
541 system against an intruder or a modification of the host physiological status. These changes
542 in the microbiota communities would have consequently strong effects or consequences on
543 the host fitness.

544 In this specific interaction between the snail *Biomphalaria glabrata* and the *Schistosoma*
545 agent of the Schistosomiasis disease, it would be interesting to see if microbiota bacterial
546 communities would have a impact on the transmission of the parasite. A decrease of the
547 vector transmission enables a control of disease expansion. A better knowledge of the
548 immune response, the microbiota composition and finally the potential tripartite
549 interactions between all actors will enable a better control of this widespread human
550 parasitic disease.

551

552

553 **Reference:**

- 554 Bouchut A, Roger E, Gourbal B, Grunau C, Coustau C, Mitta G. 2008. The compatibility polymorphism
555 in invertebrate host/trematode interactions : research of molecular determinants. *Parasite*:304-
556 309.
- 557 Broderick NA, Buchon N, Lemaitre B. 2014. Microbiota-Induced Changes in *Drosophila melanogaster*
558 Host Gene Expression and Gut Morphology Microbiota-Induced Changes in *Drosophila*
559 *melanogaster* Host Gene. *5*:1-13.
- 560 Brummel T, Ching A, Seroude L, Simon AF, Benzer S. 2004. *Drosophila* lifespan enhancement by
561 exogenous bacteria. *Proceedings of the National Academy of Sciences* 101:12974-12979.
- 562 Buchon N, Broderick NA, Chakrabarti S, Lee W-j, Buchon N, Broderick NA, Chakrabarti S, Lemaitre B.
563 2009. Invasive and indigenous microbiota impact intestinal stem cell activity through multiple
564 pathways in *Drosophila*. *Genes & Development* 23:2333-2344.
- 565 Chandler JA, Lang J, Bhatnagar S, Eisen JA, Kopp A. 2011. Bacterial communities of diverse *Drosophila*
566 species: Ecological context of a host-microbe model system. *PLoS Genetics* 7.
- 567 Chaston JM, Dobson AJ, Newell PD, Douglas AE. 2016. Host Genetic Control of the Microbiota
568 Mediates the *Drosophila* Nutritional Phenotype John. *Applied and Environmental Microbiology*
569 82:671-679.
- 570 Cirimotich CM, Dong Y, Clayton AM, Sandiford SL, Souza-Neto JA, Mulenga M, Dimopoulos G. 2011.
571 Natural microbe - mediated refractoriness to *Plasmodium* infection in *Anopheles gambiae*.
572 *Science* 332:855-858.
- 573 Clark RI, Salazar A, Yamada R, Fitz-gibbon S, Alcaraz J, Rana A, Rera M, Pellegrini M, William W,
574 Walker DW. 2015. Distinct shifts in microbiota composition during *Drosophila* aging impair
575 intestinal function and drive mortality. *Cell Rep.* 12:1656-1667.
- 576 Coustau C, Gourbal B, Duval D, Yoshino TP, Adema CM, Mitta G. 2015. Advances in gastropod
577 immunity from the study of the interaction between the snail *Biomphalaria glabrata* and its
578 parasites: A review of research progress over the last decade. *Fish & Shellfish Immunology*.
- 579 Ducklow HW, Boyle PJ, Mangel PW, Strong C, Mitchell R. 1979. Bacterial flora of the schistosome
580 vector snail *Biomphalaria glabrata*. *Applied and Environmental Microbiology* 38:667-672.
- 581 Franzenburg S, Walter J, Künzel S, Wang J, Baines JF, Bosch TCG, Fraune S. 2013. Distinct
582 antimicrobial peptide expression determines host species-specific bacterial associations.
583 *Proceedings of the National Academy of Sciences of the United States of America* 110:E3730-
584 E3738.
- 585 Futo M, Armitage SA, Kurtz J. 2015. Microbiota Plays a Role in Oral Immune Priming in *Tribolium*
586 *castaneum*. *Front Microbiol* 6:1383.
- 587 Galinier R, Roger E, Moné Y, Duval D, Portet A, Pinaud S, Chaparro C, Grunau C, Genthon C, Dubois E,
588 et al. 2017. A multistrain approach to studying the mechanisms underlying compatibility in the
589 interaction between *Biomphalaria glabrata* and *Schistosoma mansoni*. *PLOS Neglected Tropical*
590 *Diseases* 11:e0005398.
- 591 Hooper LV, Littman DR, Macpherson AJ. 2012. Interactions between the microbiota and the immune
592 system. *Science (New York, N.Y.)* 336:1268-1273.
- 593 Hooper LV, Macpherson AJ. 2010. Immune adaptations that maintain homeostasis with the intestinal
594 microbiota. *Nat Rev Immunol* 10.

- 595 Joshi D, Pan X, McFadden MJ, Bevins D, Liang X, Lu P, Thiem S, Xi Z. 2017. The Maternally Inheritable
596 *Wolbachia* wAlbB Induces Refractoriness to *Plasmodium berghei* in *Anopheles stephensi*.
597 *Frontiers in Microbiology* 8:366.
- 598 Martinson VG, Douglas AE, Jaenike J. 2017. Community structure of the gut microbiota in sympatric
599 species of wild *Drosophila*. *Ecology Letters* 20:629-639.
- 600 Mitta G, Galinier R, Tisseyre P, Allienne JF, Girerd-Chambaz Y, Guillou F, Bouchut a, Coustau C. 2005.
601 Gene discovery and expression analysis of immune-relevant genes from *Biomphalaria glabrata*
602 hemocytes. *Developmental and comparative immunology* 29:393-407.
- 603 Mitta G, Gourbal B, Grunau C, Knight M, Bridger JM, Théron A. 2017. Chapter Three – The
604 Compatibility Between *Biomphalaria glabrata* Snails and *Schistosoma mansoni*: An Increasingly
605 Complex Puzzle. *Advances in Parasitology* 97:111-145.
- 606 Moné Y, Ribou A-C, Cosseau C, Duval D, Théron A, Mitta G, Gourbal B. 2011. An example of molecular
607 co-evolution: Reactive oxygen species (ROS) and ROS scavenger levels in *Schistosoma*
608 *mansoni*/*Biomphalaria glabrata* interactions. *International Journal for Parasitology* 41:721-730.
- 609 Pinaud S, Portela J, Duval D, Nowacki FC, Olive M-A, Allienne J-F, Galinier R, Dheilly NM, Kieffer-
610 Jaquinod S, Mitta G, et al. 2016. A Shift from Cellular to Humoral Responses Contributes to
611 Innate Immune Memory in the Vector Snail *Biomphalaria glabrata*. *PLOS Pathogens*
612 12:e1005361-e1005361.
- 613 Pradeu T, Eric V. 2014. The discontinuity theory of immunity. *Sci Immunol* 1:1-9.
- 614 Ramirez JL, Souza-Neto J, Cosme RT, Rovira J, Ortiz A, Pascale JM, Dimopoulos G. 2012. Reciprocal
615 tripartite interactions between the *Aedes aegypti* midgut microbiota, innate immune system
616 and dengue virus influences vector competence. *PLoS Neglected Tropical Diseases* 6:1-11.
- 617 Rodrigues J, Brayner FA, Alves LC, Dixit R, Barillas-mury C. 2010. Hemocyte Differentiation Mediates
618 Innate Immune Memory in *Anopheles gambiae* Mosquitoes. *Science* 329:1353-1355.
- 619 Ryu J-H, Kim S-H, Lee H-Y, Bai JY, Nam Y-D, Bae J-W, Lee DG, Shin SC, Ha E-M, Lee W-J. 2008. Innate
620 Immune Homeostasis by the Homeobox Gene *Caudal* and Commensal-
621 Gut Mutualism in *Drosophila*. *Science* 319:777.
- 622 Sansone CL, Cohen J, Yasunaga A, Xu J, Osborn G, Subramanian H, Gold B, Buchon N, Cherry S. 2015.
623 Microbiota-dependent priming of antiviral intestinal immunity in *Drosophila*. *Cell Host and*
624 *Microbe* 18:571-581.
- 625 Sharon G, Segal D, Zilber-Rosenberg I, Rosenberg E. 2011. Symbiotic bacteria are responsible for diet-
626 induced mating preference in *Drosophila melanogaster*, providing support for the hologenome
627 concept of evolution. *Gut Microbes* 2:190-192.
- 628 Shin SC, Kim SH, You H, Kim B, Kim AC, Lee KA, Yoon JH, Ryu JH, Lee WJ. 2011. *Drosophila*
629 Microbiome Modulates Host Developmental and Metabolic Homeostasis via Insulin Signaling.
630 *Science* 334:670-674.
- 631 Silva TM, Melo ES, Lopes ACS, Veras DL, Duarte CR, Alves LC, Brayner FA. 2013. Characterization of
632 the bacterial microbiota of *Biomphalaria glabrata* (Say, 1818) (Mollusca: Gastropoda) from
633 Brazil. *Letters in applied microbiology* 57:19-25.
- 634 Theis KR, Dheilly NM, Klassen JL, Brucker RM, Baines JF, Bosch TCG, Cryan JF, Gilbert SF, Goodnight
635 CJ, Lloyd EA, et al. 2016. Getting the Hologenome Concept Right: an Eco-Evolutionary
636 Framework for Hosts and Their Microbiomes. *mSystems* 1:e00028-00016.

- 637 Vitetta L, Coulson S, Linnane AW, Butt H. 2013. The Gastrointestinal Microbiome and
638 Musculoskeletal Diseases: A Beneficial Role for Probiotics and Prebiotics. *Pathogens* 2:606-626.
- 639 Wong ACN, Dobson AJ, Douglas AE. 2014. Gut microbiota dictates the metabolic response of
640 *Drosophila* to diet. *Journal of Experimental Biology* 217:1894-1901.
- 641 Xi Z, Ramirez JL, Dimopoulos G. 2008. The *Aedes aegypti* toll pathway controls dengue virus infection.
642 *PLoS Pathogens* 4.
- 643 Zilber-Rosenberg I, Rosenberg E. 2008. Role of microorganisms in the evolution of animals and
644 plants: The hologenome theory of evolution. *FEMS Microbiology Reviews* 32:723-735.
- 645

Discussion

Dans le terme holobionte, en opposition avec l'interaction hôte-microbiote, nous ne distinguons pas seulement les hôtes et leurs symbiotes obligatoires, mais nous mettons aussi l'accent sur la diversité des symbioses facultatives et leurs associations dynamiques au sein d'un hôte (Theis, et al. 2016). Les variations de l'hologénome peuvent survenir non seulement par des mutations ou des recombinaisons chez l'hôte ou le microbiote mais aussi par acquisition de nouvelles souches microbiennes depuis l'environnement, un changement d'abondance microbienne ou encore grâce à des transferts de gènes horizontaux entre les micro-organismes, ou entre hôte et micro-organismes (Rosenberg and Zilber-Rosenberg 2011).

L'intérêt grandissant pour la théorie de l'hologénome au détriment des génomes amène de nouveaux questionnements. En effet, la mise en évidence de sa composition ou encore de ces potentielles variations amène à se demander comment cet hologénome évolue au cours du temps et quels sont les acteurs de son évolution. En 2008 Zilber-Rosenberg et Rosenberg proposent la théorie de l'évolution de l'hologénome. Dans ce contexte, ils considèrent l'hologénome comme une unité unique de sélection dans l'évolution (Zilber-Rosenberg and Rosenberg 2008; Rosenberg and Zilber-Rosenberg 2011). Cette théorie est basée sur 4 principes de bases à savoir : (1) Tous les animaux ou plantes établissent une relation symbiotique avec leur microbiote. (2) Les micro-organismes symbiotiques sont transmis à travers les générations. (3) L'association entre l'hôte et ses symbiotes affecte la fitness de l'holobionte dans son environnement. Et enfin (4) la variation de l'holobionte peut être provoquée par des changements à l'intérieur des génomes de l'hôte ou du microbiote ; sous l'influence d'un stress environnemental, la communauté microbienne symbiotique peut changer rapidement. Ces points suggèrent que la richesse spécifique et la diversité des divers symbiotes microbiens peut jouer un rôle important tant dans l'adaptation que dans l'évolution de leur hôte. Pendant les périodes de changements rapides dans l'environnement, la communauté diversifiée des symbioses microbiennes peut aider l'holobionte à survivre, se multiplier et ainsi laisse le temps nécessaire au génome de l'hôte pour évoluer (adaptation rapide ou acclimatation aux changements brutaux de l'environnement).

L'analyse du microbiote bactérien peut se faire de diverses manières ; il peut être caractérisé sur l'organisme entier ou uniquement sur une fraction de l'individu, la plupart du temps, c'est le compartiment intestinal qui est regardé en priorité, pour son évidente abondance en micro-organismes. Pour réaliser ces analyses, plusieurs techniques se

Chapitre 3

sont succédées au cours du temps dans le but d'être toujours plus exhaustives et permettre une meilleure compréhension de la composition holobiontique de l'individu ainsi que de son fonctionnement. Les premières caractérisations de communautés bactériennes se sont faites uniquement sur la partie bactérienne cultivable. Ensuite, c'est par séquençage des ARN ribosomiques (ARNr) 16S que les études ont pu dépasser la barrière du cultivable et identifier un plus grand nombre de bactéries composant le microbiote des individus. Finalement, avec l'avènement des techniques de séquençage à haut débit, l'identification du microbiote bactérien s'effectue par séquençage massif de la région 16S à l'échelle de l'individu ou de la population, on parle d'analyse de métabarcoding. Cette technique permet de passer outre la culture bactérienne avant une identification, mais présente aussi une profondeur de séquençage très importante, ce qui permet de mettre en évidence des genres bactériens très faiblement représentés, toujours dans le but d'une caractérisation la plus exhaustive et précise possible.

La succession de ces différentes techniques a permis de caractériser le microbiote de nombreux invertébrés majoritairement des arthropodes.

Les moustiques sont les arthropodes les plus étudiés de par leur implication dans de nombreuses maladies vectorielles. Les études du microbiote bactérien intestinal ont commencé dans les années 90, dans un premier temps sur la fraction des bactéries gram-négatifs présentes chez plusieurs espèces d'anophèles (Straif, et al. 1998). Toujours chez ce moustique, des analyses massives chez *Anopheles coluzzi* et *Anopheles gambiae* présentent de grandes variations dans la composition du microbiote des juvéniles qui se stabilisent ensuite chez les individus adultes (Gimonneau, et al. 2014). Avec l'avancé des analyses massives, nous voyons apparaître la caractérisation des microbiotes bactériens de nombreuses nouvelles espèces de moustiques ou provenant de nouvelles zones géographiques, comme c'est le cas par exemple en Inde, avec la caractérisation moléculaire du microbiote intestinal d'*Aedes albopictus* et d'*Aedes aegypti* (Yadav, et al. 2015). Un séquençage des ARNr 16S, de *Aedes aegypti* a permis de mettre en évidence une co-évolution entre le microbiote intestinal du moustique et le moustique lui-même (Terenius, et al. 2012). En effet, l'identification de bactéries dans le tube digestif de ces insectes, leur isolement et leurs transplantations chez d'autres moustiques de la même espèce montrent une mauvaise adaptation chez le nouvel hôte. Toujours chez ce même moustique, le pyroséquençage du microbiote intestinal met en évidence la présence d'un Core-microbiote stable chez tous les moustiques adultes et ce dans différentes zones géographiques (David, et al. 2016). A *contrario*, chez une autre espèce de moustique, *Culex quinquefasciatus*, une étude massive de la composition du microbiote intestinal des

Chapitre 3

femelles, a permis de mettre en évidence une très grande complexité et variation de se dernier entre les individus (Chandel, et al. 2013).

Le microbiote de la drosophile, qui est aussi un invertébré modèle, bénéficie également de nombreuses études. Dès les années 2000, l'étude de l'espérance de vie de la drosophile en milieu axénique ou non, montre que les bactéries exogènes améliorent la longévité de ces dernières (Brummel, et al. 2004). Une diète prolongée perturbe le microbiote et révèle une modification des préférences de l'accouplement des drosophiles (Sharon, et al. 2011). Au final une dérégulation du microbiote affecte de manière globale l'animal, jusqu'à un dysfonctionnement de la barrière épithéliale, protégeant des infections bactériennes, par exemple, (Clark, et al. 2015). Ce dysfonctionnement indique alors que l'homéostasie intestinale est réalisée par une inter-régulation complexe de la réponse immunitaire, du microbiote intestinal et de l'activité des cellules-souches (Buchon, et al. 2009a).

Le microbiote établit aussi une relation entre le stockage de l'énergie et la masse corporelle, ce qui témoigne d'une coordination du métabolisme avec la taille du corps, démontrant les multiples impacts du microbiote sur le métabolisme et les différentes interactions possibles selon la composition du régime alimentaire et le sexe de l'hôte (Shin, et al. 2011; Wong, et al. 2014). Il est important de noter que le microbiote a des effets de grande envergure sur la physiologie de l'hôte, allant de l'immunité à la structure intestinale (Broderick, et al. 2014). En populations naturelles, une différence dans la richesse et la composition des communautés bactériennes est observée entre les différents sites de récolte des populations hôtes (Corby-Harris, et al. 2007; Wong, et al. 2013). Malgré la diversité taxonomique, écologique et géographique des hôtes, les taxons bactériens dominants sont répandus et se retrouvent dans de nombreuses espèces (Chandler, et al. 2011). Il semblerait que le régime alimentaire joue un rôle majeur dans l'acquisition et la structuration du microbiote bactérien de la drosophile, dont la composition reste tout de même réduite par rapport aux communautés bactériennes externes présentes dans leur milieu de vie, ce qui suggère un contrôle de la composition par l'hôte (Chandler, et al. 2011).

Finalement lorsque l'on compare des souches de laboratoires et des souches naturelles, la composition du microbiote bactérien semble très différente (Chandler, et al. 2011). Lorsque l'on étudie les effets des sources alimentaires et des espèces hôtes sur ces communautés, ces facteurs n'ont pas d'effet en laboratoire, mais affectent fortement le microbiote des drosophiles naturelles (Staubach, et al. 2013). L'abondance du microbiote résident est influencée par le génotype de l'hôte, démontrant que le contrôle génétique

Chapitre 3

de ce dernier contribue à la relation avec son microbiote (Chaston, et al. 2016; Martinson, et al. 2017).

Toujours chez les Arthropodes, plusieurs études ont été réalisées sur les *Malacostracae*. L'hémolymphe de nombreux invertébrés apparaît comme non-stérile et composée d'une communauté microbienne hémolympatique. L'équipe de Wang qui travaille chez des crustacés ont montré que ces communautés, étaient endémiques et présentes chez des animaux en bonne santé (Wang and Wang 2015). Ces études montrent aussi que la prolifération de ce microbiote particulier est régulée par des C-type lectines et l'expression de peptides anti-microbiens (AMP) (Wang, et al. 2014; Wang and Wang 2015) par ailleurs, la caractérisation de la composition du microbiote bactérien du cloporte, *Armadillidium vulgare* ; par pyroséquençage des ARNr 16S d'individus provenant de lignées de laboratoire et de terrain, a permis de révéler une diversité bactérienne élevée. Ils démontrent chez ce modèle des communautés bactériennes distinctes entre les individus des différentes populations, ce qui indique un lien étroit entre le microbiote associé à l'hôte et les bactéries environnementales, probablement en raison de son écologie nutritionnelle (Dittmer, et al. 2016). En 2017, le microbiote intestinal de 5 espèces de puces de mer (amphipodes, *Talitridae*) fut caractérisé par métabarcoding des ARNr 16S, et indique une spécificité des communautés bactériennes aux différentes espèces. Cette diversité de microbiote bactérien pourrait refléter en partie les différents micro-habitats et les préférences alimentaires des hôtes (Abdelrhman, et al. 2017). Il ne faut pas oublier que le fond génétique des hôtes ainsi que leur capacité de contrôle du système immunitaire peuvent aussi influencer la diversité du microbiote bactérien.

Chez *Biomphalaria glabrata*, des études du microbiote bactérien cultivable ont déjà été réalisées (Ducklow, et al. 1979; Silva, et al. 2013), mais nous proposons aujourd'hui une étude plus exhaustive de ce microbiote bactérien par métabarcoding 16S ainsi que la caractérisation de son microbiote viral.

Nous rapportons pour la première fois la présence de virus résidents chez *Biomphalaria sp.* (Galinier, Tetreau, et al. 2017). En effet, à partir de données de transcriptomique massive, nous avons pu mettre en évidence 5 virus résidents dans différentes souches de mollusques, nommés BV1 à BV5 (fig. 10). Parmi eux, quatre appartiennent à la super-famille de picorna-like virus, des virus ARN simple-brin polyadénylés, tandis que le cinquième est proche de la famille des Totiviridae, des virus ARN double-brins non-polyadénylés. Ces résultats étant soutenus par une analyse phylogénétique basée sur les séquences de protéines polymérase dépendante de l'ARN

Chapitre 3

(RdRP) et hélicase. La distribution et l'abondance de ces virus varient fortement entre les 5 souches de mollusques étudiés (fig. 10). En effet, nous avons analysé la présence de ces virus dans 4 souches de *B. glabrata* provenant d'origines géographiques différentes (de Belo-Horizonte au Brésil (BgBAR), de Recife au Brésil (BgBRE), de Guacara au Venezuela (BgVEN) et enfin de Lamentin en Guadeloupe (BgGUA)) ainsi que chez une souche de *B. pfeifferi* provenant du Sultanat Oman (BpOMAN). L'analyse de la distribution (fig. 10) montre que seul BV1 est présent dans les 5 souches de mollusques. Les quatre autres virus ne sont pas répartis de manière égale dans toutes les souches. Certains d'entre eux, tels que BV2 et BV3, semblent avoir une origine géographique spécifique. En effet, nous pouvons imaginer que BV2 ait une origine africaine et du sud de l'Amérique latine quand BV3 proviendrait plutôt du nord de l'Amérique latine. Tandis que BV4 et BV5, pourraient être spécifiques soit du continent africain, soit uniquement de l'espèce *B. pfeifferi*. Si l'on s'intéresse à l'abondance de ces différentes virus à travers les différents souches de mollusques, BV1 apparaît clairement le virus le plus abondant chez *B. glabrata*, alors qu'il s'agit de BV4 chez *B. pfeifferi*.

Il faut maintenant aller plus loin dans la caractérisation de ce microbiote viral, des travaux futurs devront étudier la prévalence virale au niveau individuel, mais aussi dans les populations sur le terrain. De plus, le rôle joué par ces virus sur l'homéostasie des mollusques doit également être étudié et plus particulièrement lors de l'infection par le parasite *S. mansoni* pour savoir si la présence de ces virus pourrait avoir une quelconque valeur adaptative pour l'hôte.

À la suite de cette étude nous avons caractérisé pour la première fois le microbiote bactérien de *B. glabrata* de manière exhaustive ainsi que l'influence du stress immunitaire causé par l'infection de *B. glabrata* par *S. mansoni* sur les communautés bactériennes, à l'échelle individuelle, par à une approche de séquençage métabarcoding 16S (fig. 10). Dans un premier temps nous avons mis en évidence le microbiote bactérien de *B. glabrata* brésilien (BgBRE) à l'état naïf et établi la partie de ce dernier correspondant au Core-microbiote, c'est à dire à la proportion du microbiote commun et stable entre l'ensemble des individus d'une population (fig. 10). Nous avons pu mettre en évidence que le microbiote bactérien de BgBRE était relativement stable au cours du temps. En effet, des mollusques naïfs prélevés du milieu d'élevage avec 25 jours de décalage présente un microbiote bactérien extrêmement similaire.

Par la suite nous avons étudié la perturbation et les modifications du microbiote engendrées au cours d'infections par *S. mansoni* (fig. 10). Des études immunitaires

Chapitre 3

réalisées chez *B. glabrata*, montre des stratégies immunitaires différentes de la part du mollusque selon l'origine des parasites utilisés. En effet, lors d'une infection sympatrique, nous observons une forte immunosuppression de la réponse immunitaire du mollusque, alors que lors d'une infection allopatrique, c'est une activation de cette réponse immunitaire qui est observée (Portet *et al.* soumis - chapitre 1). De plus, lorsque le mollusque est infecté une première fois puis ré-infecté une seconde fois par le même parasite, les processus immunitaires ne sont pas du tout les mêmes. En effet, suite à une primo-infection, une réponse immunitaire cellulaire est observée alors que lors de la seconde infection c'est une réponse immunitaire humorale qui est activée (Pinaud, *et al.* 2016). Dans ce contexte, nous étudions l'influence de ces différentes réponses immunitaires sur la dynamique du microbiote de BgBRE. Pour cela, nous avons infecté BgBRE avec deux souches parasitaires, son parasite sympatrique, *Schistosoma mansoni* brésilien (SmBRE) et un parasite allopatrique, *Schistosoma mansoni* du Venezuela (SmVEN), et ce au cours de la cinétique comprenant une primo-infection ainsi qu'un challenge homologue et hétérologue.

Lors de la primo-infection, nous n'observons pas de différence significative de nombre d'OTU observés, de leur richesse ou encore dans leur répartition en comparaison avec les individus naïfs. Par contre lors de la ré-infection, nous observons une baisse de la diversité globale du microbiote. Il semblerait que ce soit l'accumulation d'infection qui finisse par affecter les communautés bactériennes de l'hôte. Par ailleurs, quelque soit le parasite utilisé, le même effet de perturbation du microbiote est observé, l'origine sympatrique ou allopatrique du parasite ne semble pas impacter différemment le microbiote différemment. Il faut tout de même noter que le microbiote bactérien tend à retourner à la normale 25 jours après la première infection (fig. 10).

En parallèle, nous avons aussi étudié la réponse immunitaire anti-microbienne du mollusque en utilisant une approche de transcriptomique massive (RNAseq). Des études réalisées chez d'autres invertébrés, comme par exemple chez les crustacés montre une régulation des communautés bactériennes hémolympatiques par le système immunitaire de l'hôte grâce à des C-type lectines ou des AMP (Wang, *et al.* 2014; Wang and Wang 2015). Chez *Biomphalaria glabrata*, les C-types lectines ne présentaient pas de variations d'expression associées aux modifications des communautés bactériennes. Nous nous sommes alors focalisés sur les protéines déjà identifiées comme anti-microbienne, à savoir les LBP/BPI, les achacines ainsi que l'unique famille d'AMP présent chez *B. glabrata* les biomphamacines. Les résultats montrent que les biomphamacines sont les

Chapitre 3

seules protéines anti-microbiennes étudiées à présenter une corrélation entre leur expression et les différentes modifications du microbiotes (**fig. 10**). En effet, parmi les 6, certaines sont sur-exprimées lors des différentes primo-infections, puis leur expression revient à un niveau basal à 25 jours et enfin suite aux différents challenges, une sur-expression est à nouveau observable. Il semblerait que seule cette catégorie de molécules anti-microbiennes jouent un rôle dans la régulation du microbiote.

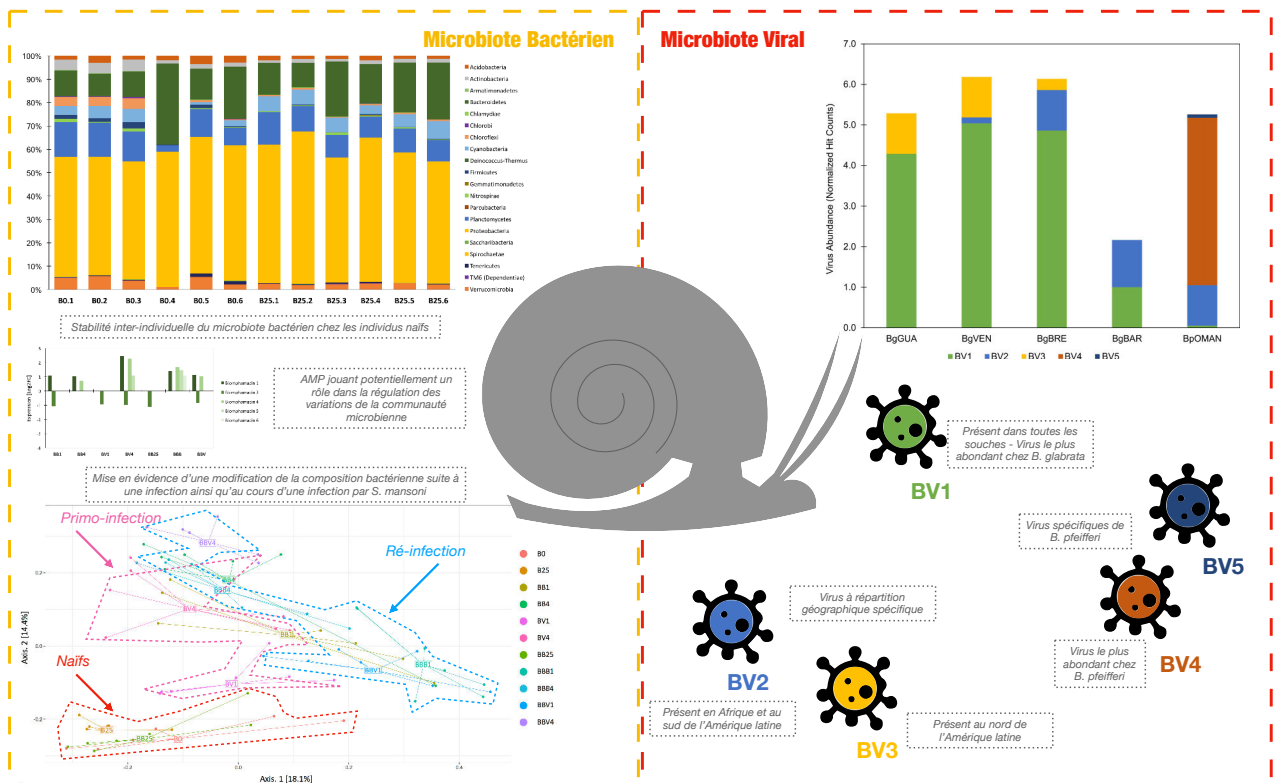


Figure 10 : Caractérisation du microbiote et étude de son influence sur l'interaction *Biomphalaria* et *Schistosoma*

Figure adaptée d'après PORTET et al. en préparation et (Galnier, Tetreau, et al. 2017). Synthèse des résultats de la caractérisation du microbiote bactérien chez les individus naïfs et évolution du microbiote au cours des différentes infections. A noter que le groupe naïf comprend des individus naïfs récupérés à deux temps au cours de l'expérience ($t=0$ au début des expériences et $t=25$ jours correspondant à la ré-infection des mollusques parasites), ainsi que des mollusques infectés depuis 25 jours présentant des caractéristiques et compositions du microbiote proche des individus naïfs. Synthèse des résultats de la caractérisation du microbiote viral. Identification de 5 virus résidents présentant une répartition et des abondances variables entre les souches de mollusques étudiées.

Maintenant pour aller plus loin dans la mise en évidence du microbiote, des études de sa caractérisation doivent être réalisées chez des mollusques présentant des niveaux de compatibilité différents envers *Schistosoma sp.*. Ces dernières permettront de mettre en évidence s'il existe une spécificité d'association mollusque-microbiote en fonction du phénotype de compatibilité vis à vis du parasite. Pour cela différentes souches de mollusques présentant des niveaux de compatibilités différents avec le parasite devront

Chapitre 3

être utilisées, et ce dans le but de mettre en évidence à la fois leur microbiote bactérien et leur microbiote viral. Des études similaires ont été réalisées chez *Aedes sp.* et montrent une spécificité du microbiote intestinal au niveau des espèces (Abdelrhman, et al. 2017). Quant est-il pour des souches d'une même espèce ?

Une fois les microbiotes des différentes souches mis en évidence, une étude de la dynamique de ces communautés microbiennes au cours de l'infection parasitaire sera nécessaire, dans le but de mettre en évidence si le polymorphisme de compatibilité influence les modifications de ce dernier.

Finalement, le microbiote pourrait être utilisé pour étudier une potentielle plasticité phénotypique du mollusque vis-à-vis de son parasite. Pour cela, un transfert de microbiote pourrait être réalisé entre des mollusques présentant, par exemple, différentes compatibilités vis-à-vis du parasite. Une modification de la compatibilité suite à un transfert de microbiote permettrait de mettre en évidence un lien direct entre capacité immunitaire et microbiote chez le mollusque. Il faut tout de même noter que des expériences de transfert de microbiote ont déjà été réalisées chez des moustiques, et montrent une co-évolution entre l'hôte et ses communautés bactériennes associées (terenius 2012). En effet, le microbiote transféré présente une mauvaise adaptation à son nouvel hôte. On pourrait également perturber le microbiote en utilisant des antiviraux ou des antimicrobiens afin de perturber la communauté microbienne et étudier les phénotypes de compatibilité chez ces individus dont le microbiote est déstabilisé.

À ce jour toutes les études que nous avons réalisées sur le microbiote de *Biomphalaria* prennent en compte le mollusque dans sa globalité. Néanmoins, plusieurs communautés microbiennes peuvent être différenciées dans un organisme. En effet, chez certains invertébrés, une distinction peut être faite entre le microbiote intestinal, le plus abondant, le microbiote épithélial ou de surface et enfin le microbiote associé à l'hémolymphe, aussi appelé hémomicrobiote. En effet, des études réalisées sur des organismes tels que les crustacés (Wang, et al. 2014; Wang and Wang 2015) montrent une communauté bactérienne hémolympatique stable.

Chez *Biomphalaria glabrata*, nous avons réalisés des études préliminaires de cultures en milieux solides et de séquençage ainsi que d'amplifications 16S par PCR des bactéries présentes dans l'hémolymphe (*données non montrées*). Les résultats suggèrent que nous n'avons pas de communauté bactérienne installée dans l'hémolymphe chez *Biomphalaria glabrata*.

Chapitre 3

Pour finir toutes ces études sont réalisées sur des souches de laboratoire. Les mollusques sont maintenus au laboratoire selon des techniques qui permettent de conserver au mieux les diversités génétiques de chaque souche, en milieu confiné. En effet, chaque souche de mollusque est élevée indépendamment et les différentes souches ne se croisent jamais. Les cycles parasitaires se font toujours en interactions sympatriques. Mais les mollusques ne sont jamais soumis aux modifications de l'environnement, comme c'est le cas en milieu naturel, avec par exemple l'alternance des saisons qui entraîne des épisodes de pluies plus ou moins intenses et fréquents, ou de sécheresses, ce qui pourrait modifier les composantes du milieu tels que le pH ou la température de l'eau (au moins en surface). Ces différentes modifications environnementales pourraient entraîner des variations du microbiote, dont on sait que la composition est influencée par le milieu, comme c'est le cas chez la drosophile (Chandler, et al. 2011; Staubach, et al. 2013). En effet, le régime alimentaire (Staubach, et al. 2013) et la composition bactérienne du milieu (Chandler, et al. 2011) influencent le microbiote de la drosophile. Ces variations ne sont pas prises en compte dans nos études et ce qui pourrait apporter un biais dans les conclusions que nous pourrions faire sur l'association entre le microbiote du mollusque et sa compatibilité.

Pour cela, dans un premier temps, l'étude de souches de laboratoires permet de contrôler certains paramètres plus facilement et de limiter les variables pouvant influencer les résultats et ainsi compliquer l'analyse de ces derniers. Mais à la suite de ces expériences sur des souches de laboratoires, des études sur des souches provenant de milieux naturels seront essentielles pour confirmer les hypothèses apportées notamment sur l'impact du microbiote sur le polymorphisme de compatibilité et par conséquent sur l'interaction immunitaire entre *B. glabrata* et *S. mansoni*. Ceci est une étape essentielle afin de savoir si l'approche holobiontique des interactions pourrait être une nouvelle et prometteuse voie de recherche dans le développement de nouvelles stratégies de lutte et/ou de contrôle de la bilharziose sur le terrain.

Discussion

Discussion

L'objectif de ce travail de thèse s'intègre dans un projet plus large de la compréhension de l'interaction hôte / parasite, et plus particulièrement au niveau immunologique, dans notre modèle d'étude impliquant l'escargot d'eau douce *Biomphalaria glabrata* et le trématode parasite *Schistosoma mansoni*.

Ce modèle d'étude présente un intérêt à la fois dans un but de recherche fondamentale mais aussi de recherche appliquée. En effet, l'interaction entre le mollusque et le parasite présente un large éventail de compatibilités, certains couples hôte / parasite seront compatibles, c'est à dire que le parasite pourra se développer dans son hôte, mais d'autres seront dit incompatibles, cette fois-ci le mollusque éliminera le parasite. Ce large éventail de compatibilité est appelé le polymorphisme de compatible (Langand and Morand 1998). Ce polymorphisme de compatibilité peut être observé tant au niveau populationnel qu'individuel (Webster and Davies 2001; Théron, et al. 2005). De plus on observe un large continuum de compatibilité entre les souches de mollusque et de parasite, lorsqu'on réalise des croisements expérimentaux en laboratoire (Theron, et al. 2014). Ces résultats mettent en évidence, non seulement de l'adaptation locale entre le mollusque et son parasite durant laquelle le mollusque développera de nouveaux outils pour répondre aux pressions de sélections de son environnement et le parasite développera lui, des outils, pour conserver ses capacités d'infections de l'hôte et pourvoir continuer son cycle de vie, « courir pour rester à la même place », ce phénomène est soutenu par la théorie de « la reine rouge » (Van Valen 1974). De plus, le polymorphisme de compatibilité repose sur une concordance des déterminants moléculaires clés dans l'interaction entre le mollusque et le parasite il s'agit de l'hypothèse des "matching molecular variants" (Théron, et al. 2005). Ce modèle d'étude présente aussi un intérêt appliqué, car en effet *B. glabrata* est l'hôte intermédiaire de *S. mansoni*, trématode parasite agent responsable de la bilharziose, seconde endémie parasitaire humaine mondiale derrière le paludisme. Cette maladie tropicale négligée touche 240 millions de personnes à travers le monde et est responsable de 200 000 morts par an (King, et al. 2005). Reconnu comme un problème de santé publique, l'OMS souhaite trouver des moyens de l'éradiquer, pour cela, une meilleure compréhension de l'interaction entre l'hôte intermédiaire et l'agent pathogène pourrait ouvrir la voie à de nouvelles stratégies de lutte. En effet, des nouveaux outils de médiations vectorielles pourraient être développés, afin de limiter l'infection des mollusques comme c'est déjà le cas chez les

Discussion

insectes contre moustique dans le but d'éradiquer la malaria ou la punaise pour éliminer la maladie de Chagas (Iturbe-Ormaetxe, et al. 2011; Taracena, et al. 2015; Tennessen, et al. 2015).

Dans ce contexte, le but de cette thèse est de décortiquer le phénomène du polymorphisme de compatibilité dans l'interaction entre les deux protagonistes. Pour cela, plusieurs approches ont été utilisées. Nous avons cherché à mettre en évidence les bases moléculaires de ce phénomène, puis essayé d'aller plus loin dans la caractérisation de molécules immunitaires du mollusque impliquées dans la reconnaissance des pathogènes et finalement nous avons voulu caractériser le microbiote associé à l'hôte qui pourrait lui aussi contribuer au polymorphisme de compatibilité et à l'issue de l'interaction existante entre *B. glabrata* et son parasite *S. mansoni*.

La relation entre *B. glabrata* et *S. mansoni* présente un haut niveau de complexité. Un complexe immun entre les deux protagonistes a pu être identifié au sein du laboratoire, il implique les FREP et les SmPoMuc de parasite (Moné, et al. 2010), ce qui démontre un réel dialogue moléculaire entre l'hôte et son parasite. Nous avons cherché à mettre en évidence l'influence de ce dialogue moléculaire dans la compatibilité entre les souches de mollusques et de parasites venant de différentes localités géographiques. Pour cela des études génomiques, transcriptomiques, protéomiques et épigénétiques ont permis une meilleure compréhension des deux catégories de molécules (chapitre 1 - publication 1). Ces études ont été réalisées sur un panel de souche de mollusques et de parasite présentant des niveaux de compatibilités différents. Chez les SmPoMuc, les résultats mettent en évidence qu'une grande diversité de mucines couplée à une faible expression semble être la stratégie adoptée par les souches les plus infectieuses. Ces résultats mettent en évidence des stratégies d'évitement et de contournement de la réponse immunitaire du mollusque par le parasite (chapitre 1 - publication 2). En parallèle des études de transcriptomiques ont permis de mettre en évidence que la souche de mollusque la moins susceptible présentait non seulement un plus grand nombre de variants immunitaires mais aussi une forte expression des FREP (chapitre 1 - publication 2). Chez le mollusque, la stratégie semble plutôt se tourner vers une forte expression des FREP, les diversifications de ces dernières n'étant pas clairement corrélées avec une meilleure capacité de reconnaissance. Ceci est un résultat particulièrement inattendu; en effet, la diversification de ces récepteurs immuns était supposée permettre une meilleure reconnaissance des pathogènes. Dans notre modèle il ne semble pas que ce soit le cas, puisque le mollusque BgBAR qui est le plus incompatible avec l'ensemble des souches

Discussion

de Schistosome est celui qui exprime la plus faible diversité de FREPs. Toutefois, l'ensemble de ces résultats mettent en évidence que le complexe immunitaire FREP/SmPoMuc pourrait jouer un rôle clé dans le polymorphisme de compatibilité entre *B. glabrata* et *S. mansoni*. En parallèle de ces études sur le continuum de compatibilité entre les mollusques et les parasites, dans le contexte évolutif entre les hôtes et les parasites, nous nous sommes intéressés aux interactions sympatriques et allopatriques entre les deux partenaires (chapitre 1 - publication 3). En effet, lors des études précédentes sur l'interaction immunitaire entre le mollusque et le parasite, une souche compatible et une souche incompatible étaient sélectionnées dans le but d'observer les réponses extrêmes et donc de mettre en évidence des mécanismes de compatibilité ou d'incompatibilité. Cependant, dans le large panel de souche dont nous disposons au laboratoire, nous avons des interactions sympatriques et compatibles mais aussi allopatriques et compatibles. Dans ce contexte, nous avons cherché à mettre en évidence une différence dans la réponse immunitaire entre une interaction reposant sur une histoire évolutive courte (500 ans d'évolution sympatrique en milieu naturel) et une première rencontre dans un contexte d'interaction allopatrique (chapitre 1 - publication 3). Il apparaît que lors d'une interaction sympatrique une forte immunosuppression réprime toute la réponse immunitaire du mollusque alors qu'en allopatrie le mollusque répond activement à l'infection. Ces résultats mettent en évidence un phénomène d'adaptation locale entre le mollusque et le parasite. Cependant, cette adaptation locale n'est pas observable au niveau phénotypique en ce qui concerne la prévalence et l'intensité parasitaire car les deux interactions sont au final compatibles, le parasite continuera son développement dans les tissus du mollusque. Cet aspect de l'adaptation locale est plutôt surprenant, nous pouvons imaginer que les capacités intrinsèques du parasite lui permettent de finalement contrer la réponse immunitaire du mollusque et se développer. Ces résultats montrent l'importance d'approcher les concepts évolutifs à différentes échelles, pour mettre en évidence des phénomènes évolutifs tels que l'adaptation locale dans une vision intégrative des interactions.

Malgré l'importance du complexe immunitaire FREP/SmPoMuc dans l'interaction immunitaire et le polymorphisme de compatibilité entre *B. glabrata* et *S. mansoni*, celui-ci n'explique pas à lui seul l'intégralité du dialogue moléculaire. En effet, à la suite de cette découverte des expériences d'inactivations fonctionnelles, ont permis de démontrer que les FREP étaient bien impliquées dans l'incompatibilité du mollusque, une baisse de la prévalence est observée suite à l'inactivation de ces lectines (Hanington, et al. 2012; Pinaud, et al. 2016). Mais ces études révèlent aussi que ce ne sont pas les seules

Discussion

molécules impliquées dans la réponse immunitaire du mollusque. En effet, ces invalidations entraînent des changements de phénotypes des mollusques, qui passent d'incompatibles à seulement partiellement compatibles (Hanington, et al. 2012; Pinaud, et al. 2016). Des études plus exhaustives des molécules immunitaires de mollusque sont donc nécessaires. Dans le cadre de ma thèse, nous nous sommes donc intéressés à une molécule mise en évidence dans le complexe FREP/SmPoMuc et impliquée dans la réponse immunitaire chez d'autres invertébrés, la BgTEP. Depuis sa mise en évidence en 2010 (Moné, et al. 2010), nous avons cherché à mieux la caractériser afin de comprendre son fonctionnement ainsi que son implication dans la réponse immunitaire du mollusque (chapitre 2 - publication 1). La BgTEP présente une structure tridimensionnelle très similaire de l'AgTEP1, de plus, elle semble aussi jouer un rôle d'opsonine comme c'est le cas chez le moustique. Cependant des études réalisées dans les années 90 sur l'interaction entre le mollusque et le parasite ont mis en évidence une molécule possédant un domaine α -2-macroglobuline présentant une activité anti-protéase contre le schistosome, qui s'apparenterait à la BgTEP identifiée en 2010 (Bender and Bayne 1996). D'après nos résultats, nous pouvons émettre l'hypothèse d'une bi-fonctionnalité de la molécule, rôle d'opsonine, elle guide alors la phagocytose et l'encapsulation des pathogènes, mais également un rôle actif de lyse des pathogènes métazoaires. En parallèle de cette étude sur une molécule ciblée, une approche de protéomique a permis de mettre en évidence de manière globale les molécules impliquées dans la reconnaissance de divers pathogènes suite à des interactions expérimentales proches des conditions réelles d'interactions (chapitre 2 - publication 2). Ces résultats démontrent une complexité de la reconnaissance immunitaire, reposant sur des molécules spécifiquement impliquées en réponse à un type de pathogène et d'autres communes à tous les pathogènes.

Le polymorphisme de compatibilité qui régit l'interaction entre *B. glabrata* et *S. mansoni* peut être influencée par d'autres facteurs que l'immunité. En effet, des facteurs abiotiques comme la température du milieu jouent un rôle sur la compatibilité des mollusques (Knight, et al. 2015), mais des facteurs biotiques peuvent aussi jouer un rôle dans l'interaction hôte / parasite. Pendant ma thèse nous avons décidé dans un premier temps, de caractériser le microbiote de *B. glabrata*, puis de regarder l'influence d'un stress comme une infection parasitaire sur les communautés microbiennes associées au mollusque (chapitre 3). Ces études ont permis de démontrer la présence de cinq virus à ARN résidents dans les mollusques, présentant une abondance et une répartition différentes entre les souches étudiées (chapitre 3 - publication 1). En parallèle le

Discussion

microbiote bactérien du mollusque révèle une stabilité inter-individuelle mais aussi dans le temps, mais présente une baisse de sa diversification et de sa richesse suite aux infections parasitaires. De plus, l'expression de la seule famille d'AMP présentent chez *B. glabrata* (Adema, et al. 2017), semble corrélée aux modifications observées sur le microbiote (chapitre 3 - publication 2). Ces premiers résultats ne nous permettent pas de mettre en évidence un rôle direct du microbiote, cependant nous pouvons émettre des hypothèses quant à l'influence d'un stress sur les communautés bactériennes associées. De plus, nous ne pouvons pas non plus préciser la cause et la conséquence de ces variations de communautés microbiennes et d'expression d'AMP.

En effet, plusieurs théories sur le rôle du système immunitaire sur le microbiote ont été avancées cette dernière décennie, deux grandes pensées s'affrontent. La première, formulée en 2010, présente un rôle constant du système immunitaire de l'hôte dans le maintien du microbiote (Hooper, et al. 2012). En 2014, selon la théorie de la discontinuité décrite dans les systèmes vertébrés, le système immunitaire répond aux changements brutaux suite à une stimulation antigénique mais il est rendu tolérant par des stimulations faibles et continues, auxquelles nous pourrions apparenter le microbiote (Pradeu and Eric 2014). Dans un cas nous avons donc un maintien du microbiote et dans l'autre plutôt une tolérance. Au moment d'une infection nous pouvons imaginer que le maintien du microbiote par le système immunitaire de l'hôte ne se fera plus correctement, en conséquence le microbiote ne sera plus régulé et de forts changements de communautés pourront être observés. Dans ce contexte, les modifications du microbiote seront donc une conséquence de l'infection parasitaire et de la réponse de l'hôte. *A contrario*, si nous avons une tolérance de microbiote par le système immunitaire, une infection par un pathogène ne devrait pas entraîner de modification de ce dernier, à moins qu'il ne participe lui-même à la défense de l'holobionte contre l'intrus. En effet, une relation tripartite entre l'hôte, son microbiote et le pathogène est possible, et a déjà été démontrée dans des modèles invertébrés (Xi, et al. 2008; Sansone, et al. 2015; Joshi, et al. 2017).

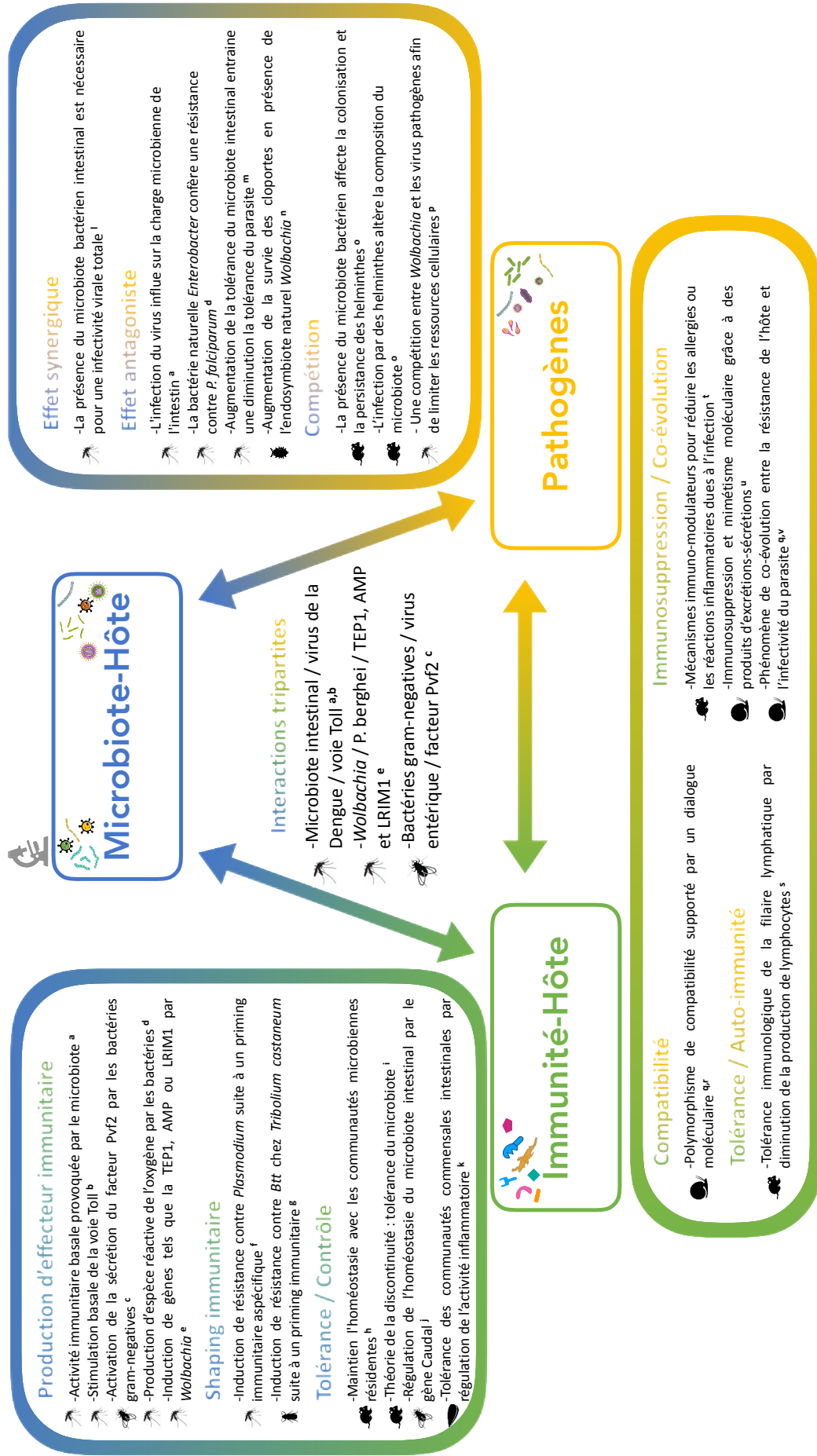
La relation tripartite entre le système immunitaire de l'hôte, son microbiote et les pathogènes, déjà mise en évidence chez les invertébrés présente une grande complexité (**fig. 11**). En effet, chacune des trois entités peut avoir une influence sur les autres de manière singulière et réciproquement.

Discussion

Entre le microbiote et l'immunité de l'hôte, trois grandes interactions peuvent être observées (fig. 11). Le microbiote peut influencer la production d'effecteurs d'immunitaires qui participeront ensuite à la réponse immunitaire de l'hôte. Dans ce contexte là nous retrouvons chez *Aedes sp.* une stimulation basale de l'activité immunitaire (Ramirez, et al. 2012), notamment de la voie Toll (Xi, et al. 2008). Chez *Anopheles sp.* une production d'espèce réactive de l'oxygène est directement réalisée par les bactéries du microbiote intestinal (Cirimotich, et al. 2011). Finalement *Wolbachia*, bactérie endosymbiotique de nombreux arthropodes, induit chez *Anopheles sp.* l'expression de gènes immunitaires tels que la TEP, LRIM1 ou encore la défensine1 (Joshi, et al. 2017). Le microbiote peut aussi influencer la réponse immunitaire en la préparant à mieux répondre à de futures attaques, on parle de shaping immunitaire. Chez *Anopheles sp.* le microbiote intestinal est capable de réaliser un priming aspécifique basé sur la différenciation cellulaire naturellement induite et observée lorsque *Plasmodium sp.* franchi la barrière épithéliale de l'intestin (Rodrigues, et al. 2010). Un autre exemple, chez *Tribolium castaneum* le microbiote intestinal est essentiel dans la défense contre *Bacillus thuringiensis* *bv. tenebrionis* (Futo, et al. 2016).

Enfin le système immunitaire peut tolérer ou contrôler le microbiote, chez les vertébrés deux théories s'opposent, le maintien de l'homéostasie entre l'organisme et ses communautés bactériennes résidentes nécessite un contrôle par le système immunitaire (Hooper, et al. 2012). Dans le cadre de la théorie de la discontinuité, le microbiote de l'organisme peut être toléré par le système immunitaire, car toujours présent à bas bruit (Pradeu and Eric 2014). Des exemples de tolérance et de contrôle ont été mis en évidence chez les invertébrés. Chez la drosophile, un gène homéotique intestinal, Caudal, régule le mutualisme des communautés commensales de l'intestin en réprimant l'expression de facteurs de transcription essentiel dans la production d'AMP (Ryu, et al. 2008). Un cas de tolérance des communautés commensales intestinales par le système immunitaire est observé chez les moules, par régulation de l'activité inflammatoire du système immunitaire (Vitetta, et al. 2013). Dans ce cadre-là, le microbiote aura un impact indirect sur le pathogène, par l'intermédiaire du système immunitaire de l'hôte.

Entre le microbiote et les pathogènes, trois grandes interactions peuvent aussi être observées (fig. 11). Un effet synergique peut avoir lieu entre le microbiote et le pathogène, comme c'est le cas lors d'une infection d'*Aedes sp.* par le virus de la dengue, ici la flore microbienne assure une meilleure infectivité (Carissimo, et al. 2015).



Discussion

Figure 11 : Relation tripartite entre l'immunité de l'hôte son microbiote et les pathogènes

Représentation de grandes influences de chacune des trois entités sur les deux autres. Chaque lettre correspond à la référence de la publication. a (Ramirez, et al. 2012), b (Xi, et al. 2008), c (Sansone, et al. 2015), d (Cirimotich, et al. 2011), e (Joshi, et al. 2017), f (Rodrigues, et al. 2010), g (Futo, et al. 2016), h (Hooper, et al. 2012), i (Pradeu and Eric 2014), j (Ryu, et al. 2008), k (Vitetta, et al. 2013), l (Carissimo, et al. 2015), m (Habtewold, et al. 2017), n (Braquart-Varnier, et al. 2015), o (Reynolds, et al. 2015), p (Moreira, et al. 2009), q (Galinier, Roger, et al. 2017), r (Portet, et al. 2017), s (King, et al. 1992), t (Maizels and McSorley 2016), u (Guillou, et al. 2007), v (Moné, et al. 2010).

Le microbiote peut également avoir un effet antagoniste sur les pathogènes, comme c'est le cas chez *Anopheles sp.*, une augmentation de la tolérance du microbiote par le système immunitaire entraîne une diminution de la tolérance de l'infection par *Plasmodium sp.* (Habtewold, et al. 2017). De plus, toujours chez *Anopheles gambiae*, *Enterobacter*, naturellement présente chez le moustique, confère une résistance à l'infection par *Plasmodium sp.* en jouant un rôle sur son développement (Cirimotich, et al. 2011). De plus, la présence de *Wolbachia* chez *Armadillidium vulgare* a un effet bénéfique sur la survie de ce dernier au moment d'une infection par des bactéries intracellulaires (Braquart-Varnier, et al. 2015).

Mais le pathogène peut aussi jouer un rôle antagoniste sur le microbiote, lors d'une infection de *Aedes sp.* par le virus de la dengue, le pathogène déclenche une réponse immunitaire qui entraîne une diminution de la charge microbienne chez l'hôte (Ramirez, et al. 2012). Finalement, une compétition peut être observée entre le microbiote et le pathogène. Chez les vertébrés, lors d'une infection par des parasites helminthiques, on observe une altération de la composition du microbiote intestinale ce qui affectera ensuite la colonisation et la persistance des parasites dans le tubes digestifs de leur hôte (Reynolds, et al. 2015). Chez les invertébrés, de la compétition a aussi pu être mise en évidence, chez *Anopheles sp.*, chez qui l'endosymbiote *Wolbachia* a la capacité de limiter les ressources requises pour le développement des virus pathogènes (Moreira, et al. 2009). Le microbiote ou le pathogène peuvent donc avoir une influence directe, par des effets antagonistes, synergiques ou même de compétitions mais cette dernière peut aussi être indirecte selon les cas.

Entre le système immunitaire et les pathogènes, trois grandes interactions peuvent aussi être observées (fig. 11).

Finalement, une relation directe entre l'immunité de l'hôte, son microbiote et le pathogène fut mis en évidence chez les invertébrés, que l'on appelle relation tripartite (fig. 11). En effet chez *Aedes sp.* l'implication du microbiote intestinal dans la stimulation de l'immunité basale notamment de la voie Toll permet une meilleure réponse contre le virus

Discussion

de la dengue (Xi, et al. 2008; Ramirez, et al. 2012). Chez *Anopheles sp.* les *Wolbachia* intestinale active des molécules immunitaires telles que la TEP1, LRIM1 ou encore des AMP, ce qui permet une meilleure résistance contre *Plasmodium berghei* (Joshi, et al. 2017). Finalement chez la drosophile aussi une relation tripartite fut mise en évidence entre les bactéries intestinales gram-négatifs qui activent la transcription de facteur tel que Pvf2 ce qui entraîne une meilleure résistance contre les virus entériques. Ces trois exemples montrent une action du microbiote contre le pathogène mais de manière indirecte en passant toujours par le système immunitaire de l'hôte.

L'immunité est donc toujours au coeur de l'interaction entre l'hôte et le pathogène. Nous avons pu mettre en évidence un polymorphisme de compatibilité reposant sur une interaction complexe entre des molécules immunitaires de l'hôte et des molécules d'attaques du parasite. De plus, une adaptation locale au niveau moléculaire existe entre *B. glabrata* et *S. mansoni* dans laquelle la réponse immunitaire efficace du mollusque est précocement réprimée par le parasite. Lors de cette interaction des facteurs parasitaires doivent être impliqués, parmi lesquels nous pourrions retrouver les microARN, exprimés par le parasite spécifiquement pour bloquer la réponse du mollusque.

Dans l'interaction entre *B. glabrata* et *S. mansoni*, nous constatons l'existence de souche de mollusque qui sont compatibles et d'autres qui sont incompatibles. Une souche compatible sera composée de mollusque majoritairement sensible au parasite donné. A *contrario*, une souche incompatible contiendra une majorité de mollusque résistant au parasite en question. Il faut noter que cette compatibilité et incompatibilité varie en fonction des souches mises en interaction. En effet une souche de mollusque peut être compatible vis-à-vis d'une souche de parasite et incompatible vis-à-vis d'une autre. Dans tous les cas, que la souche soit compatible ou incompatible, le mollusque est capable de reconnaître l'intrus et ensuite de déclencher une réponse immunitaire ou non. Cette seconde étape de la réponse immunitaire peut être influencée par de nombreux facteurs provenant à la fois de l'hôte mais aussi du parasite. En effet, les capacités intrinsèques de chacun des deux protagonistes ainsi que leur histoire co-évolutive, ou adaptative entre eux pourront influencer la réponse immunitaire de l'hôte.

La reconnaissance des pathogènes est essentielle pour l'hôte et ce produit obligatoirement au moment de l'invasion. En effet, que l'on soit en condition sympatrique, dans laquelle le mollusque n'active pas de réponse immunitaire ou en condition allopatrique, dans laquelle à lieu une véritable réponse immunitaire, la première étape

Discussion

sera toujours la reconnaissance, ce qui permettra alors à l'hôte de définir la meilleure stratégie immunitaire.

Jusqu'ici un seul complexe immunitaire a été mis en évidence entre la réponse du mollusque et une molécule de parasite (Moné, et al. 2010), mais la reconnaissance impliquée dans l'interaction entre les deux protagonistes semble plus complexe que cela. Cette reconnaissance complexe est composée de molécules spécifiques aux divers pathogènes que le mollusque peut rencontrer (bactéries, levures, parasites métazoaires) mais aussi des molécules de reconnaissances communes à tous les pathogènes, présentant parfois différents variant (chapitre 2 - publication 2). Dans cette reconnaissance complexe, il semblerait que non seulement des molécules a activité immunitaire directe soient impliquées mais pas uniquement. En effet, des molécules impliquées dans la structuration de la matrice extracellulaire sont aussi identifiées dans la reconnaissance des pathogènes. Chez *Anopheles gambiae*, la description d'un nouveau rôle de l'actine, protéine très abondante présentant un rôle important dans l'architecture tissulaire et dans le mouvement cellulaire, fut mise en évidence en 2015. En effet, il apparait que non seulement l'actine présente la capacité d'interagir avec un complexe de facteurs immunitaires qui lui permettra de se fixer à la surface des pathogènes bactériens et entrainer leur phagocytose mais aussi un rôle de facteur immunitaire extracellulaire qui aura un effet antagoniste sur l'intrusion des *Plasmodium sp.* chez le moustique (Sandiford, et al. 2015).

Pour mieux comprendre les interactions hôtes / parasites et mettre en évidence la réponse immunitaire efficace pour contrer l'intrusion des pathogènes, des études sont menées pour sélectionner des souches résistances, qui pourraient aussi jouer un rôle important dans le contrôle de la propagation des maladies vectorielles sur le terrain. Chez *Biomphalaria glabrata*, dès les années 60 une souche, BS90, a été sélectionnées, en laboratoire, pour sa capacité a être plus résistante à l'infection par *S. mansoni* (Paraense and Correa 1963). Depuis, d'autres études de sélection ont été réalisées, notamment sur *Biomphalaria glabrata* guadeloupéen présentant un profil résistant avec une résilience de pathogènes (Allan et al., Annexe 3). Mais toutes ces sélections ne sont que partielles. De nouvelles sélections devraient être menées en sélectionnant des caractères de résistances précis. Nous savons par exemple que la réponse cellulaire joue un rôle important dans la réponse immunitaire contre les pathogènes. En effet, nous savons que les parasites, une fois neutralisés, sont encapsulés par les hémocytes, et que par conséquent une activité hémocytaire plus importante permettrait peut-être une meilleure

Discussion

réponse et donc une résistance plus importante à l'infection. A ce jour, la réponse cellulaire des invertébrés est assez mal détaillée, y compris dans les espèces modèles comme la drosophile ou encore des espèces vectrices de nombreuses maladies comme les moustiques. Chez *Biomphalaria glabrata*, une seule étude de caractérisation hémocytaire existe à ce jour (Cavalcanti, et al. 2012). Elle fut réalisée par une approche de microscopie électronique et se base sur des différences de caractéristiques morphologiques. Toujours dans le but d'une meilleure compréhension de la relation entre *B. glabrata* et *S. mansoni*, il est aujourd'hui nécessaire de réaliser une caractérisation brute des hémocytes à travers les différentes souches de mollusque présentant des niveaux différents de compatibilité. En effet, la mise en évidence du rôle importante de la réponse cellulaire dans l'immunité du mollusque laisse penser qu'une différence de cette dernière pourrait jouer un rôle dans le polymorphisme de compatibilité existant entre *B. glabrata* et *S. mansoni*. Dans ce cadre le nombre, la composition ou encore la capacité de prolifération des hémocytes peuvent être différents selon la susceptibilité du mollusque. Cette découverte pourrait mettre en évidence une implication de la composition hémocytaire dans le continuum de compatibilité qui existent entre *B. glabrata* et *S. mansoni*. La composition hémocytaire des différentes souches pourrait ensuite être comparée à celle d'une souche sélectionnée précédemment comme étant résistante, comme BS90. Et finalement, de nouvelles sélections sur des souches déjà sélectionnées comme étant résistantes (BS90) pourrait être réalisées, en se basant par exemple sur des capacités cellulaires d'encapsulation et ainsi améliorer la résistance de ces dernières.

Bien entendu, il ne faut pas oublier de prendre en compte la physiologie de l'animal lors de la sélection de résistance. En effet, il faut que la sélection ne se fasse pas au détriment d'autres traits biologiques (trade-off), qu'il n'y est pas un coût à la résistance pour le mollusque et pas conséquence que ces souches résistantes soient très peut compétitrices vis-à-vis des autres mollusques sur le terrain. Si c'est le cas les approches de médiation vectorielle avec ces souches résistantes ne seront pas réalisables.

En parallèle, toujours dans le but d'une meilleure caractérisation hémocytaire chez *B. glabrata* la mise en évidence de marqueurs de surfaces spécifiques de certains types cellulaires pourrait être une avancée considérable pour mieux comprendre le rôle de chacun lors d'invasion de pathogènes. Dans ce contexte, des travaux sont en cours au laboratoire reposant sur un tri cellulaire des hémocytes en solution en fonction de leur taille et leur granularité, par cytométrie en flux. Puis une analyse des protéines de surfaces des cellules triées pourrait permettre la mise en évidence de marqueurs spécifiques à une population hémocytaire donnée.

Discussion

Nous pouvons imaginer qu'une meilleure compréhension de la réponse cellulaire du mollusque pourrait amener à la sélection de mollusque possédant une réponse immunitaire plus performante lors d'attaque parasitaire, avec par exemple des capacités d'encapsulation plus importante, qui pourrait fournir des solutions de lutte vectorielle sur le terrain afin de limiter la transmission des parasites.

Dans le cadre de l'amélioration de la lutte vectorielle sur le terrain, nous pouvons aussi imaginer que la sélection et la sur-expression d'un gène impliqué dans la réponse immunitaire de l'hôte peut permettre de diminuer les risques de transmissions. Dans ce contexte, chez *Anopheles gambiae*, l'augmentation de l'expression de la TEP1, connu comme un facteur anti-parasitaire, chez des moustiques transgéniques, fut réalisée. Il apparaît que l'augmentation de cette dernière n'octroie pas une meilleure résistance aux moustiques, suite à l'infection par plasmodium (Volohonsky, et al. 2017). Ces résultats mettent en évidence que la réponse immunitaire et la résistance des hôtes sont des phénomènes complexes, qui ne reposent pas uniquement sur un facteur ou une molécule immunitaire mais bien sur un ensemble de réactions conduisant à la protection. En effet, la participation de molécule telle que la TEP dans la réponse immunitaire n'en fait pas un acteur unique. Il semblerait donc que la lutte vectorielle sur le terrain par la sur-expression d'un gène immunitaire ne semble pas être une solution, mais elle permettent de mieux comprendre en laboratoire ce qu'il est ou non envisageable de faire sur le terrain.

Les capacités de la réponse immunitaire peuvent aussi être acquises au cours des temps évolutif, grâce à l'interaction avec le milieu de vie. Dans certain cas, nous pouvons imaginer que le microbiote, au cours de l'évolution peut apporter des avantages sélectifs à son hôte grâce à du transfert horizontal de gènes. En effet, chez *B. glabrata*, par exemple, une toxine formant des pores, généralement retrouvée dans des bactéries du genre *Clostridium* ou *Aeromonas*, est apparue chez le mollusque et lui confère une capacité de résistance directe contre *S. mansoni* (Galinier, et al. 2013) ainsi qu'en réponse à d'autres pathogènes (chapitre 2 - publication 2). Il apparaît que des bactéries du genre *Clostridium* et *Aeromonas* soient présentes dans le microbiote du mollusque, ce qui suggère la possibilité d'un transfert horizontal passé entre le microbiote et le mollusque et qui a été démontré pour les Biomphalysines (Pinaud et al., Annexe 1). D'autres transferts horizontaux peuvent être observés chez les invertébrés, cette fois-ci plutôt entre des pathogènes et l'hôte. En effet, chez les insectes des séquences d'ADN de polydnavirus ont été retrouvées à plusieurs endroits dans le génome de lépidoptères indiquant un transfert horizontal entre les deux (Gasmi, et al. 2015). De plus lors de l'étude approfondie

Discussion

de ces séquences d'ADN, il apparaît que les polydnavirus, spécifiques des guêpes parasitoïdes d'insectes, soient le support de transfert de gènes entre deux ordres d'insectes les hyménoptères et les lépidoptères. En effet, par exemple il apparaît par exemple que les lectines de type C retrouvées chez les lépidoptères soient le résultats d'un transfert via un polydnavirus depuis les hyménoptères (Gasmi, et al. 2015).

Longtemps nous avons considéré que les symbiotes n'avaient pas d'impact sur leur hôte, mais il faut noter que certains peuvent avoir des effets délétères sur ces derniers. En effet, l'alpha-protéobactérie *Wolbachia*, endosymbiote le plus répandu dans le monde animal, qui infecte essentiellement les arthropodes notamment le crustacé *Armadillidium vulgare*, transmet verticalement à travers les oeufs de l'hôte peut modifier la biologie de son hôte de diverses manières. Une des influences les plus connue de *Wolbachia* sur ces hôtes est l'induction de la manipulation de la reproduction, avec la féminisation, l'incompatibilité cytoplasmique des spermatozoïdes etc. (Werren, et al. 2008). Depuis, d'autres influences ont été mises en évidence. En effet, *Wolbachia* présente des effets délétères sur l'immunocompétence des femelles infectées (Braquart-Varnier, et al. 2008). On observe chez ces femelles une baisse de la quantité des hémocytes qui pourrait entraîner une diminution de la survie de ces dernières. De plus, des études plus récentes montrent une modulation de l'immunité chez les femelles infectées y compris dans les ovaires, organe crucial de transmission verticale de l'endosymbiote (Chevalier, et al. 2011).

Ces interactions entre les hôtes, leurs microbiotes et les pathogènes peuvent donc conférer des avantages sélectifs à l'hôte dans l'évolution de ces systèmes. Ces résultats mettent en évidence l'importance de connaître le microbiote de l'hôte ainsi que celui du parasite. De plus il est essentiel de mettre en évidence tous les acteurs de l'interaction entre les deux protagonistes dans une vision intégrative des interactions des molécules aux populations.

Généralement, la recherche se concentre soit sur la réponse de l'hôte à l'infection parasitaire, soit sur les mécanismes de virulence parasitaire. Plus récemment, les études intégrées sur les interactions hôtes-parasites ont permis des progrès significatifs dans la biologie théorique et appliquée. L'hôte et le parasite sont associés à une myriade de microorganismes qui pourraient bénéficier de l'amélioration des aptitudes de leurs partenaires. Les illustrations de telles interactions multi-joueurs complexes sont apparues récemment à partir d'études réalisées dans divers taxons (Dheilly, Poulin, et al. 2015). Les microorganismes associés à l'hôte et au parasite peuvent participer à l'interaction hôte-parasite en interagissant directement ou indirectement avec l'autre partenaire.

Lorsque l'on s'intéresse à des maladies causées par des agents pathogènes transmissibles par vecteurs, outre la connaissance du modèle, nous cherchons à mettre en place des outils de lutte efficaces pour diminuer la transmission et ainsi diminuer les prévalences de ces maladies. Ici, le but ultime des études faites sur les microbiotes, est de pouvoir prétendre à créer un moyen de lutte supplémentaire grâce des outils de transgénése. Le terme « transgénése » décrit des manipulations génétiques réalisées sur les micro-organismes symbiotiques de l'hôte, y compris son microbiote. Des études approfondies de la composition du microbiote intestinal de l'anophèle ont été réalisées dans le but de modifier ensuite ce dernier pour lutter contre la transmission du paludisme en Iran (Dinparast Djadid, et al. 2011; Dheilly, Duval, et al. 2015). Des premiers tests de transfections ont été réalisés à l'échelle d'un pays, l'Australie, dans des zones endémiques de la dengue (Iturbe-Ormaetxe, et al. 2011). Des moustiques sont transfectés avec *Wolbachia*, bactérie endosymbiotique héréditaire, dont certaines souches qui sont capables d'envahir des populations de moustiques et de s'y maintenir tout en réduisant la durée de vie des adultes, qui affectent la reproduction des moustiques et interfèrent dans la réplication des agents pathogènes qui leur sont associés. Ces premiers tests mettent en évidence des avancées prometteuses dans les approches de bio-contrôle des maladies transmises par ces vecteurs (Iturbe-Ormaetxe, et al. 2011). Finalement, des tests, en laboratoire, de modifications du microbiote intestinal de *Rhodnius prolixus* ont vu le jour pour lutter contre une autre maladie vectorielle, la maladie de Chagas (Taracena, et al. 2015). En effet, des bactéries, *Escherichia coli*, ont été modifiées dans le but d'exprimer des ARN interférents capables d'inhiber une protéine de l'œuf essentielle, la *Rhodnius* heme-binding (RHBP), ainsi qu'une enzyme antioxydante exprimée tout le long de la vie de l'insecte. L'effet d'interférence de l'ARN est systémique et temporel, mais la modification du microbiote des insectes pour induire des ARN interférents systémiques peut entraîner une stratégie de transgénése tout à fait nouvelle et originale dans la lutte anti-vectorielle durable (Taracena, et al. 2015).

Nous pouvons donc imaginer, dans notre modèle d'étude des approches similaires pour lutter contre la transmission de la maladie par le mollusque. En effet, une meilleure compréhension de la réponse immunitaire du mollusque couplée à l'étude approfondie de son microbiote (bactérien, viral ou eucaryote) ainsi que son rôle au cours d'une infection peut amener à la création de mollusque transgénique. En effet, dans la lutte vectorielle nous pouvons imaginer deux approches, une modification des capacités immunitaires du

Discussion

mollusque qui entrainerait une plus grande résistance au moment de l'infection, dans ce cas-là nous aurions un lien direct entre la modification transgénique et son influence sur l'interaction. Mais nous pouvons aussi imaginer une modification du microbiote du mollusque qui entrainerait alors une modification de l'interaction entre l'hôte et le pathogène, dans ce cas là, l'influence de la modification transgénique sur la résistance serait indirecte.

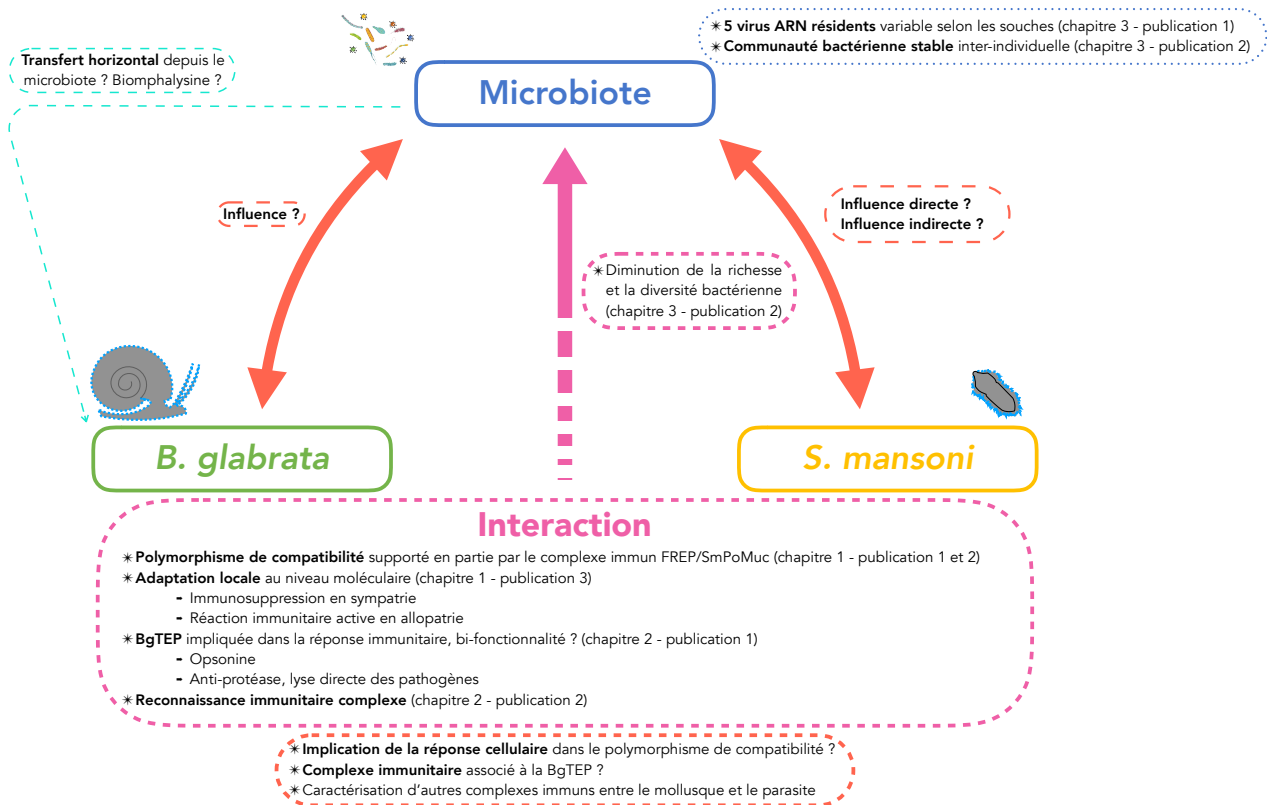


Figure 12 : Schéma de conclusion du décryptage du polymorphisme de compatibilité

Représentation schématique des découvertes réalisées au cours de cette thèse, principalement dans l'interaction entre l'immunité de l'hôte et le parasite mais aussi sur la caractérisation du microbiote associé à l'hôte. Les différents points encadrés en rouge sont les questions principales qui restent sans réponses, nécessitant de futures investigations.

En conclusion, le but de ce travail de thèse était de décrypter le polymorphisme de compatibilité entre *B. glabrata* et *S. mansoni* à différents niveaux d'intégration (fig. 12). Nous avons mis en évidence dans ce contexte l'implication d'un complexe immunitaire dans la compatibilité ainsi que l'existence d'un phénomène d'adaptation locale entre les deux protagonistes. Toujours dans le but de décortiquer ce polymorphisme de compatibilité, nous avons mis en évidence la complexité de la reconnaissance immunitaire du mollusque ainsi que le rôle important de la BgTEP dans cette réponse immunitaire. Finalement, en se plaçant à un autre niveau d'observation de l'interaction entre le mollusque et le parasite, nous avons pu caractériser le microbiote bactérien et viral de

Discussion

l'hôte et ainsi que mettre en évidence l'influence d'un stress d'infection sur sa composition.

Le but de ces recherches peut être à la fois replacé dans le contexte d'études fondamentales, dans ce cas nous cherchons à mieux comprendre l'immunité des invertébrés au sens large, pour cela nous utilisons un modèle complexe, présentant des niveaux de compatibilités variables selon les souches voir même selon les individus. Mais ce n'est qu'une étape préliminaire avant de réaliser des études à but appliqué. En effet, notre modèle d'étude n'est autre que l'interaction entre l'hôte intermédiaire et le parasite responsable de la seconde endémie parasitaire humaine mondiale la bilharziose. Dans ce contexte une lutte contre la transmission est nécessaire, pour cela de nouveaux moyens parmi lesquels la lutte vectorielle, sont mis en oeuvre. La transposition de nos résultats pourrait être réalisée sur des souches de terrains avant peut-être de mettre au point des mollusques transgéniques dans lesquels le parasite ne pourrait pas continuer son cycle de vie. En effet, les différentes approches réalisées au laboratoire sur des isolats de souches d'origines géographiques différentes apportent des découvertes essentielles et fondamentales qui pourraient par la suite être transposées sur le terrain et finalement aider à lutter contre la maladie. Dans ce cadre-là, le projet de thèse apporte une vision intégrative du polymorphisme de compatibilité existante entre *B. glabrata* et *S. mansoni* (fig. 12). Il met en évidence, non seulement l'importance de la reconnaissance mais aussi de la réponse immunitaire tant cellulaire qu'umorale et ceci dans une vision holobiontique des interactions. Il faut maintenant aller plus loin dans le décryptage de cette interaction tout en gardant en considération l'influence de nombreux facteurs et ce à différentes échelles. En effet, l'interaction entre *Biomphalaria glabrata* et *Schistosoma mansoni* présente un haut niveau de complexité qui ne la rend tout à fait passionnante et ouvre de nombreuses perspectives de recherches futures.

Annexes

Annexe 1

Publication : The aerolysin-like proteins family acquired from bacteria are key diversified immune weapons in an invertebrate

PINAUD Silvain, TETREAU Guillaume, PORTET Anaïs, CHAPARRO Christian, LASSALLE Damien, GALINIER Richard, GOUBAL Benjamin, DUVAL David

*Papier en préparation soumission prévue pour Automne 2017

Ma participation à ces travaux :

La biomphalysine, β -pore forming toxin, présentant un rôle direct dans l'élimination des parasites, fut identifiée au laboratoire en 2013. Avec la publication du génome de *B. glabrata*, c'est en réalité 23 biomphalysines qui ont été identifiées. Cet article cherche à démontrer l'origine de cette famille de toxine. Ce type de toxine est généralement retrouvée dans 3 familles de bactéries, *Clostridium*, *Aeromonas* et *Pseudomonas*. Dans le cadre de cette article, j'ai réalisé une analyse sur les données de métabarcoding dont nous disposons dans le cadre de mes travaux pour essayer de mettre en évidence la présence de ces 3 familles bactériennes dans le microbiote des mollusques naïfs. Parmi toutes les familles d'OTU identifiées, *Clostridium*, *Aeromonas* et *Pseudomonas* sont retrouvées et correspondent à 2,03% des familles totales. Nous avons donc pu émettre l'hypothèse d'un transfert horizontal entre des bactéries du microbiote et le mollusque.

The aerolysin-like proteins family acquired from bacteria are key diversified immune weapons in an invertebrate.

PINAUD Silvain, TETREAU Guillaume, PORTET Anaïs, CHAPARRO Christian, LASSALLE Damien, GALINIER Richard, GOURBAL Benjamin, DUVAL David

Abstract (< 250 words)

Putative horizontal acquisition was previously described from bacteria to the snail vector of schistosomiasis (*Biomphalaria glabrata*) for a β -pore forming toxin (β -PFT) named biomphalysin. Despite feature homologies with aerolysin, a well-known bacterial virulence factor, this β -PFT displayed rather an hemolytic and anti-schistosomal activities. Genomic exploration reveals new aerolysin-like genes, leading to the identification of a total of 23 intron-less biomphalysin genes located into 20 different scaffolds. Deduced amino acid sequences revealed an important range of sequence identity between members while a high degree of structural homology is predicted. Contrasting patterns of tissue-specific expression of the different members suggest potential non-redundant functions. The taxonomic distribution implies that some β -PFTs were probably acquired horizontally several times from bacteria to eumetazoa. These results suggests : 1) selection pressure on structure to keep the function of acquired genes; 2) amplification of several members; 3) domestication of variants to target different sets of pathogens including schistosomiasis trematode.

Keywords: Horizontal gene transfer, biomphalysin, neofunctionalization, immunity

Introduction

Horizontal gene transfer defined as DNA transfer between different species plays a major role in genome evolution (Soucy, Huang, 2015) (Daubin Szollosi, 2016). The acquisition of gene as those involved in antibiotic resistance between different bacteria have been widely described (Smith Feng, 1992, Koonin Makarova, 2001). HGT is not only just confined to

prokaryotes. Indeed the development of massive genome sequencing of non model organisms and their comparison has revealed the presence of many genes acquired by lateral transmission (Hotopp, 2011)(Sharma Das de T, 2015)(Harrison Rowley, 2016) (Crisp Boschetti, 2015). Inter-kingdom gene transfer has been reported from bacteria to metazoan (Sun, Li, 2015) (Emameh, barker, 2016) (Boto, 2014) or plant recipients (Gao, Ren, 2014) but also between multicellular organisms (El baidouri Carpentier, 2014) (Wijayawardena, 2013). Acquisition of new genes by HGT may confer a selective advantage of the recipient in the adaptation to abiotic and/or biotic environmental condition (Keeling, 2009). Many studies have described novel *inheritance of acquired characteristics by HGT leading to colonize new niches*. *Domestication of fungal cellulase genes by some nematodes was essential for successful plant parasitism* (Jones JT, Furlanetto C, 2005)(Palomares, Hirooka, 2016). In bdelloid rotifers, genome integration of about 4000 foreign genes encoding various biochemical functions like the degradation of toxic compounds or the desiccation resistance has led to improve the fitness in a rapidly changing environment (Eyres Boschetti 2015, Boschetti Carr, 2012). Also, the acquired mosquito-polyubiquitin gene by the fungus *Zancudomyces culisetae* could provide an advantage to colonize the host digestive tract by modulating its host immune response and/or by conferring a competitive advantage to others intestinal microbiota (Wang White, 2016). More and more studies have reported the HGT into animals and can be considered as a driver in adaptative evolution (Crips, Boschetti, 2015, Lacroix, Citovsky, 2016). Among the most transferred genes, the bacterial toxin genes belonging to the aerolysin family are detected in many metazoans and could be useful in both defence and predation mechanism (Moran, Fredman, 2012). The plant cytolytic enterolobin isolated from seeds may protect against herbivore grazing or insect attack (Castro Faria neta, 1991). In cnidarian, hydralysin and Nvlysin 1b are suspected to play a major role in protecting against predators or killing prey (Sher Fishman, 2005)(Moran, Fredman, 2012). The $\beta\gamma$ -CAT, an aerolysin like protein isolated from the frog mucus, contributes to bacterial clearance and the host pro inflammatory response (Xiang Y Yan C, 2014). The parasitic mushroom *Laetiporus sulphureus* express a lytic pore-forming lectin (LSL) whose its role in host- parasite relationship remain to be determined (mancheno tateno, 2010). Another metazoan aerolysin-like described in the Schistosomiasis vector snail *Biomphalaria glabrata* and called biomphalysin was shown to bind to parasitic membranes and exhibit cytotoxic activity toward *S. mansoni* sporocysts. (Galinier, et al. 2013). A RAS genotyping study has reported a correlation between

biomphalysin expression and snail resistance to schistosoma parasite supporting a central role in snail immune response (Tennessen, Bonner, 2015). Also, recently we have shown a higher expression of its bacterial-originated toxin gene involved in snail innate immune memory (Pinaud et al, 2016). Interestingly, many isoforms of this biomphalysin were characterized by a deep RNA sequencing supporting the potential presence of additional copies.

Consequently in this study, we report the first genomic, structural *and functional* characterization of expansion of this β PFT biomphalysin family in *Biomphalaria glabrata*. Several HGT are behind the first step of these aerolysin-like expansion in the *Biomphalaria* genome. Despite lack of similarity at the amino acid level between the different members due to a diversifying selective pressure, the whole structural conformation has been retained. However, some fine differences of folding could be predicted on the small domain suspected to be involved in selective binding to target receptor. Finally, an interactome approach confirm (a GUILLAUME)

Material and Methods

Identification of Biomphalysins by FISH

Bge cell line are cultivated in XXX at 26°C during X days. (Odoemelum, et al. 2010)

Identification of Biomphalysins from *Biomphalaria glabrata* genome

*Detail the procedure to identify them all. First InterPro search, then manual BLAST of retrieved sequences and/or of aerolysin domain sequence on RNA-seq and/or genome of *Biomphalaria glabrata*.*

Explain the naming (from the first identified (=Biomphalysin 1) to the most distant (=Biomphalysin 23)).

Resequencing

Gene were obtained by PCR amplification with probes covering all the translated sequences (SUPP XX) and sanger sequencing (GenoScreen, Lille France). Consensus sequences were obtained for the 23 members of biomphalysin then deposit on NCBI GenBank data server (XX, Supp XX).

Phylogenetic analysis

Confirmed sequenced sequences for the 23 members of biomphalysin were subjected to a phylogenetic analysis. The gene and protein sequences corresponding to the crystallized aerolysin from *Aeromonas hydrophila* was used as an outgroup. Sequences were aligned using ClustalW and then a phylogenetic tree was generated using the neighbor joining method (Poisson substitution model; uniform substitution rate; gaps/missing data treatment: pairwise deletion) with MEGA 6.06 software (Tamura, et al. 2013). A bootstrap analysis of 2000 replications was carried out to assess the robustness of the branches on the trees inferred from the neighbor joining method. Bootstrap values are shown on each branch of the trees generated.

The aerolysin and MTX2 domain (IPR005830 and IPR004991) were identified in the protein sequence of each biomphalysin by searching against the Conserved Domains Database (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) (Marchler-Bauer, et al. 2011). These sequences were used as a query for a BLAST search against the NCBI database to identify similar sequences in other species. A total of 87 sequences were used for the phylogenetic analysis performed as described previously. They included 39 sequences from Gastropoda, 16 from Hydrozoa & Anthozoa, 18 from Arachnida, and 14 from bacteria. The accession numbers and corresponding names of each sequence used are indicated in Supplementary Table XX GUILLAUME for all phylogenetic trees performed.

Secondary structure prediction and analysis

Structure prediction, alignments, etc. Refer to the Fig. number for consistency.

Search for transposable elements (TE)

A 10Kb region surrounding each biomphalysin was screened for the presence of TEs through a similarity search using BLAST with the Biomphalaria specific database of repeat elements as well as using RepeatMasker. We performed a visual inspection for conserved sequences in

these 10Kb regions surrounding the biomphalysin genes by producing a dotplot using gepar v. 1.4 (ref in comment).

Transcription factor motif search

Transcription factor motifs were searched using the 2016 version of the JASPAR (ref) database of transcription factors and the Motif Occurrence Detection Suite MOODS v. 1.9.2 (ref) by using the provided script (moods_dna.py) and the data was parsed, filtered and summarized by in house developed scripts. The search for conserved domains was carried out by using MEME and searching for motifs between 12 and 50 bases long. Shorter motifs did not produce significant results except for tata boxes and a poly C stretch.

Relative transcript abundance in whole snails using RNAseq experiment.

High quality reads are used to be aligned on sequences of 23 members biomphalysin. Briefly, two pools of 20 uninfected snails were sampled and RNA was extracted using Trizol® Reagent (Sigma Life Science, St. Louis Missouri, USA) prior to Illumina sequencing. Best quality reads (Phred >29) from both libraries (Naive1 & Naive2) were aligned as already described (Pinaud et al, 2016) on each of the 23 biomphalysins using Bowtie2 (v2.2.4; Langmead B, Salzberg S. Fast gapped-read alignment with Bowtie 2. *Nature Methods*. 2012, 9:357-359.) and samtools (v0.1.18, Li H.*, Handsaker B.*, Wysoker A., Fennell T., Ruan J., Homer N., Marth G., Abecasis G., Durbin R. and 1000 Genome Project Data Processing Subgroup (2009) The Sequence alignment/map (SAM) format and SAMtools. *Bioinformatics*, 25, 2078-9. [PMID: [19505943](#)]), which were run on a local Galaxy Project server (@galaxyproject, **The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2016 update.** *Nucleic Acids Research* (2016) 44(W1): W3-W10 doi:10.1093/nar/gkw343). Raw counts were normalized with upper-quartile division per libraries (Dillies *et al.* 2012).

Tissue representation of biomphalysin transcripts

Seven organs (Mantel, Head-Foot, Hemocytes, Albumen gland, Stomach, Hepatopancreas and Ovotestis) from 5 individual *Biomphalaria glabrata* snails (± 8 mm) were recovered by dissection and immediately transferred in frozen nitrogen for mRNA total extraction thanks to Norgen Biotek (Cat. #50300, Thorold, Ontario, Canada) manufacturer protocol. DNase treatment (optimize at 10mn in 37°C) was carried on (Cat. K1681, ThermoFischer, USA) to

eliminate trace of contaminant genomic DNA. Total RNA (500 ng) was undergo reverse transcription (RT) using Maxima H Minus First Strand cDNA synthesis kit (Cat. K1681, ThermoFischer, USA) and manufacturer's protocol. Equimolar random hexamer primer and oligo (dT)₁₈ primer was used. Absence of genomic DNA and quality of cDNA synthesis was verified by using myoglobin primers (forward : 5'-GAT GTT CGC CAA TGT TCC C-3' and reverse : 5'-AGC GAT CAA GTT TCC CCA G-3') with intron/exons targeting. All Biomphalysin primers couples were designed then validated by genomic amplification and sanger sequencing (SUP XX). For tissues-specific expression PCR amplifications were performed on the 7 organs in biological quintuplicate on cDNAs (diluted 5-fold with nuclease-free water). PCR reactions were performed with GoTaq® G2 Hot Start Polymerase kit (Cat. M7405, Promega, Madison, USA) and performed with thermocycler brands advices (Eppendorf, Hamburg, Germany). Protocol have following conditions: 4 minutes at 95°C for the polymerase activation then 35 cycles with 20s initial denaturation at 95°C, 30s annealing at 48°C and 30s at 72°C of extension with 5mn at 72°C of final extension. PCR products were separated by electrophoresis on 1.5% agarose gels.

Codon Usage and GC content

Codon usage are compared between sequences from all the 23 Biomphalysins, 13 aerolysins/Pertussis Toxin for bacterial kingdom and 25 random selected *Biomphalaria glabrata* predicted transcript (1600-1800 pb) (SILVAIN SUPP). Nucleotide sequence are compared thanks to codon usage tools of CAIcal server ((Puigbò, et al. 2008) ; <http://genomes.urv.es/CAIcal/>). Mean codon usage are compared for each triplets between Biomphalysins transcripts, *Biomphalaria* transcripts and Bacteria transcripts by PCA (Principal Component Analysis) using ClustVis web tool (<http://biit.cs.ut.ee/clustvis/>) from BIIT group (Metsalu, Vilo, 2015). Local transcriptome of *Biomphalaria glabrata* (Pinaud et al. 2016) and the same 23 sequences of biomphalysins and 13 of aerolysins-like sequences are used to calculated the GC content with using geecee tools (v5.0.0) on our local Galaxy server and plot on histogram.

In-silico biomphalysin-targets of miRNA

All the miRNA described up to now in *Biomphalaria glabrata* (Adema CM et al, 2017), were used to predicted miRNA/mRNA interactions and decipher putative miRNA targeting of

Biomphalysin. Genomic portions surrounding biomphalysin positions up to 500 nucleotides are recovered as putative 5' and 3' UTR. UTRs are cleaned in confronting putative sequence to RNAseq reads to delimit Transcription starting site (TSS) and polyadenylation signal, as well as available. Sequences of 5' UTR, CDS and 3'UTR are used to predict miRNAs targets using Miranda software (10.1186/gb-2003-5-1-r1 and [10.1371/journal.pbio.0020363](https://doi.org/10.1371/journal.pbio.0020363)) with high strictness (-en -20 -sc 150). Graphical representation was generate and details of prediction are included in SUPP XX.

Microbiota diversity

Twelve naive snail have been recovered without shell and grind individually in nitrogen. The total DNA was extracted to the DNeasy Blood and Tissue Kit (Qiagen). Paired-end sequencing with 250 bp read length was performed on the Illumina MiSeq PE (GenomeQuébec, Montréal, Québec, Canada) using the v2 chemistry according to the manufacturer's protocol. The FROGS pipeline ((Find Rapidly OTU with Galaxy Solution) implemented on a galaxy instance (<http://sigenae-workbench.toulouse.inra.fr/galaxy/>) was used for data processing (Escudié et al., 2015).

Results

From one Biomphalysin to a family of 23 genes

To contribute to snail 's genome characterization, a metaphase FISH analysis on mollusc cell line (Bge) was performed using the Biomphalysin full length rDNA probe. Numerous hybridization signals were obtained on several non-homologous chromosomes. It is noteworthy that the hybridized loci were detected **near the centromere of the largest metacentric chromosome RONALDO.**

We have searched for nucleic sequences related to the Biomphalysin gene by BLAST search against a *de novo* assembly of RNA seq data from uninfected snails (Dheilly et al., 2015)(local server). Only one partial sequence of 395 bases (Locus_25218_Transcript_19/20) was obtained sharing 80% identity. However, a tBLASTn searches revealed 10 other hits with potential homology to Biomphalysin with an E-value score between 1e-79 and 5e-38. As most of them harboured an aerolysin or epsilon toxin signatures (IPR005830 and IPR004991, respectively), an InterProScan and sequence similarity search were conducted to investigate the presence of additional aerolysin-like genes against the preliminary assembly v4.3 of the *B. glabrata* genome. A total of 23 sequences including the known Biomphalysin renamed Biomphalysin 1 has been identified on 20 different genomic scaffolds (Table S1). Biomphalysin 1 and 2 as Biomphalysin 11 and 22 and Biomphalysin 20 and 21 appear as gene pairs resulting from a duplication since both pairs are located in tandem on large scaffolds 10 (1 Mb), scaffold 2594 (72 kb) and 2201 (88 kb), respectively. No conserved regions were detected through visual inspection of the dotplot produced by Gepard (Table S2). This confirmed the results of blast searches against the repeat database as well as searches using repeatmasker which do not detect significant traces of transposable elements in the 10 Kb vicinity of biomphalysin genes. Repetitive elements such transposable elements do not seem to have been involved in gene duplication and expansion.

Surprisingly, all 22 supplementary genes identified contain no intron making an evolutionarily conserved characteristic of this novel gene family.

Biomphalysins represent a highly diverse family in *Biomphalaria glabrata*

To rule out eventual errors in genome assembly and sequencing, specific PCRs on snail genomic DNA were performed with primer pairs flanking the predicted coding DNA sequence of Biomphalysin genes (Table S3b, **Silvain**). The putative translated regions are on average

1725 in length (1698 -1758 bp). The nucleic identity within this novel family is comprised between from 43.2 to 94.7 % (Table S2). The deduced amino acid sequences of new Biomphalysins showed 33.1 to 95.1 % identity compared to Biomphalysin 1 and 16.9 to 23.7% with the bacterial aerolysin toxin (Table S4). The lower identity was found between Biomphalysin 9 and 22 (29.2%), while the highest were observed for the pair Biomphalysins 1 and 2 encoded by genes located on the same genomic scaffold (95.1%). Their level of similarity is also very dissimilar from each other, ranging from 32.6% between Biomphalysin 5 and Biomphalysin 23 to 97.7% between Biomphalysin 1 and 2. Contrariwise, all of those proteins have a low similarity to aerolysin (23.6 – 30.2 %) (Table S5). The deduced amino-acid sequences of the 23 biomphalysins were compared by performing a phylogenetic analysis (Fig. 4). Analyses performed with gene and protein sequences generated similar results. At least three clusters of biomphalysins could be observed and they were strongly supported by high bootstrap values (over 95%) (Fig. 3). The first cluster included biomphalysins from 1 to 8 including 10, another one biomphalysins from 11 to 18 and the last one biomphalysins from 19 to 23. Biomphalysins 9 was isolated from the 21 other biomphalysins and were the closest biomphalysins to the aerolysin sequence used as an outgroup (Fig. 3).

Biomphalysins exhibit common domains and cluster-specific features

Biomphalysin proteins are composed of approximately 575 residues (from 565 min to 585 max). All members harbour a predicted N-terminal signal peptide by 17 -23 amino acids that adopt an alpha-helical conformation. A structural feature common to all aerolysin like toxins is the presence of many β -sheet elements (about 40 %). Most of them are present in the common core also called aerolysin domain consisting of five β strands with an insertion loop corresponding to the pore forming transmembrane domain (TMD) boxed in blue dotted (Fig. 4). This predicted membrane spanning domain flanking by two charged residues regions are found for all biomphalysin toxins (Figure S1A). Moreover, an alternating pattern of hydrophobic and hydrophilic residues located in this beta-hairpin loop has been retained in all highlighting their ability to perforate the lipid layer of target organisms (Figure S1B and C) (Rai, Chattopadhyay, 2015). Interestingly, all biomphalysins show sequence conservation in domain involved in oligomerization and cytolytic activity. Compared to Biomphalysin 1, Asp₂₃₅ and Cystein₂₅₅ display a crucial role in the oligomeric assembly into a ring like structure (Rossjohn Feil, 1998) (Bucley, Wilmsen, 1995). Tryp₄₆₆ and Tryp₄₆₈ mutation modify in

aerolysin toxin its ability to oligomerize (Gsiou vender Goot, Ausio, 1993) whereas Tryp₄₂₀ and His₄₂₈ could be involved in binding to a membrane receptor (Green Buckley, 1990) (Gsiou vender Goot, Ausio, 1993). The C terminal part tend to be divergent and could be associated to a differential lytic activation (Zhang Pridgeon, 2013).

All biomphalysin proteins share a similar general organization despite a low degree of protein similarity. Each has a signal peptide necessary for secretion, a transmembrane domain shared by members of β -PFT (Sher Fishman, 2005) (Degiacomi iacovache, 2013)(Xiang Yan, 2014)(Jia, liu, 2016) involved in crossing cellular membranes and forming an amphiphatic β barrel by oligomerization (Iacovache De Carlo, 2016)(bokori Brown, 2016).

Species-specific diversity of aerolysin-like proteins among invertebrates (Guillaume)

To determine whether such an expansion and/or diversification of the aerolysine-like genes occurred in other invertebrates, we also searched them in several Sequence Databases of NCBI (Nucleotide, Protein, EST). Then, a phylogenetic analysis was performed using sequences of the aerolysin domain of aerolysin-like proteins from 3 different bacteria genus (*Aeromonas*, *Clostridium* and *Vibrio*) and different invertebrates, including the Gastropoda *B. glabrata* and *Aplysia californica*, the Hydrozoa *Hydra vulgaris* and *H. viridissima*, the Anthozoa *Nematostella vectensis* and *Exaiptasia pallida*, and the Arachnida *Ixodes scapularis* (Fig. 5). Bacterial toxins all clustered together, with three different sub-clusters corresponding to the three bacterial genus used, and they were clearly separated from invertebrate aerolysin-like sequences (Fig. 5). Increasing the number of bacterial sequences used in the analysis did not induce any modification of the structure of the tree (data not shown/supplementary OUI à mettre GUILLAUME et l'ensemble des sequences Accession number dans le fichier excel). Although bacterial toxins were clustered separately, they were not rooting the tree and were located between the group formed by *Ixodes* and *Hydra sp.* and the group containing Gastropoda and Anthozoa.

For all invertebrate species analyzed, with the exception of *B. glabrata*, all aerolysin-like proteins identified in the same species were clustered together, with relatively high bootstrap support (ranging from 60 to 100%) (Fig. 5). Interestingly, the phylogenetic closeness of species did not induce a higher phylogenetic similarity in the sequences of aerolysin-like proteins. Indeed, aerolysin-like proteins from the Gastropoda *A. californica* were more similar to those from the Anthozoa *E. pallida* and *N. vectensis* than to most of the biomphalysins from the

Gastropoda *B. glabrata* (Fig. 4). Similarly, Anthozoa and Hydra, which are two classes from the Cnidarian phylum, were found at the opposite of the tree generated with sequences of aerolysin domains from aerolysin-like proteins. This suggests that the multiple aerolysin-like proteins identified in these invertebrate species resulted from a species-specific diversification.

In *B. glabrata*, only the biomphalysin 9 was not clustered with the 22 other biomphalysins and rather grouped with aerolysin-like proteins from Anthozoa (Fig. 5), supporting the hypothesis that biomphalysins originated from at least two distinct horizontal transfer events.

Biomphalysins result from an old domestication of aerolysin-like genes

In most cases, recent horizontal transfer can be determined by a significant GC% and codon composition between donor and recipient DNA molecules. Thus, The GC content of nucleotide sequences of biomphalysins (N = 23), *Biomphalaria* sequences (± 1700 pb; N = 25) and bacterial toxin sequences (N = 13) are calculated. GC% content distribution indicates that *Biomphalaria glabrata* transcripts spread from 0.2 to 0.6 with peak of normal distribution at 39. GC ratio of biomphalysin transcripts were much lower (GC mean : 41.0 ± 3 %), compared to the bacterial toxin nucleic sequence (GC mean : 55.8 ± 8 %). Concerning the codon composition, principal component analysis (PCA) was realized with mean codon use value for each triplet in *Biomphalaria glabrata* transcripts, Biomphalysin and toxin-like from bacteria. We showed that most variance (78%) can be due to the difference between eukaryotic and prokaryotic transcripts. In others terms the nucleotide and codon composition in biomphalysin transcripts is closer of snail transcripts than bacterial toxin transcripts. Combined, GC% and codon use between biomphalysin and bacterial toxin genes share distinct signature and suggest a domestication of biomphalysin genes by *Biomphalaria*.

HGT is known to be enhanced by an immediate proximity between the potential donor of gene and its recipient. So, to find the possible bacterial origin of biomphalysin coding gene, we have explored the microbiota bacterial diversity by 16S rRNA-targeted Next-Gen sequencing. The diversity of OTUs (Operational Taxonomic Units) characterized in naive snail we recovered 324 OTUs family. Among them, several key families of bacteria known to produce β -pore forming toxins, including *Aeromonadaceae*, *Clostridiaceae* and *Pseudomonadaceae*, which correspond on average to 2.03% of total OTUs observed (Table S6).

This suggests that biomphalysins could have originated from horizontal transfer from bacteria present in the snail microbiota population.

Highly conserved three-dimensional structures despite a high variation in sequence conservation

Despite the apparent divergence and the low sequence homology between these biomphalysins, their structure can be similar. Indeed, we have previously reported that the antiparasitic biomphalysin 1 displayed the same structural feature with the bacterial aerolysin toxin. Because of similar prediction for all these diverse sequences of biomphalysin member could be indicative for a function to be characterized, we have performed a structural modeling for each biomphalysin proteins with I-Tasser server using the best aligned template against the Protein Data Bank database. All biomphalysins shows a structural similarity close to the aerolysin structure with a TM score ranging from 0.66 to 0.91 (Table S7). The predicted structure are made up of two distinct lobes, a small lobe also defined Domain I and the large lobe divided into three structural domains (Domain II, III and IV) so qualified of aerolysin domain. Also, difference in primary structure gives rise to highly similar conformation between the different members with an average TM score of 0.70 ± 0.03 (Table S5). Hence, conservation analysis of residues positions among the biomphalysin predicted structure was performed with the ConSurf software. Conservation score mapped onto the 3D structure of biomphalysin 1 reveals that most conserved residues are located to the Domain II known to be involved in oligomerization (Buckley, 1995) and receptor binding to aerolysin (MacKenzie 1999)(Abrami 2000) and the Domain III necessary for the channel forming stabilization (Rossjohn, 1998) and for toxin insertion into lipid bilayers via the transmembrane β hairpin loop (Iacovache,2006). In deep, conserved residues are involved in highly-organized form to maintain a proper topological arrangement characteristic of a β -PFT. Indeed, the most conserved shape the two α helices and the different β strands of Domain II and III. Indeed, the structure of this aerolysin domain appears to be under selection despite a divergent sequence with an average TM score about 0.93 ± 0.02 (Table S7). Surprisingly, many variable amino acids are found in loop regions and particularly in the insertion loop even if the stretches of alternating hydrophobic and hydrophilic residues are conserved (Fig S1).

As structural information reveals the presence of variable loops mainly located in the Domain II which could contribute to specific interactions, a predicting protein-ligand binding sites was

performed based on predicted biomphalysin three dimensional structure. For all snail toxins, a mannose 6 phosphate binding domain was identified involving crucial residues such arginine, glycine and tryptophan (Figure 9) (Zelent odili S, 2008)(Degiacomi iacovache, 2013)(Antonik, Volkov, 2016). The carbohydrate binding pocket are located in the vicinity of the two α helices present in Domain II, a well-characterized site in aerolysin toxin involved in binding the mannose residue of the glycosyl phosphatidylinositol (GPI)-anchored receptors (Abrami Velluz, 2002).

Small lobe shares high structure similarity with recognition domains A completer DAVID

Needs a Table with the Tm (or other) scores showing which small lobe of which biomphalysin is close to which known domains. Highlights that most if not all matching domains are recognition domains.

Figure 10 : Structural prediction of the Biomphalysin small lobes

A. Three-dimensional structure prediction of Biomphalysin 8, 12 and 17 was performed using using I-Tasser server using the crystal structure of proaerolysin as pattern. A C score was determined for each prediction (see Table S5). Surface representation was realized with PyMol software. Small lobe was shown in red, the large lobe in green and the TMD domain in blue.

B. Structural similarities the small lobe of biomphalysin proteins 8, 12 and 17 were investigated against RCSB Protein Data Bank. A TM-score was calculated between the small lobes and the best hit found by I-Tasser server. A score greater than 0.5 reveals significant alignment, whereas a TM-score less than 0.17 indicates random similarity. 3D superposition of each small lobe of Biomphalysin 8, 12 and 17 and the respective best hit were performed by TM align and rendered with PyMol. The best structural alignment was reported between the predicted isolated structure of the small lobe in red and the significant template hit identified in yellow.

Each biomphalysin is expressed and exhibits a specific pattern of tissue-specific gene expression

High quality reads of RNAseq experiment using two pools of 20 naïve snails are used (Pinaud et al. 2017) to be aligned with high-strictness on the 23 members of biomphalysin family,

counted and normalized by upper-quartile for each library. All of the 23 can be detected in whole naïve snails with different expression level (Figure 7A). Biomphalysin 1, 2, 4, 20 and 21 are the most represented (about 3.0 UQ normalized count for Biomphalysine 1) whereas biomphalysin 5, 14 or 16 are lower presents (about 0.1 UQ normalized count for Biomphalysine 14). Presence of all biomphalysin transcripts in snail highlights that all biomphalysins are assimilated in host genome without features dedicated to pseudogenes.

To explore settings in which Biomphalysins may play a role, we analyzed their transcript representation by RT-PCR in seven different tissues (stomach, albumen gland, ovotestis, hepatopancreas, hemocytes, mantel, head-foot) revealed a mosaic pattern of expression of these genes in naïve snails (Figure 7B). While some biomphalysins, such as biomphalysins 2, 10, 14, 18 and 23, are expressed ubiquitously in all tested tissues, others were specifically expressed in some tissues, such as biomphalysin 7 in the hepatopancreas and stomach or the biomphalysin 17 in the mantel and stomach. Moreover, a high variation between tissues in the number of biomphalysins expressed can be observed (Figure 7B). The highest number of biomphalysins expressed was observed in the stomach, with 22 out of the 23 biomphalysins, while albumen gland and hemocytes solely displayed 11 and 12 biomphalysins expressed, respectively. Such high variability in tissue expression suggests that the different biomphalysins could exhibit different functional and translate a contrasting functions in different tissues. Many transcriptional factor binding sites in biomphalysin gene promoters have been mapped but the search for conserved motifs in all 23 biomphalysin promoter regions did not show any conservation throughout the whole group (Table S8). The diverse and different cis response elements found in the promoter region suggest that each individual gene could be regulated spatially and temporally by specific transcription factors reinforcing a functional divergence in this family (Table S9). However, among these transcription factors, Sox5 and AT-rich interactive domain-containing protein 3A (Arid3a) are the most represented. Both are involved in activation of immune gene transcription (ma, Lancto, 2010)(Tidwell, Schmidt, 2011)(Ward Ratliff, 2016) and B or T lymphocyte differentiation (Kurkewich, Klopfenstein, 2016)(Suto Tanaka, 2015). Consequently, expansion of biomphalysin genes could be associated with a functional diversification of the snail innate immune system to exhibit efficient response to different pathogens.

Diversification of functionalization Parler de l'interactome Guillaume

In-silico biomphalysin-targets of miRNA

To integrate acquired gene in a larger genetic network, the organism recipient must not only reformat the gene to use it in function of this own codon use but also set up a fine regulatory mechanism. As Biomphalysins could display a key role in immunity as receptor but also as effector, we have investigated if their expression could be regulated by miRNAs, a key component of immune regulation (Gracias, Katsikis, 2011). Sequences of 5' UTR, CDS and 3'UTR of all biomphalysins are examined using Miranda software to determine if they could be targeted by miRNAs described in *Biomphalaria glabrata* genome (Adema et al, 2017). All biomphalysins transcripts can be controlled by these small non-coding RNA molecules. One transcript can be affected by several miRNA as the 3'UTR of biomphalysin 3 targeted by two miRNAs bgl-miR-1992-3p and bgl-miR-34-5p. The same miRNA like bgl-miR-133-5p can interact with 7 sites located on different biomphalysin transcripts (Table S10). This finding in miRNA-mediated biomphalysin regulation underlying their genomic assimilation but also their functional integration into regulatory networks.

Discussion

Work in progress

Acknowledgements

We thank Nathalia Arancibia for her technical assistance. Thanks to Marie Buysse for technical help in molecular biology as Master student.

This work was supported by French National Research Agency (ANR) with the ANR JCJC INVIMORY (number ANR-13-JSV7-0009). The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

A COMPLETER par les auteurs

Figure and legends.

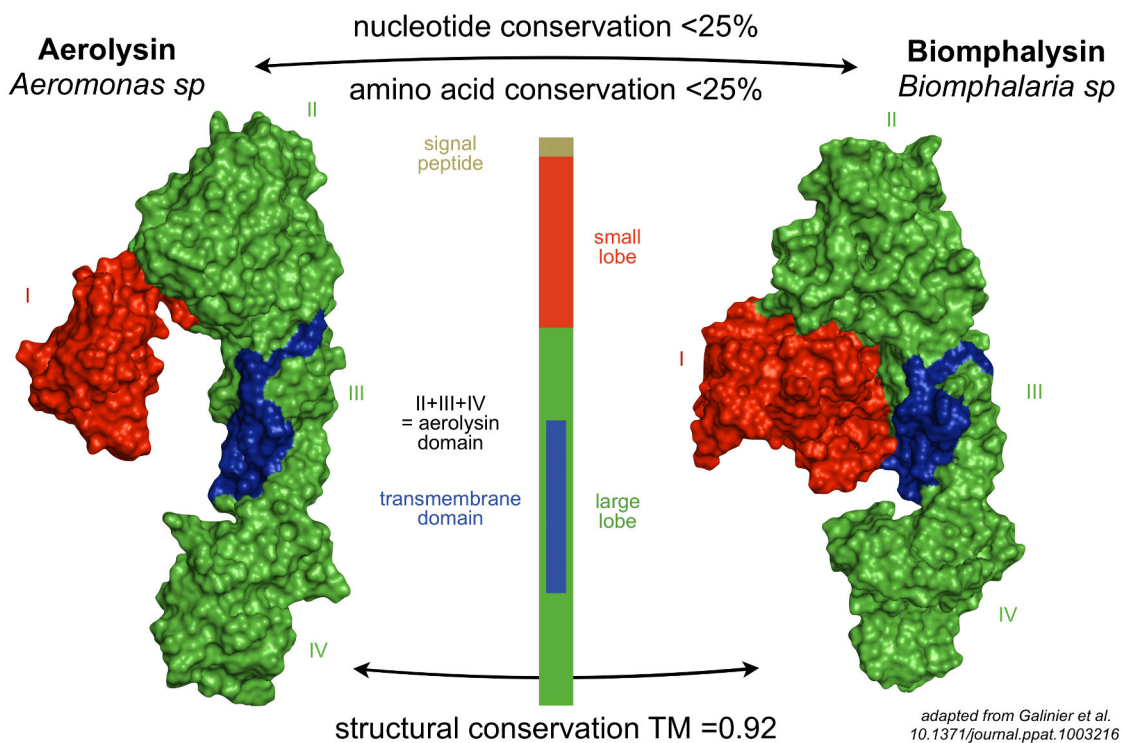


Figure 1. Biomphalysin: an aerolysin-like protein from *Aeromonas sp.* All typical domain features from aerolysins can be identified in biomphalysin, which includes the aerolysin

domain ((IPR005830) in one large lobe (green box), one small lobe (red box) and a signal peptide (brown box).

Fig 2. FISH

wik

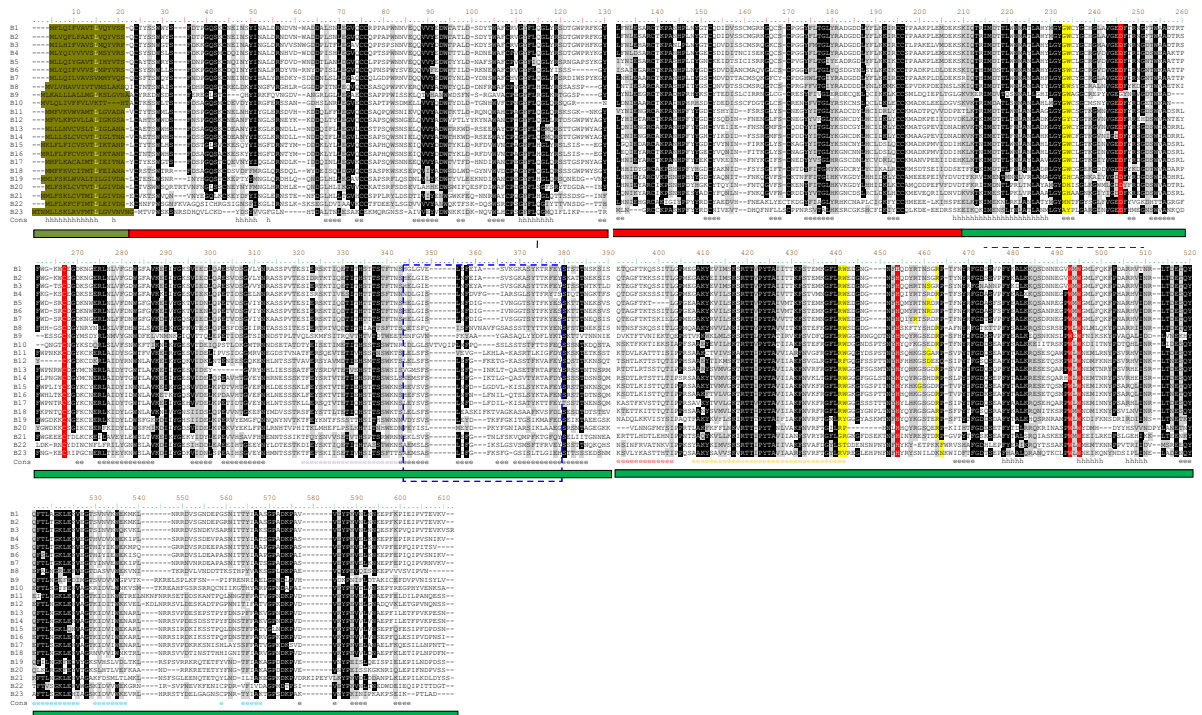


Figure 3 : Sequence alignment and predicted topology of Biomphalysin proteins Richard, montage?

Multiple alignment of the Biomphalysin proteins were aligned using CLUSTALO. Conserved amino acid residues identified in 75 % of all sequences are highlighted with a black box. Amino acids containing similar chemical properties are shown in a gray box. Key amino acids whose roles are described as important for oligomerization and lytic activity are indicated in white on a red background. The positions of secondary structure elements were predicted using the server PROMALS3D. α helices and β strands are indicated respectively by h and e. The putative peptide signal indicated in brown was predicted on SignalP 4.1 server. The Transmembrane Domain boxed in blue dotted was predicted using the PRED –TMBB server. The structural domains are represented as boxes. The signal peptide is in brown, the small domain in red and the large domain containing the aerolysin motif (IPR005830) in green.

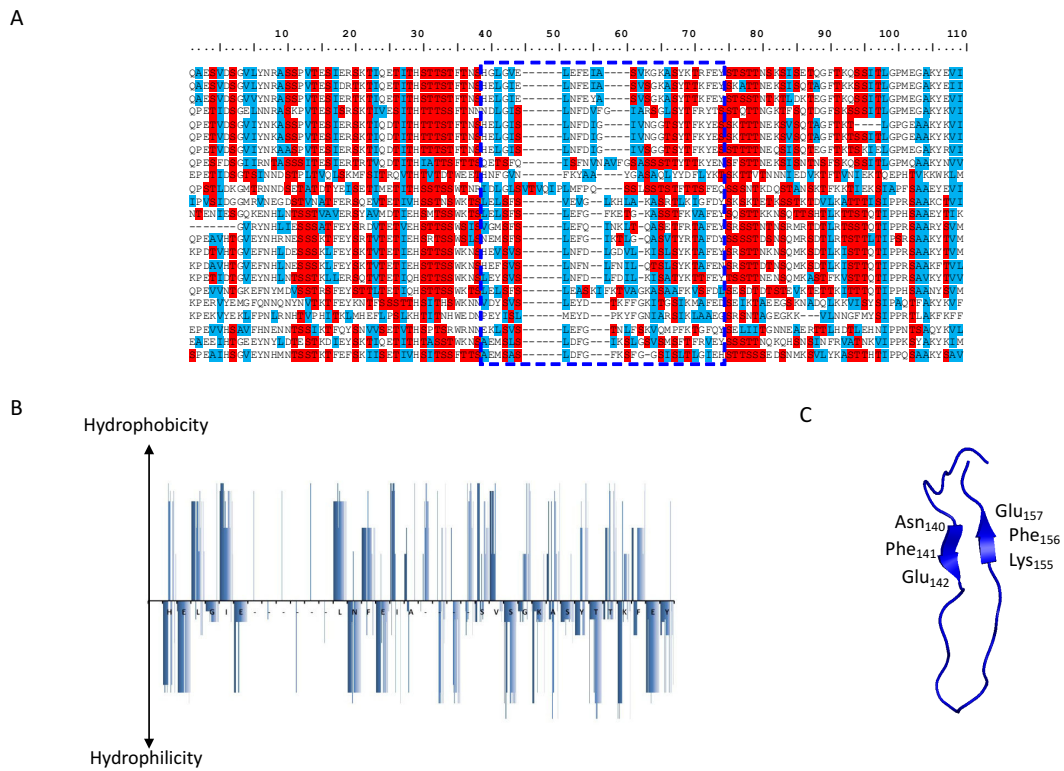


Figure S1 : TMD characterization in biomphalysin proteins

A. Multiple alignment of the region surrounding the Transmembrane domain of all Biomphalysin proteins was performed using the HHpred server. The Transmembrane Domain boxed in blue was predicted using the PRED –TMBB server and confronted to the TMD of aerolysin protein (AFP82959) from *Aeromonas hydrophyla*. Hydrophilics (Ser and Thr) and Hydrophobics (Val, Ile, Leu and Ala) are shown in red and blue respectively.

B. A hydropathy plot of the predicted TMD from all Biomphalysin proteins was done by the method of Kyte and Doolittle (1982). An alternation of hydrophilic and hydrophobic residues was observed for all predicted TMD. For example, TMD sequence of biomphalysin 2 is given in this plot.

C. Structure of the TMD of biomphalysin 2 was predicted by the I Tasser server from the complete sequence of the protein. Close view on TMD was generated by PyMOLWin. Beta sheets represented by arrows forms a beta hairpin similar to the loop region in domain III of aerolysin toxin. All Biomphalysins contains a transmembrane beta hairpin required to drive membrane insertion.

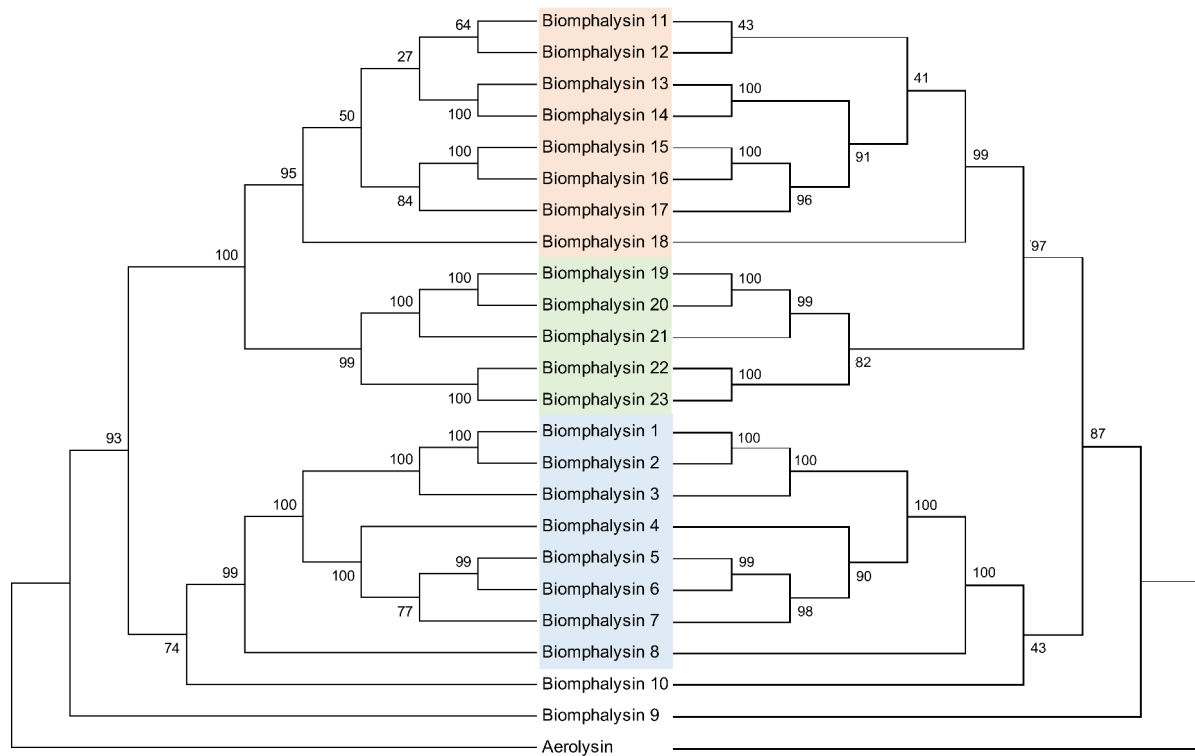


Figure 4 : Phylogenetic analysis of the gene (left) and protein (right) sequences from the 23 biomphalysins from *B. glabrata*.

The three clusters identified in this analysis are highlighted in blue, red and green for the clusters I, II and III that contain biomphalysins 1 to 8, 11 to 18 and 19 to 23, respectively. The aerolysin sequence from *A. hydrophila* was used as an outgroup. A bootstrap analysis of 2000 replications was carried out on the trees inferred from the neighbor joining method. Bootstrap values are shown at each branch of the trees. The accession number of each gene and protein used is listed in Supplementary Table AccNb. GUILLAUME.

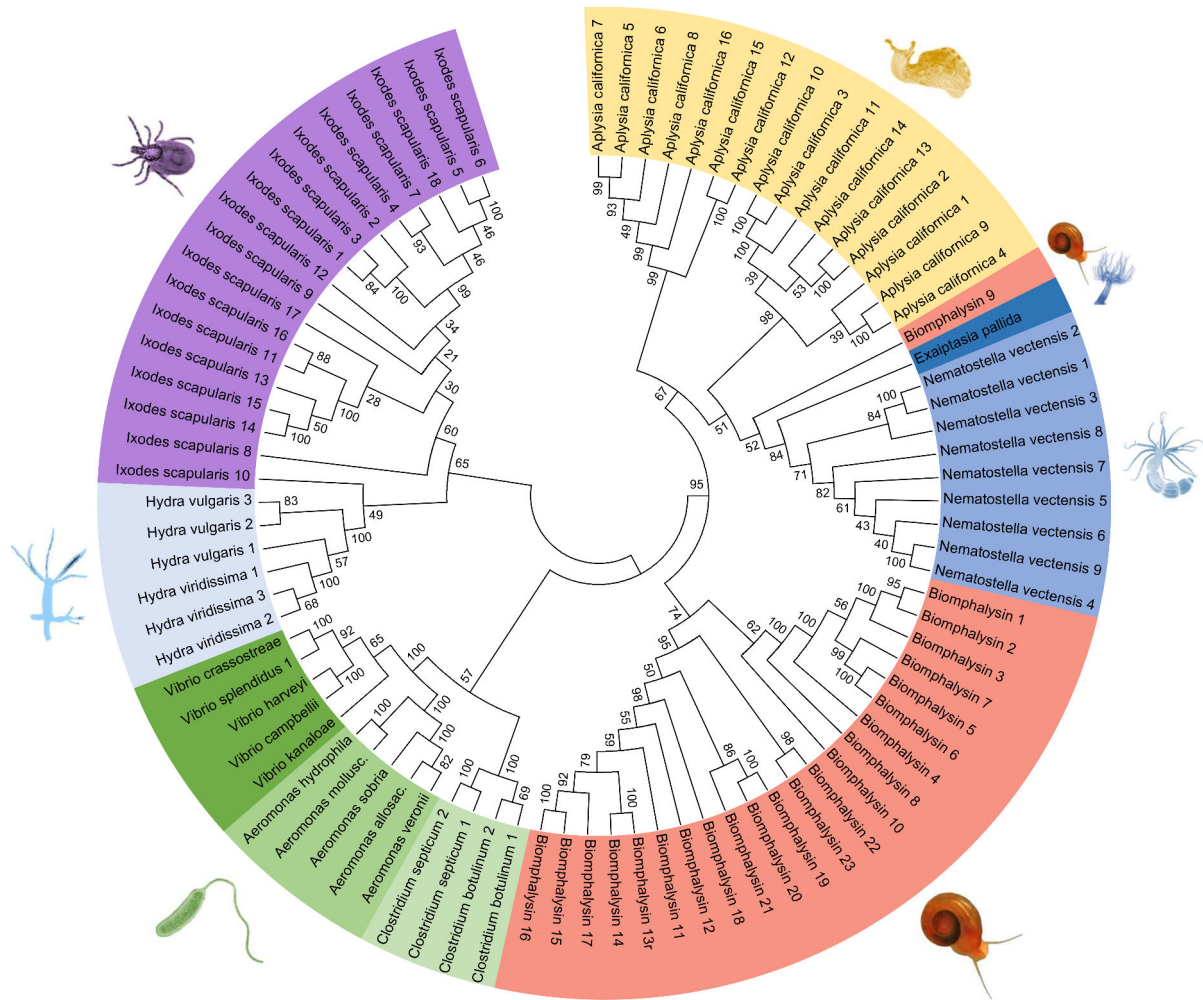


Figure 5. Phylogenetic analysis of the aerolysin domain (IPR005830) from 19 different species.

Arthropoda (*Ixodes scapularis*) is highlighted in purple, *Supplementary Fig. Correl* Gastropoda in red (*Biomphalaria glabrata*) and yellow (*Aplysia californica*), Cnidaria in dark blue (Anthozoa – *Exaiptasia pallida*), medium blue (Anthozoa – *Nematostella vectensis*) and in light blue (Hydrozoa – *Hydra viridissima* and *H. vulgaris*) and bacteria in dark green (*Vibrio sp.*), medium green (*Aeromonas sp.*) and light green (*Clostridium sp.*). A bootstrap analysis of 2000 replications was carried out on the trees inferred from the neighbor joining method. Bootstrap values are shown at each branch of the trees. The accession number of each gene and protein used is listed in [Supplementary Table AccNb](#) listed in [Supplementary Table AccNb](#). **Guillaume a corriger par rapport au bon numero de la table.**

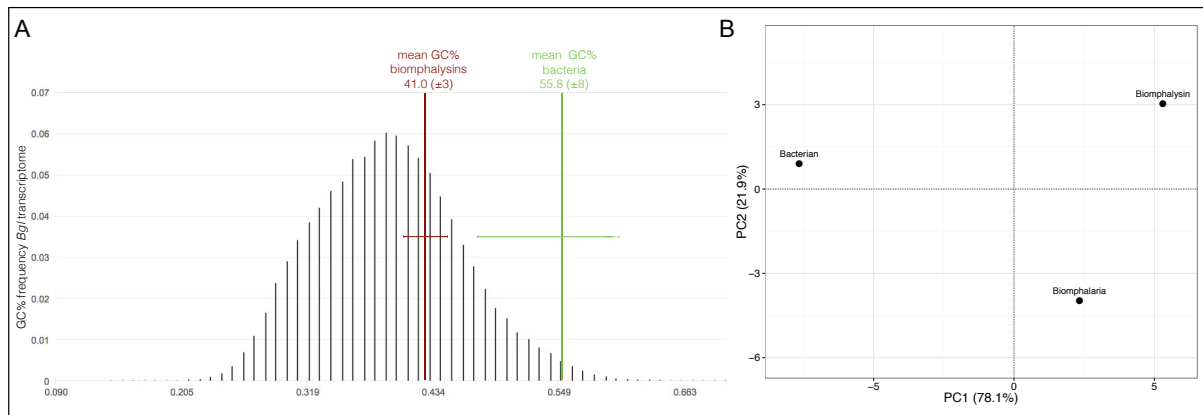


Figure 6 : Nucleotide support of domestication

A : Box-plot representation of GC content and (B) principal component analysis on codon usage (A) comparing nucleotide sequences of biomphalysins (N = 23), of other *Biomphalaria* sequences (± 1700 pb; N = 25) and of bacterial toxin sequences (N = 13). GC% content distribution indicate that *Biomphalaria glabrata* transcripts spread from 0.2 to 0.6 with peak of normal distribution at 39. Mean GC% of biomphalysins transcripts is 41.0 (± 3) while bacteria transcript reveal 55.8 (± 8) indicating a process of assimilation of the snail nucleotide code usage rather than historical bacterial one.

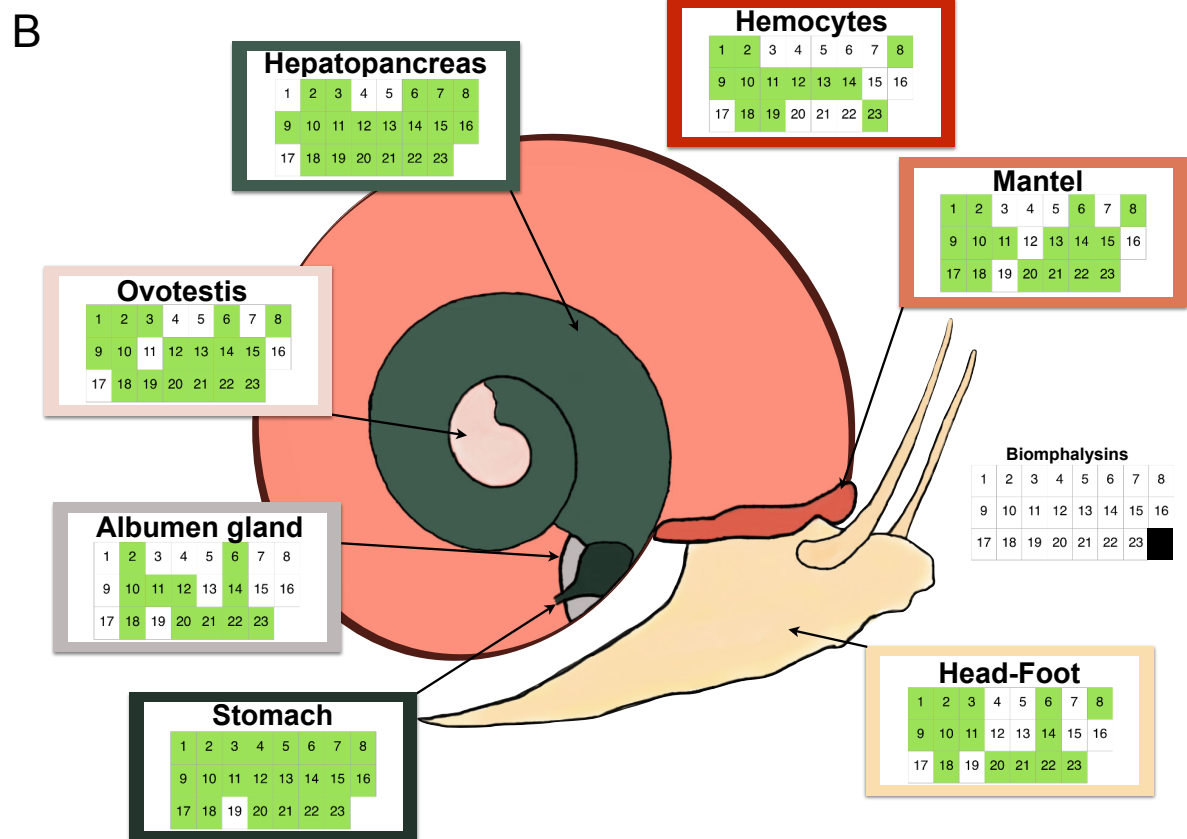
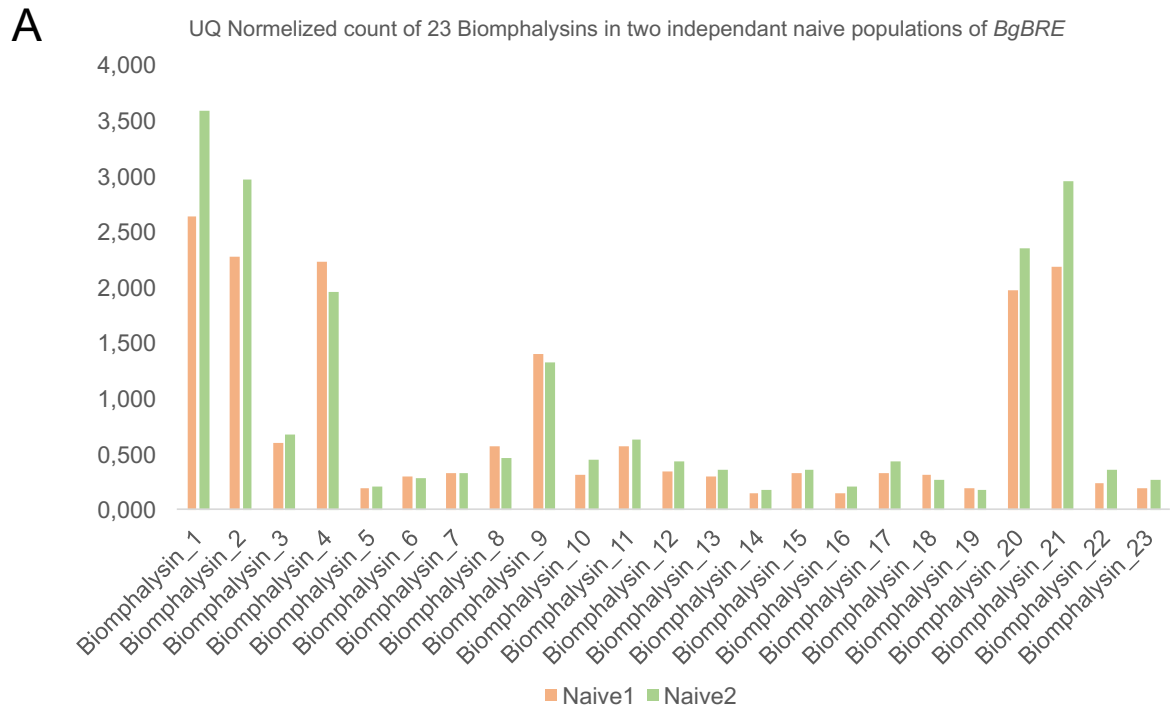


Figure 7 : Expression analysis of the biomphalysin family.

A. Constitutive expression of biomphalysin on whole snails

Transcripts abundance for all 23 biomphalysins retrieved from RNAseq experiment performed on two pools of 20 naive snails (Naive1/Naive2). Libraries from Naive1 and Naive2 samples are

highlighted in orange and green, respectively. Counts were normalized by upper-quartile (UQ) division for each library. All the 23 members are expressed in snails with high differences between each other's. Biomphalysin 1, 2, 4, 20 and 21 seems to be the most represented members in the snail.

B. Patterns of expression of biomphalysins in different snails tissues.

Each table represents the presence of the 23 biomphalysin transcript in a specific tissue by rt-PCR. From biomphalysin 1 in the upper left case to biomphalysin 23 in the lower right case of the table. A green-colored case correspond to a biomphalysin expressed in the tissue at least in one snail on 5 tested while an uncolored case indicates that the biomphalysin is not expressed in any biological replicate tested. Biomphalysin transcripts show mosaic expression in the different tissues for naïve snails.

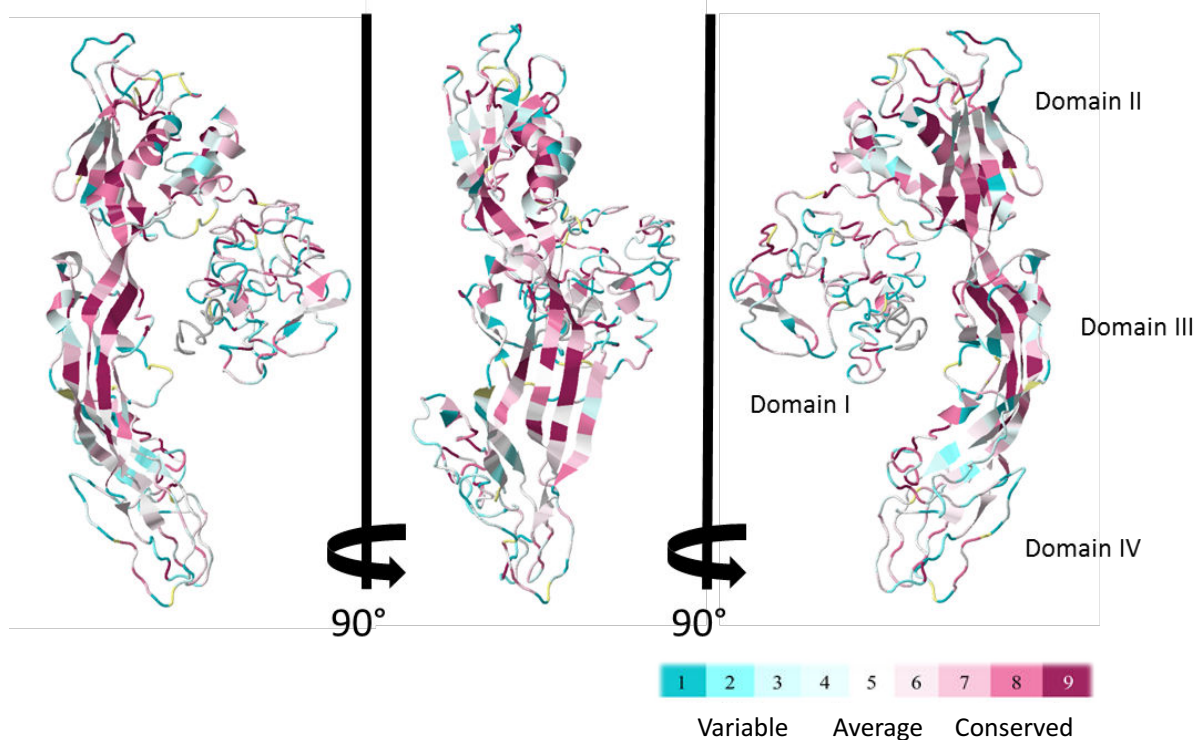


Figure 8 : ConSurf sequence conservation score of biomphalysin proteins.

Consurf image of biomphalysin was generated using alignment of all 23 biomphalysin proteins and plotted on the predicted structure of biomphalysin 1. Residues are colored according to conservation predictions analysed by the ConSurf server. Variable residues are shown in cyan and most conserved are in dark red. Images are rotated 90° about the y axis.

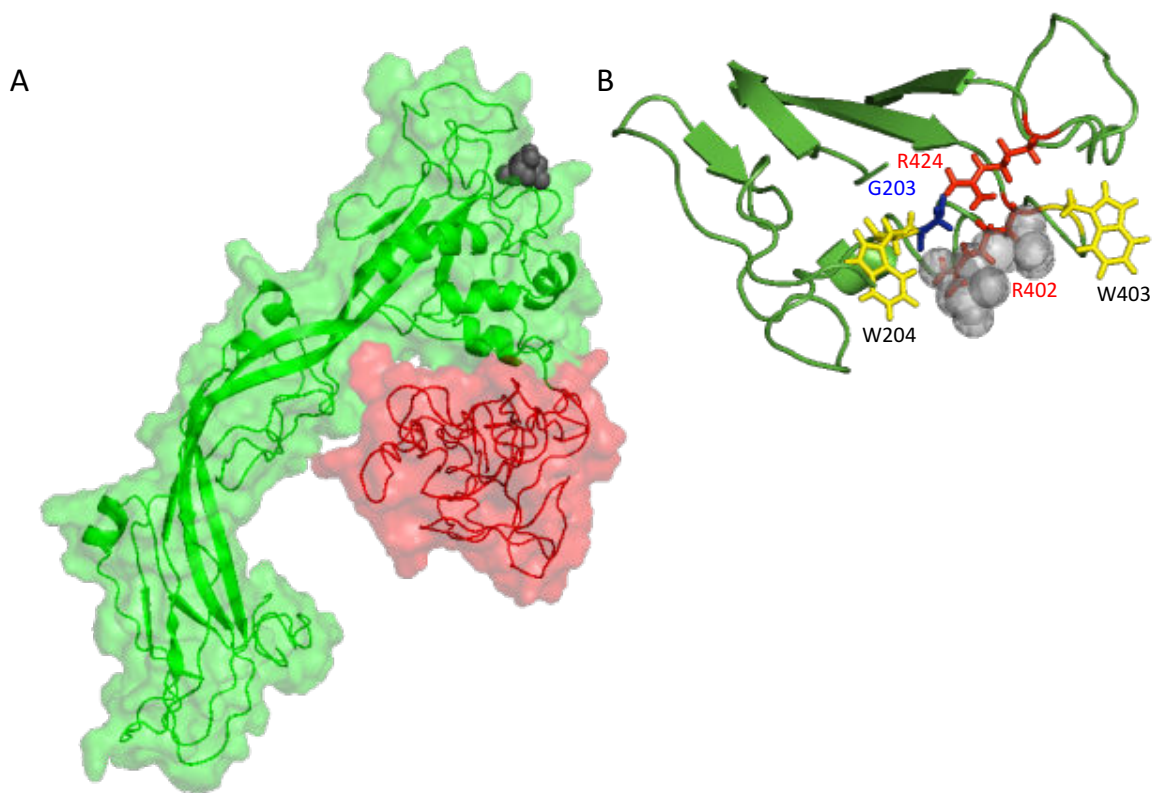


Figure 9 : Carbohydrate binding site prediction on biomphalysin proteins.

To predict biomphalysin proteins –ligand binding site, the meta-server COACH was performed using two comparative methods, TM and S-SITE. Ligand binding site prediction were generated from three dimensional structure of biomphalysin proteins. A. Surface representation of biomphalysin 16 protein interacting with alpha D mannose 6 phosphate (PDB : M6P). M6P are shown as spherical structure with gray carbon, phosphate and oxygen atoms. Ribbon representation of biomphalysin 16 was superimposed on a transparent surface map. B. Closeup of the binding site. Alpha D mannose 6 phosphate bound to the predicted pocket are located on the domain II of biomphalysin protein. The predicted residues involved in mannose binding are shown.

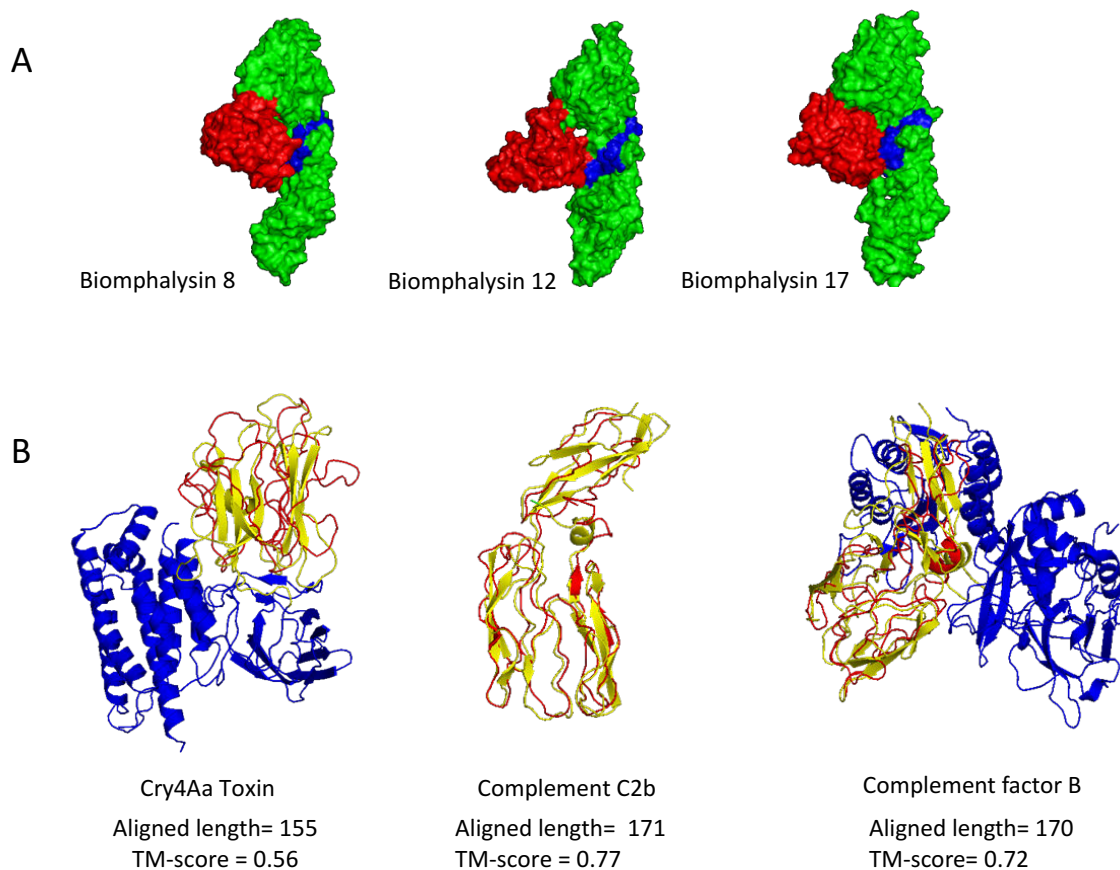


Figure 10 : Structural prediction of the Biomphalysin small lobes

C. Three-dimensional structure prediction of Biomphalysin 8, 12 and 17 was performed using I-Tasser server using the crystal structure of proaerolysin as pattern. A C score was determined for each prediction (see Table S5). Surface representation was realized with PyMol software. Small lobe was shown in red, the large lobe in green and the TMD domain in blue.

D. Structural similarities the small lobe of biomphalysin proteins 8, 12 and 17 were investigated against RCSB Protein Data Bank. A TM-score was calculated between the small lobes and the best hit found by I-Tasser server. A score greater than 0.5 reveals significant alignment, whereas a TM-score less than 0.17 indicates random similarity. 3D superposition of each small lobe of Biomphalysin 8, 12 and 17 and the respective best hit were performed by TM align and rendered with PyMol. The best structural alignment was reported between the predicted isolated structure of the small lobe in red and the significant template hit identified in yellow.

The peptides of the MS/MS spectra

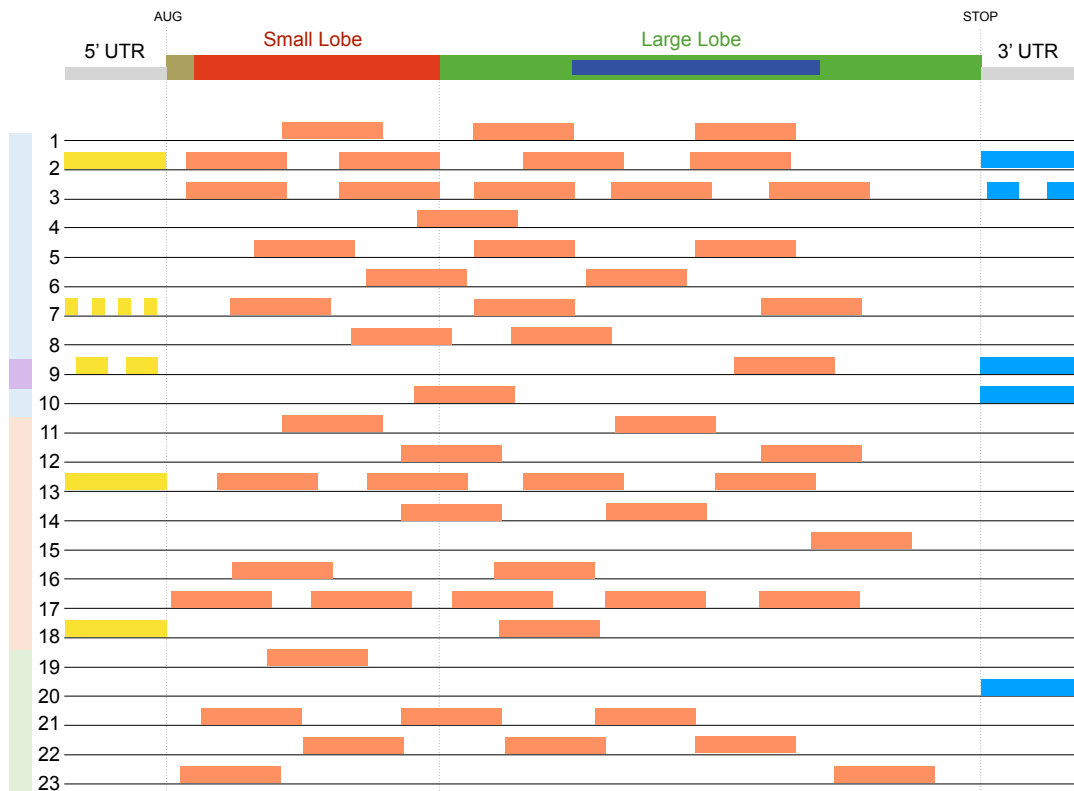


Figure MirRNA,

All the 23 members of biomphalysin can be potentially targeted by *Biomphalaria glabrata* miRNAs. Targeting can be carried from one to 7 different miRNAs. In the same way, targeting can be localise either on UTRs or CDS.

Supplementary material

Table S1 : Accession number and genomic localization of biomphalysin genes family

Table S2 : Identity score of nucleic and proteic alignment between all biomphalysins members **Table S3 : No transposable elements close/in biomphalysin. GEPARD Cristian**

Table S4 : Structural and protein sequence similarity between all biomphlaysin members and aerolysin toxin.

Table S5 : Scores of structural alignment and prediction of each domain separately (small lobe, aerolysin domain and whole sequence) and full list of the domain matched by the analysis with the small lobe as a query.

Table S6 ou Table 1 : Best hit predicted for a structural alignment with the isolated small lobes of each biomphalysin.

Supplementary Table AccNb. Accession numbers of the genes and proteins used for the phylogenetic analysis shown in Fig 5. **Guillaume**

Ambrose KV, Koppenhofer AM, Belanger FC. 2014. Horizontal gene transfer of a bacterial insect toxin gene into the *Epichloe* fungal symbionts of grasses. *Sci Rep* 4:5562.

Boissier J, Moné H, Mitta G, Bargues MD, Molyneux D, Mas-Coma S. Schistosomiasis reaches Europe. *The Lancet Infectious Diseases* 15:757-758.

Clayton AM, Dong Y, Dimopoulos G. 2014. The *Anopheles* innate immune system in the defense against malaria infection. *J Innate Immun* 6:169-181.

Coustau C, Kurtz J, Moret Y. 2016. A Novel Mechanism of Immune Memory Unveiled at the Invertebrate-Parasite Interface. *Trends Parasitol.*

Deleury E, Dubreuil G, Elangovan N, Wajnberg E, Reichhart JM, Gourbal B, Duval D, Baron OL, Gouzy J, Coustau C. 2012. Specific versus non-specific immune responses in an invertebrate species evidenced by a comparative de novo sequencing study. *PLoS One* 7:e32512.

Dong Y, Das S, Cirimotich C, Souza-Neto JA, McLean KJ, Dimopoulos G. 2011. Engineered *Anopheles* immunity to *Plasmodium* infection. *PLoS Pathog* 7:e1002458.

Dunning Hotopp JC, Estes AM. 2014. Biology wars: the eukaryotes strike back. *Cell Host Microbe* 16:701-703.

Etienne G.J. Danchin EAG, Sophie Mantelin, Adokiye Berepi, John T. Jones. 2016. Horizontal gene transfer from bacteria has enabled the plant-parasitic nematode *Globodera pallida* to feed on host-derived sucrose. *Molecular Biology Evolution.*

Galinier R, Portela J, Mone Y, Allienne JF, Henri H, Delbecq S, Mitta G, Gourbal B, Duval D. 2013. Biomphalysin, a new beta pore-forming toxin involved in *Biomphalaria glabrata* immune defense against *Schistosoma mansoni*. *PLoS Pathog* 9:e1003216.

Marchler-Bauer A, Lu S, Anderson JB, Chitsaz F, Derbyshire MK, DeWeese-Scott C, Fong JH, Geer LY, Geer RC, Gonzales NR, et al. 2011. CDD: a Conserved Domain Database for the functional annotation of proteins. *Nucleic Acids Research* 39:D225-D229.

Molehin AJ, Rojo JU, Siddiqui SZ, Gray SA, Carter D, Siddiqui AA. 2016. Development of a schistosomiasis vaccine. *Expert Review Of Vaccines* 15:619-627.

Moran Y, Fredman D, Szczesny P, Grynberg M, Technau U. 2012. Recurrent horizontal transfer of bacterial toxin genes to eukaryotes. *Mol Biol Evol* 29:2223-2230.

Odoemelam EC, Raghavan N, Ittiprasert W, Miller A, Bridger JM, Knight M. 2010. FISH on chromosomes derived from the snail model organism *Biomphalaria glabrata*. *Methods Mol Biol* 659:379-388.

Pinaud S, Portela J, Duval D, Nowacki FC, Olive MA, Allienne JF, Galinier R, Dheilily NM, Kieffer-Jaquinod S, Mitta G, et al. 2016. A Shift from Cellular to Humoral Responses Contributes to Innate Immune Memory in the Vector Snail *Biomphalaria glabrata*. *PLoS Pathog* 12:e1005361.

Portela J, Duval D, Rognon A, Galinier R, Boissier J, Coustau C, Mitta G, Theron A, Gourbal B. 2013. Evidence for specific genotype-dependent immune priming in the lophotrochozoan *Biomphalaria glabrata* snail. *J Innate Immun* 5:261-276.

Puigbò P, Bravo IG, Garcia-Vallvé S. (Puigbò2008 co-authors). 2008. E-CAI: a novel server to estimate an expected value of Codon Adaptation Index (eCAI). *BMC Bioinformatics* 9:1-7.

Szydlowski L, Boschetti C, Crisp A, Barbosa EG, Tunnaciff A. 2015. Multiple horizontally acquired genes from fungal and prokaryotic donors encode cellulolytic enzymes in the bdelloid rotifer *Adineta ricciae*. *Gene* 566:125-137.

Tamura K, Stecher G, Peterson D, Filipowski A, Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution* 30:2725-2729.

Xiang Y, Yan C, Guo X, Zhou K, Li S, Gao Q, Wang X, Zhao F, Liu J, Lee WH, et al. 2014. Host-derived, pore-forming toxin-like protein and trefoil factor complex protects the host against microbial infection. *Proc Natl Acad Sci U S A* 111:6702-6707.

Yoshino TP, Wu XJ, Gonzalez LA, Hokke CH. 2013. Circulating *Biomphalaria glabrata* hemocyte subpopulations possess shared schistosome glycans and receptors capable of binding larval glycoconjugates. *Exp Parasitol* 133:28-36.

Publication : Polymorphic sets of immune receptors support specificity in the innate immune memory of the Schistosomiasis vector snail, *Biomphalaria glabrata*

PINAUD Silvain, PORTET Anaïs, ALLIENNE Jean-François, SAINT-BEAT Cécile, ARANCIBIA Nathalie, GALINIER Richard, DUVAL David, GOURBAL Benjamin

*Article en préparation soumission dans Molecular Ecology prévue pour Septembre 2017

Ma participation à ces travaux :

La mémoire immunitaire innée chez *B. glabrata* fut mise en évidence au laboratoire en 2013. Il est ensuite montré lors de la réponse immunitaire du mollusque un shift entre une réponse cellulaire lors d'une première infection à une réponse humorale lors d'un challenge en 2016. Ce papier s'inscrit dans la continuité de ces travaux et cherche à mettre en évidence la présence d'une spécificité dans cette réponse immunitaire mémoire. Pour cela une étude transcriptomique fut réalisée, à laquelle j'ai participé, qui met en évidence un grand nombre de récepteurs impliqués dans cette réponse immunitaire. Par ailleurs, lors du challenge immunitaire la réponse cellulaire d'encapsulation n'étant plus activée, une étude hémocytaire fut entreprise pour mettre en évidence des changements potentiels de ces cellules. Dans ce cadre, des études de cytométrie en flux ont été réalisées, auxquelles j'ai participé, et ont permis de mettre en évidence que la morphologie des cellules hémocytaires était inchangée entre la réponse immunitaire cellulaire lors de la première infection et la réponse immunitaire mémoire. Ces travaux ont permis d'émettre l'hypothèse d'un entraînement des cellules, au cours de la première infection, à mieux répondre lors du challenge.

Polymorphic sets of immune receptors support specificity in the innate immune memory of the Schistosomiasis vector snail, *Biomphalaria glabrata*

PINAUD Silvain, PORTET Anaïs, ALLIENNE Jean-François, SAINT-BEAT Cécile, ARANCIBIA Nathalie, GALINIER Richard, DUVAL David, GOURBAL Benjamin

Abstract (<250)

Invertebrate immune response may be primed in a sustainable manner, leading to the failure of a secondary encounter with the same pathogen. This phenomenon called innate immune memory was described in numerous clade of invertebrates. Underlying molecular support was often lacking and delayed its comprehension. Present study focuses on the Schistosomiasis vector snail *Biomphalaria glabrata*, in which first molecular support of priming was described as depending on a shift from a cellular immune response to a humoral immune response. *Biomphalaria* was described to exhibit a genotype dependant immune memory where increasing genetic distance between parasites used for primary and secondary infection decreased the associated resistance. This work proposes an in-depth study of molecular support of specificity in the innate immune memory response. As proposed in vertebrates, *Biomphalaria* regulate different sets of innate immune receptor repertoire to face up different strains of parasite during immune memory. Combined with previously published works, these results attempt to suggest combinatory use of PRRs in invertebrates to distinguish different strains of parasite during acquisition of innate immune memory. Immunization experiment bring results that snail can become resistant after exposition to parasite extract. Hemolymph transfer bring clue that specificity in this vector snail is so far higher than expected and this circulating hemolymphatic compartment display a strain-specific toxicity.

This work brings first evidence of the molecular support of the innate immune memory specificity in invertebrates.

Introduction

Immune memory is no longer a vertebrate feature but a shared way, for living organisms, to limit physiological cost caused by pathogens frequently met during their whole life. Storing information of pathogen represent a way to answer to a secondary occurring infections at lowest cost (Contreras-Garduno, 2016 and Kurtz & Armitage, 2006).

Living organisms have evolved complex immune system to confront pathogens biological-traits and diversity, from prokaryote ((Marraffini and Sontheimer 2008)) to eukaryote including plants ((Dangl, Horvath et al. 2013, Calil and Fontes 2016)) or animals, and within animals from invertebrates ((Moret and Schmid-Hempel 2000, Moret and Schmid-Hempel 2001, Haine, Moret et al. 2008, Cirimotich, Dong et al. 2011, Portela, Duval et al. 2013)) to vertebrates ((Du Pasquier 2004, Eason, Cannon et al. 2004, Cooper and Alder 2006)). These new capabilities for the innate immune system to store pathogens information through resistance acquisition were referred as “trained immunity” for vertebrates (Netea, Quintin et al. 2011) or “immune priming” or “innate immune memory” for invertebrates (Milutinovic and Kurtz 2016). All this acquired resistances have been shown to be immuno-globulin (Ig) independent without any homologous molecules in invertebrates. Also, question of: do immuno-globulin (Ig) independent immune memory represent a way of remembering about pathogen resistance across living kingdom, remains debated (Netea and van der Meer 2017)

Specificity of this innate immune memory can be assessed by tested different type of pathogens through a homologous (same pathogens from primary and secondary immune challenge) or heterologous (different pathogens between both infections) set of infection and reinfection. A high specificity is observed when a homologous re-exposure to a pathogen lead to a higher protection than a heterologous one (Jorge Contreras-Garduno, Humberto Lanz-Mendoza et al. 2016, Milutinovic and Kurtz 2016). For example, *Drosophila* display high specificity against bacterial infection. When infected with *Streptococcus pneumoniae* or *Beauveria bassiana*, flies were found protected against reinfection with the same microorganisms, but not against other pathogens (Pham, Dionne et al. 2007). Same thing in the copepod with a tapeworm parasite, authors reported a significant reduction of reinfection rate with homologous reinfection (Kurtz and Franz 2003). Also, shrimp display better protection against a viral strain previously used as oral vaccination (Witteveldt, Cifuentes et al. 2004).

If the existing observations of specific immune priming in invertebrates are mainly phenomenological, the potential underlying molecular and/or cellular mechanisms of such immune specificity remained poorly widen.

In the present paper, we propose to investigate this question in a suitable model involving the Lophotrochozoan snail, *Biomphalaria glabrata*, and its metazoan parasite, the trematode *Schistosoma mansoni*. *Biomphalaria* snails are pinpointed invertebrates as vector of Schistosomiasis, which is the second most widespread human parasitic disease after malaria, causing a substantial public-health burden with about 200 million humans infected in 74 countries and more than 200,000 deaths annually (King, Dickman et al. 2005).

Recently, a high degree of immune specificity has been shown in *Biomphalaria* snail through homologous and heterologous challenges (Portela, Duval et al. 2013). While homologous experiments show protection of 100%, heterologous experiments display a protection that decreased with increasing genetic distance between the parasites used for primo-infections and challenges (Portela, Duval et al. 2013). The first molecular bases of immune priming have been also described for one Brazilian *Biomphalaria/Schistosoma* sympatric interaction. A Transcriptomic effort, combined with a comparative proteomic approach brought first evidences of a shift in the immune response during the acquisition of resistance (Pinaud, Portela et al. 2016). This immune shift occurred at two different scales. First, it could be observed at the phenotypic scale, a shift from a cellular immune response (hemocyte encapsulation), following primo infection, to a humoral immune response (without encapsulation and production of plasmatic factors) following secondary immune challenge (Pinaud, Portela et al. 2016). Second at the molecular scale, we identified a shift in transcript expression where a transcriptomic reprogramming was associated to acquisition of resistance to reinfection in *Biomphalaria glabrata* (Pinaud, Portela et al. 2016).

Herein, we bring along first exploration of the molecular support of genotype-dependant innate immune memory specificity by comparing homologous versus heterologous multi-scale host-parasite interactions among high compatible host/parasite couples (Figure 1). Hemocyte flow cytometry associated with vaccination and *in-vitro* toxicity assays of the cell-free plasmatic compartment bring new clues for a high immune specificity potential in *Biomphalaria* snails.

Innate immune response may induce very highly-specific and long-lasting resistance in both vertebrate and invertebrate as already proposed in the combinatory of pathogens sensors theory (Hargreaves and Medzhitov 2005, Nish and Medzhitov

2011). Our results represented one of the first description of the activation of a diversified repertoire of putative innate immune receptor following acquisition of innate immune memory in a non-model invertebrate species. Altogether these results also suggest that the complex combinations or multimerizations of diversified innate immune receptors and effectors may support the genotype-dependant immune memory specificity observed in *Biomphalaria* snails.

Material & Methods

Ethical statement.

The laboratory and experimenters possessed an official certificate of the French Ministry of National Education, Research, and Technology, CNRS and DRAAF Languedoc Roussillon for experiments on animals, animal housing, and animal breeding (# A66040; decree # 87–848, October 19, 1987; and authorization # 007083).

***Biomphalaria* snail and *Schistosoma* parasites**

One strain of snail has been used for all experiments and belongs to *Biomphalaria glabrata* originating from Brazil (BgBRE). Its sympatric strain of parasite *Schistosoma mansoni* from Brazil (SmBRE) has been used for homologous interaction while heterologous interactions were conducted with a strain of the same specie: *Schistosoma mansoni* from Venezuela (SmVEN); or with another specie: *Schistosoma Rodhaini* from Burundi (SROD). Infectivity phenotypes for all interactions were previously described as highly compatible with prevalence values from 80% to 100% ((Theron, Pages et al. 1997, Portela, Duval et al. 2013)).

Innate immune memory experimental protocols

Duration of the immune memory was tested by primo-infecting BgBRE snails (n=100) with 10 SmBRE miracidia. Then at 15, 25, 56, 72, 140, 162 days after primo-infection, snails were challenged with 10 SmBRE miracidia. For RNAseq, BgBRE snails were primary individually exposed (immune priming) to 10 SmBRE miracidia then secondarily individually exposed (immune challenge) to 10 miracidia of each strain (SmBRE, SmVEN, SROD) 25 days later. For proteomic and *in-vitro* toxicity assays, priming was realised on BgBRE snails at 15 days after primo-infection with 10 SmBRE miracidia.

High through put approach protocols

For the RNAseq approach, pools of 20 BgBRE snails were recovered as previously described ((Pinaud, Portela et al. 2016)) at 1, 4 and 15 days after the immune challenges then equimolar amounts of each of these time points were grouped together into 3 separate strain samples named BRE, VEN and ROD. Two pools of 20 uninfected snails (naive1 and naive2) were sampled and used as controls. Statistical analyses were performed as already described ((Pinaud, Portela et al. 2016)) following the pipeline on our Galaxy Project server. Briefly, best quality reads are filtered (phred >29) then reads were aligned with Bowtie (v2.0.2, quality mapping 255) and PCR duplicates were removed thanks to SAM tools. DESeq2 software was used to compare duplicates samples of uninfected snails (naive1 and naive2) with our three immune challenge condition (BRE, VEN and ROD) and all statistically different transcripts are recovered (P value <0.05) and annotated firstly manually (Blast X and Interproscan against nr database) then using Bast2GoPro for all the other available databases. A heatmap was constructed from log₂ fold change of each

transcripts using Hierarchical Ascending Clustering (HAC) with Pearson correlation, as applied by the Cluster 3.0 (Eisen, Spellman et al. 1998) and JavaTreeView software packages.

Vaccination

All developmental stages of *Schistosoma* parasites were recovered. Miracidia and cercaria (swimming free-living stages) were recovered in water and directly pellet by centrifugation. Primary sporocysts (Sp1) were recovered by *in-vitro* transformation from miracidia as previously described ((Roger, Mitta et al. 2008)). Secondary sporocysts (Sp2) were recovered in the digestive gland of 30 days infected snails (Sp2 representing 70% of the digestive gland at this point) and healthy glands from naïve snails were used as control (Sp2 Control). All the samples are natively extracted in 150 µL TBS-Tween 0.05% buffer. Three cycles of frozen-melting (-80°C to 4°C) were followed by four pulses of sonication (Vibra-cell 75185) at 20% max amplitude for 20 seconds at 4°C; then proteins were dosed (2D Quant kit, GE Healthcare life sciences) and stored at -80°C. A pool combining equimolar amount of all 4 parasite stages protein extracts (named pool) was constituted and used for vaccination experiments to test for parasite antigen synergistic effects.

For homologous vaccination, 500 BgBRE naïve snails are individually anaesthetised (2h in 0.05% Pentobarbital sodium 200mg/ml (Vetoquinol Dolethal) in 26°C drilling water) and injected in the head-foot region with 1µg in 10µL TBS-Tween 0.05% buffer of each parasite protein extract (Miracidia, Sp1, Sp2, Sp2Control, Cercaria and Pool) then snails were individually exposed to 10 SmBRE miracidia 15 days after vaccination. A TBS-Tween buffer injection was used alone to test for needle injection effect (Control). For heterologous vaccination, 200 BgBRE naïve snails are

individually injected in the same conditions as previously described with Miracidia and Sp1 extracts from SmVEN and SROD, then individually exposed to 10 SmBRE miracidia 15 days after vaccination. Fifteen days after infection, snails are fixed in Raillet-Henry solution to quantify parasite prevalence as previously described (Portela, Duval et al. 2013).

Cell-free hemolymph transfer and resistant phenotype

Naïve BgBRE snails were individually primary-exposed to 10 miracidia (SmBRE) and hemolymph was recovered from 30 primed snails 15 days after infection (sample was named 15DP for 15 days primed hemolymph). Then snails were individually secondary exposed again to 10 miracidia (SmBRE), and hemolymph from pool of 30 individuals, were sampled at 1, 4 and 15 days after the immune challenge (sample were named 1DC, 4DC and 15DC). Finally, hemolymph from 30 naïve snails were sampled as control (named naïve). All hemolymph samples were centrifuged (2,500 rpm for 15 min at 4°C) to pellet hemocyte cells and supernatant referred as cell-free hemolymph was kept at -80°C until used.

Three hundred naïve snails were individually injected with 10 µL of each cell-free hemolymph samples (Naïve, 15DP, 1DC, 4DC and 15DC) and saline solution (Chernin's balanced salt solution, CBSS) was used in control injection. Fifteen days after injection, all snails were individually exposed to 10 miracidia (SmBRE), and 15 days after infection, snails were fixed in Raillet-Henry solution to estimate parasite prevalences (number of infected snails in the exposed population).

***In vitro* cell-free hemolymph toxicity assay: a validation of immune specificity**

Naïve BgBRE snails were individually primary exposed either to 10 miracidia SmBRE or SmVEN, then secondary exposed to homologous 10 miracidia SmBRE or SmVEN infection. Cell-free hemolymph was recovered 1 day after immune challenged as described above and kept at -80°C. *In vitro* primary sporocysts (Sp1) were produced as previously described ((Roger, Mitta et al. 2008)) for all the three strains (SmBRE, SmVEN and SROD). *In vitro* toxicity assay were done by putting Sp1 in contact, at 26°C, with 500µL of cell-free hemolymph from naïve snails or from 1 day post-challenged snails, or with CBSS culture medium. Mortality was assessed after 4 hours of contact under a light microscope, with the Sp1 considered “dead” when we failed to observe motility and/or beating of the flame-cell flagella.

Hemocytes flow cytometry analysis

Naïve BgBRE snails were individually primo-infected with 10 miracidia (SmBRE). Hemolymphs from 6 individual snails (biological replicates) were sampled from the head-foot region at 1 day, 4 days and 15 days (1DP, 4DP and 15 DP) after primo-infection. Then SmBRE primed snails were individually exposed to a secondary infection (immune challenge) with 10 miracidia either SmBRE or SmVEN. Then hemolymphs from 6 individuals (biological replicates) were sampled at 1 day (1DC). All samples were directly treated after recovery. Forty microliters of hemolymph per snails are mixed with 360µL of 2% filtered formol-CBSS solution containing 4x concentration of SYBR green I. After short and gentle vortexing, samples were incubated in the dark at 26°C then flash-frozen in liquid nitrogen before being stored at -80°C. Estimation of debris/cells was performed using Propidium Iode staining at 0.05µg final and analyzed in 617nm wave length reading. All readings

were carried out in FacsCanto (MRI platform, Montpellier), then data were analysed on FlowJo v10 software on Mac v10.12. All tracks are merged by samples / hours.

Immune-relevant gene expression following vaccination

BgBRE snails were injected with parasite protein extracts as described above in the vaccination section, then 4 pools are constituted with hemocyte samples recovered from 3 individual snails. mRNAs were extracted using TRIZOL® (Sigma Life Science, USA) according to the manufacturer's instructions and subsequently reverse transcribed to first strand cDNA as described by manufacturer and treated with dsDNase (Maxima First Strad cDNA Synthesis Kit with dsDNase, Thermo Scientific, USA). Real-time RT-PCR analysed was performed in the LightCycler 480 System (Roche) with a volume of 10 µL comprised 20ng cDNA diluted with MilliQ-H2O in 2 µL, 5 µL of No Rox SYBR Master Mix blue dTTP (Takyon) and 1.5 µL of each 2X primers (see supplementary Table X for details on primers and genes tested). The cycling program was as followed: 95°C for 4 minutes, followed by 45 cycles of 95°C for 10 sec, 60°C for 20 sec and 72°C for 20 sec. Expression levels of targeted genes were normalised relative to S19 gene.

Results

Innate immune memory pattern in *Biomphalaria glabrata*

We test the acquired immune memory after 15 days following primary infection with 10 SmBRE miracidia. Among primed snails (N=74, 100% primo-infected), 0% of tested snails have been re-infected following immune challenge with again 10 SmBRE miracidia at 15 days after primary infection. The duration of the innate immune memory was also tested by re-exposing primed snails to 10 SmBRE miracidia, at 15, 25, 56, 72, 140, 162 days after primo-infection. Primed snails were

all died at 162 days after primo-infection thus it was impossible to re-infect snails for this time-point. For all the others time-points tested, challenge snails were always resistant to 10 SmBRE re-exposure. Results were as follow: at 15days post-priming 100% were resistant (15/15 snails), at 25d 100% (15/15 snails), at 56d 100% (15/15 snails), at 72d 94% (14/15 snails), and at 140d 100% (15/15 snails). These results demonstrated that innate immune memory was acquired early following infection (from 15 days after infection) and thereafter was maintained all the snail life span.

Transcriptomic support of innate immune memory specificity

A RNAseq approach was used to identified differentially represented transcripts following challenges with homologous or heterologous parasite infections, respectively SmBRE, SmVEN or SROD. Venn Diagram **(FIG 2.A)** showed that among 4202 differentially represented transcripts, 22% (representing 924 different transcripts) appeared as common whichever was the strain used for immune challenges. Strain-specific transcripts were massively identified for homologous SmBRE challenge (45.8% representing 2013 transcripts) then more lightly for heterologous immune challenge with SmVEN (9.6% and 405 transcripts) or SROD (5% and 210 transcripts). The heatmap of these differentially represented transcripts brings more precise information by highlighting 14 different transcript clusters **(FIG 2.B)**. Clusters 1 to 7 corresponded to over-represented transcripts and clusters 8 to 14 to down represented one's. Both clusters 1 and 14 corresponded to differentially represented transcript shared between all the strains used for immune challenge. Cluters 7 and 8 corresponded to the two most represented clusters with respectively 1215 and 1124 transcripts for the SmBRE immune challenge. Concerning this innate immune memory response more globally, with 3339 transcripts differentially

regulated, the homologous re-infection with SmBRE induces the largest response while SmVEN strain induce regulation of 1838 immune related-transcript and SROD only 1525 transcripts. Interestingly, lot of immune related-transcripts can be identified in these several clusters. Mainly three families of immune-related transcripts can be defined, (i) the immune receptors that potentially bind pathogens factors, (ii) immune effectors, able to directly have deleterious effect on the pathogens or activation effect on host immune response and (iii) other immune related-transcripts triggering maturation activation of immune complex. In this way, the putative immune receptors are widely distributed across clusters but with differential distribution between the several parasite strains. Immune effectors, are weakly distribute on the heat-map. Biomphalysin, a beta-Pore Forming Toxin secreted in snail plasma, able to directly kill the parasite is present in cluster 1. Cluster 1 is shared by all conditions of infection, and thus Biomphalysin could explain part of the humoral phenotype observed in innate immune memory process. Biomphalysin can be observed specifically upregulated following *S. Rodhaini* infection and downregulated following *S. mansoni* Venezuela one's. Alignment of Biomphalysin transcripts suggests that more than one variant of Biomphalysin family members were involved in this memory response (data not shown). Aquaporin have been previously described as key actor of immunoregulation during schistosomes infections (Zhang, Zhu et al. 2015) or macrophage deficiency (Zhu, Feng et al. 2011). Two variants up-regulated in the cluster 1 and one in the 7 (common and Bre specific) and can be related with activity of hemocytes. Anti-microbial peptides (AMPs) and their related members of antimicrobial protein are also included in the effector superfamily. The *B. glabrata* lbp/bpi (lipopolysaccharide binding protein/bactericidal permeability increasing protein) are mainly observed (22 variants) down-regulated in response of the three

parasite strains (clusters 14). Two members can be observed specifically up-regulated in response of the SmBRE strain (cluster 1) while one is up-regulated in response shared by SmBRE and SROD strains (cluster 4). The mytimacin, a macin AMP, previously described in the mussel *Mytilus*, is up-regulated for one variant in the SmBRE specific response (cluster 7) while one variant is up-regulated in the shared response between SmBRE and SROD (Cluster 4). Concerning the third group of immune related-transcript, several immune regulator and non-canonical immune-related protein are recovered. The glutamate-synthase is up-regulated whatever the strain used for challenged and can support the energetic effort for resistance as previously described in the metabolic switch supporting innate immune memory in vertebrate (Netea). Collagen, Hemagglutinin, Haemoglobin or SCO-spondin as extra-cellular matrix (ECM) component present mosaic regulation depending of the parasite strain used for challenge with at the same time common regulation of collagen or hemagglutinin variants for the shared clusters 1 and 14 or a specific down-regulation of 11 variants of haemoglobin for SmBRE challenge. These results support the hypothesis of a complex restructuring of ECM occurring during the immune shift from cellular to humoral immune response in innate immune memory. The serpin family, a serine-protease inhibitor family implicated mainly in inflammatory processes, have heterogeneous regulation across conditions, 26 variants are downregulated for all parasite challenges (cluster 14). Notwithstanding, 4 isoforms of serpin b6-b are up-regulated exclusively following the homologous challenge (cluster 7). Cadherin and protocadherin belonging to a superfamily of transmembrane proteins implicated in cell-to-cell binding or adhesion were also identified. Following SmBRE immune challenge, 40 different transcripts of this family are down-regulated including protocadherin fat 1, 3, 4, 20. Same patterns of

regulation are also observed for 14 transcripts of hemocytin, a highly-glycosylated adhesive protein related to hemostasis or encapsulation of foreign substances for self-defence.

To go further in the understanding of innate immune memory specificity, we put a special focus on putative immune receptors to decipher a specific repertoire activation following parasite immune challenges (Figure 2). Homologous SmBRE immune challenge leads to modulation of 82 putative immune-receptor transcripts. Cumulative fold-change regulation varies between +70.59 and -70.70 log₂ fold-changes (Figure 2A-B). The heterologous SmVEN and SROD challenges lead to modulation of 62 and 48 transcripts, respectively (Figure 3A-B). Expression modulation varies toward +35.52 and -41.37 cumulative log₂ fold-change for SmVEN and +22.77 and -80.05 log₂ fold change for SROD (Figure 2A-B). These results suggest a complex reprogramming of putative immune receptors comparing homologous and heterologous immune challenges. Expression patterns of these transcripts were strongly different between strains (Figure 2B). Solely one fourth of transcripts have similar modulation whatever was the parasite strain used for challenge (N=29, 24.4%). SmBRE-challenged snails expressed the most specific differentially represented transcripts (N=42, 35.3%) followed by SmVEN (N=19, 16.0%) and SROD (N=14, 11.8%) (Figure 2B). Annotations of such recognition immune-related transcripts bring interesting information on receptor families selected by the host to face up the diversity of pathogens used for immune challenges. In cluster 1 (shared up regulated transcripts) we identified: selectin, c-type lectin, FREP 3.3, tenascin, CD205 protein, lymphocyte antigen 75-like and macrophage mannose receptor 1-like. In cluster 14 (shared down regulated transcripts), we identified: 3 variants of Pathogenesis Recognition Proteins (PGRP), 2 variants of C1q-like 4 (C1q

complement-like), 3 variants of c-type lectin (calcium-dependant lectin), 1 variant of collectin-10, 2 variants of c-type mannose receptor-2, one CD209, a dermatopontin-3 and one transcript of Thioester-containing protein (TEP). For the strain specific response, SmBRE homologous immune challenge lead to up-regulation (cluster 6) of transcripts including macrophage mannose receptor 1-like, 3 variants of selectin, 5 of PGRP, 8 of Fibrinogen receptors (FREPs), one of galectin, 4 variants of c-type lectin, 2 of alpha-2-macroglobulin and 2 dermatopontin. For downregulation (cluster 7), one variant of macrophage mannose receptor-1 like, BgFREP1, a Toll-like receptor, a mucin-5AC, a Galectin-3 and one TEP variant were identified. For SmVEN heterologous response most of the transcripts were downregulated (cluster 5-8) including 3 variants of C1q-like, 4 variants of c-type lectin, and 5 variants of macrophage-mannose receptor. Concerning SROD heterologous challenge, 2 variants of FREPs are downregulated with a Toll-like receptor and one C-type lectin (cluster 9) while 2 variants of macrophage mannose receptor 1-like are upregulated with 8 variants of peptidoglycan binding domain-containing protein (cluster 4). This complex regulation and or selection of different kinds of putative immune receptors would potentially support the high genotype-dependant innate immune memory specificity observed in *Biomphalaria glabrata* snails.

Vaccination

For homologous vaccination, 500 BgBRE naïve snails were individually injected in the head-foot region with 1µg in 10µL TBS-Tween 0.05% buffer of each parasite protein extract (i.e., Miracidia, Sp1, Sp2, Sp2Control, Cercaria and Pool) then 15 days after vaccination, snails were individually exposed to 10 SmBRE miracidia. After fifteen days, snails were fixed in Raillet-Henry solution to calculate the parasite

prevalence. Miracidia (prevalence 82.6% (N=86)), Sp2 (66% (N=50)), Sp2 control (81.9% (N=83)), Cercaria (81.4% (N=43)) and Pool extracts (72.4% (N=98)) cannot induce a significant vaccination (Fisher exact test, $p > 0.01$) compared to TBS-Tween injections (80.3% (N=61)). However, Sp1 extract induce a significant protection (Fisher exact test, $p = 0.000145$) with a prevalence of 47.9% (N=73 individuals).

As solely Sp1 vaccination partially significantly protects snails from being re-infected, we analyzed immune specificity using vaccination with Sp1 parasite extracts. Vaccinations with Sp1 protein extract from SmBRE, SmVEN or SROD were compared to TBS-Tween injection. Fifteen days after vaccination snails were individually exposed to 10 SmBRE miracidia and 15 days after infection parasite prevalence were recorded. Exclusively homologous vaccination significantly protects from parasite infection (prevalence 47.9% (N=73), (Fisher exact test, $p = 0.000145$)). Heterologous vaccinations did not differ from TBS-Tween control injection with respectively, a prevalence of 84.8% (N=33) for SmVEN, and 83.8% (N=37) for SROD. These results confirmed the high level of specificity of *Biomphalaria glabrata* innate immune memory. Snails were protected by Sp1 extracts, other parasite stages were not sufficient to activate a significant protection. Snails were also protected when vaccinated with the same parasite extract than the one used for the infection but were not protected when vaccinated with heterologous parasite extracts.

Cell-free hemolymph transfer and resistant immune phenotype

RNAseq approach demonstrates that most of the molecules identified as differentially represented between homologous and heterologous infections corresponded to circulating immune recognition molecules or receptors. Thus, we investigate how transfer of cell-free hemolymph from primed or challenge snails to naïve snails could

transfer protection against pathogens. Three hundred naïve snails were individually injected with 10 µL of cell-free hemolymph samples recovered from naïve, primed (15DP) or challenged (1DC, 4DC and 15DC) snails. Saline solution (Chernin's balanced salt solution, CBSS) was used as control. Naïve, 15DP, 4DC and 15DC hemolymph did not significantly transfer resistance to naïve snails, compared to saline solution injection (Figure 5). Parasite prevalence results were as follow: XX % (N=XX) for CBSS injection, 76.5% (N=34) for naïve, 64.4% (N=59) for 15DP, 59.4% (N=35) for 4DC and 81.8% (N=33) for 15DC. However, transfer of cell-free hemolymph recovered one-day after the challenge (1DC) led to the acquisition of a significant protection against SmBRE infection (Fisher exact test, $p=0.000145$), prevalence of 42.9% (N=35).

These results demonstrated that, as early as 1 day after the challenge relevant recognition receptors or cytotoxic/cytolytic effectors were released into the snail hemolymph and were able to participate or activate an efficient and specific immune response event 15 days after their injection into naïve snails.

***In vitro* cell-free hemolymph toxicity assay: a validation of immune specificity**

Using the RNAseq approach we demonstrated that challenge snails specifically produced a different set of recognition receptors and effector molecules depending on their interaction with homologous or heterologous parasites. Moreover, those molecules seemed, for their most part, to be released into snail hemolymph. Thus to confirm that cell-free hemolymph could be in some way the support of innate immune memory specificity, the toxicity of cell-free hemolymph towards Sp1 of SmBRE, SmVEN or SROD was investigated *in-vitro*.

Cell-free hemolymphs from homologous or heterologous 1-day post-challenged snails were recovered and added with 100 Sp1 in CBSS culture medium. Mortality of parasites were estimated after 4 hours of contact by microscopic inspection of flame cells beating. Those results were compared to mortality recorded for parasites in contact with cell-free hemolymph from naïve snails. Two homologous assays were tested, rather Brazilian snails (BgBRE) are primed and challenged by Brazilian parasite (SmBRE) or Brazilian snails are primed and challenged by Venezuelan parasite (SmVEN). Results of triplicates (Figure 6) show that significant difference exist in homologous assays when we compare hemolymph from naïve and challenged snail (Fisher exact test $p=0.0262$ in SmBRE/SmBRE and $p=3.449E-28$ in SmVEN/SmVEN). Heterologous assays don't reveal any significant protection in SmBRE primed snails against SmVEN ($P.value=0.06147$) or SROD ($p.value=0.5596$) while significant protection is observed in SmVEN primed snails against SROD ($P.value=1.8718E-09$) but not against SmBRE ($p.value=0.0844$). Toxicity of challenged plasma is normalised by toxicity of naïve plasma then compared between homologous and heterologous assays. SmBRE homologous challenged plasma have significantly different effect on SmBRE parasites than SmVEN one (Z test, $Z=2.9605$, $p.value=0.003$) or SROD ($Z=5.9755$, $P.value=0$). Same thing in the SmVEN homologous challenged plasma that target better SmVEN than SROD despite significant different protection compared to naïve ($Z=3.9726$, $p.value=8E-05$) and SmBRE ($Z=-11.3573$, $p.value=0$).

Hemocytometry analysis

In order to describe if hemocyte population differentiations were observed during the acquisition of innate immune memory, a flow cytometry approach was

performed following primary infection and homologous or heterologous challenges. First results referred to size / granularity representation (SSC-A/FSC-A) showed a very complex profile for naïve snails with a continuous gradient of size and granularity that permit with difficulty to distinguish different hemocyte sub-populations (Supplementary 2.A1). However following primary infection, a hemocyte subpopulation seems to split to high size cells as early as 1DP and heighten at 4DP (Supplementary 2.A2 and A3). At 15DP, while snails become totally resistant to another infection, size/granularity scatter return to basal values similar to naïve snail hemocyte population profiles. Challenged snail hemocyte profiles were also almost similar from naïve, or primo-infected snail profiles (Supplementary 2. B and C). The use of fluorescent labelling (FITC-A/SSC-A) settings was useful to distinguish different hemocyte sub-populations for naïve, primed or challenged snails. As SYBR-green I is known to label DNA as well as RNA, it would be possible to separate hemocyte populations based on their DNA content but also based on cell transcriptional activity. Naïve snails seem to possess 4 different hemocyte sub-populations that displayed different level of fluorescent intensity with a similar gradient of granularity. Those populations were named P1, P2, P3 and P4 (Figure 2.A1). One day after primary infection (1DP), the number of P1 cells with the lowest fluorescent labelling seems to reduce while other sub-populations (P2, P3, P4) at higher level of fluorescence did not seem to be particularly enriched (Supplementary 2. A2). At 4DP, hemocyte populations return to a basal profile. Indeed, cell populations were very similar to naïve snail hemocyte populations (Supplementary 2. A3). At 15DP, snails became totally resistant to a secondary infection, but hemocyte population profiles remained close to naïve populations with apparently an enrichment of the P2 population of medium fluorescence labelling and medium

granularity (Supplementary 2. A4). One day after challenge, snail hemocyte population profiles were almost similar from 15DP profile particularly for the heterologous challenge. P1, P2, P3 and P4 populations could always be separated and distinguished (Supplementary 2.B1 and C1). Concerning homologous challenge, a complex hemocyte profile distribution is observed, with a more or less continuous gradient of fluorescence, rendering difficult or quite impossible to separate the 4 hemocyte populations (Supplementary 2.B1 and C1).

In summary, fluorescence/granularity scale was helpful to demonstrate few modifications of hemocyte populations following primary infection and homologous challenge. However, these results suggest that there are no strong modifications of the hemocyte populations associated with the acquisition of innate immune memory in *B. glabrata*. No cell differentiation or modifications of cell morphological features were observed. It seems that the acquisition of the innate immune memory does not pass through the acquisition of new hemocyte subtypes but rather by a transcriptional reprogramming of the yet existing hemocyte populations.

Immune relevant gene-expression following vaccination

To demonstrate that vaccination mimic natural infection and results in the activation of snail immune response, QRT-PCR were realised on hemocytes following vaccination or natural infections. Six candidate genes were selected for their putative role in the specificity of innate immune memory response in *Biomphalaria glabrata* based on whole body RNAseq experiment. All these candidates were selected because they are differentially regulated following challenge and present in shared clusters 1 and 14 (same pattern of expression whatever were the parasite strains used). As a result, vaccination induced the same pattern of response for these

immune recognition receptors. The variants of FREPs, C-type lectin and Selectin are shown to be down-regulated following primo-infection in a similar manner between the RNAseq experiment, natural infection and vaccination. Glutamate synthase have the similar pattern of response shared by natural infection and vaccination by being both up-regulated. However, expressions were not perfectly similar as TEP and Biomphalysin displayed opposite expressions comparing vaccination and natural infection. Altogether, these results suggest that vaccination mainly mimic the natural primary infection by stimulating immune system and inducing similar pattern of immune-related gene expression.

DISCUSSION

Lot of invertebrate phyla have been described to show some immune protection acquired after a first primary immune stimulation. Within invertebrates, arthropods appeared as the more investigated group (Kurtz and Franz 2003, Roth and Kurtz 2009, Rodrigues, Brayner et al. 2010, Brown and Rodriguez-Lanetty 2015) but Cephalochordata, Echinodermata, Nematoda, Cnidaria, Ctenophora and Mollusca have also been investigated (Milutinovic and Kurtz 2016). Some of these studies brought relevant results concerning high innate immune specificity processes. In invertebrates, the specificity of the innate immune response is based, most of the time, on the capacity to recognize and respond to phylogenetically distant pathogens. Virus/yeast/bacteria expressed specific antigens or specific pathogen associated molecular patterns (PAMPs) like peptidoglycans, beta-glucans or Lipo-poly-saccharides (LPS), which could be easily recognized by host immune system (Jorge Contreras-Garduno, Humberto Lanz-Mendoza et al. 2016).

However, in the *Biomphalaria glabrata/Schistosoma mansoni* model both partners are Lophotrochozoan species. This phylogenetic proximity is particularly interesting

when studying the mechanisms involved in the specificity of innate immune memory. Indeed both partners would be expected to share antigenic determinants or exposed surface molecules displaying similar features (Yoshino, Wu et al. 2012). Thus, fine scale recognition processes would be expected from the host to recognize specifically the pathogen determinants and avoid auto-immunity. Recently, a genotype-dependant immune memory has been shown in this model suggesting that snail immune response can differentiate different parasite species or strains (Portela, Duval et al. 2013). Herein we go further in the understanding of the molecular basis of such specific innate immune memory process.

Strain-specific antigen recognition?

A lot of immune-related transcript are differentially regulated during a secondary infection, whether it be homologous or heterologous. After functional annotation, we found numerous differentially expressed (DE) molecules still no annotated (1625/4202) or uncharacterised / unannotated features (150/4202). For the remaining transcripts (2427/4202, 57.7%), differences between families, genes, transcripts or variants were difficult to access. Indeed, from the transcriptome assembly, some transcripts could be the result of a unique gene in a single position in the genome, or one of the numerous products of large gene family with lot of variants. Some DE transcripts must also be discussed as a functional group representing a family with shared features while other are described as single gene.

Priming response against Schistosoma sp.

The basal immune memory response observed for all the three strains of parasite (58% of protection) was associated with transcripts grouped in cluster 1 (over-represented) and cluster 14 (under-represented). Within these clusters of

shared response whichever was the strain used for the challenge, numerous DE transcripts have already been shown as implicated in immune response pathways. Within immune receptors, the lectins family is composed by all the molecules containing a lectin domain, already described as a carbohydrate-binding protein, they remain the presumed support of recognition in *Biomphalaria*. Presence in this cluster of macrophage mannose receptor family, FREP3.3, C-type lectin and selectin indicate the importance of pathogen sensing in the secondary challenge. Especially, lectin families have been shown to be highly diversified and polymorphic to cover an important range of pathogen diversity (Dheilly, Duval et al. 2015). That may presuppose that transcript and protein diversity (after putative post-transcriptional maturation) is sufficient to face up the several parasite strains/species antigenic diversity. Numerous immune response associated-genes are frequently found in deep-sequencing analysis but poorly discussed because the lack of clear identified pathway for such molecules. Mucins and collagens are in this way discussed as component of extra-cellular matrix and must be implicated in the cell motility modification. Over-expression of a variant of these families may induce several pathways, from recruitment of competent cell to block incompetent cells as previously described for *Streptococcus* infection of macrophages (Dhar, Ng et al. 2017). Glutamate-synthase over representation is also consistent with metabolic reprogramming described in innate immune memory cells (Arts, Joosten et al. 2016). Biomphalysin is a beta-pore forming toxin (Galinier, Portela et al. 2013). That remains the sole candidate able to kill directly Schistosome pathogen the association with a still unknown plasmatic factor (Galinier, Portela et al. 2013). These highly toxic molecules have been already described in the acquisition of immune memory in *Biomphalaria* (Galinier, Portela et al. 2013). Once again, its expression against all tested strain show the high

importance of this toxin in the acquired resistance and the associated cellular to humoral immune shift in *Biomphalaria*. New results enhance the importance of such Biomphalysin molecules, originally acquired from bacteria by gene horizontal transfert (Galini r et al). Two lobes composed Biomphalysin; one of them could support the specific binding properties of Biomphalysins (unpublished data, personal communication).

Complete protection in homologous response

In the homologous response, we observed a protection of 100% following a new encounter with the same pathogen. In this efficient host immune response, immune receptors were also particularly represented with a great quantity and diversity of lectins (Fibrinogen-related proteins (FREPs), c-type lectin, galectin-4). The FREPs were particularly interesting. Indeed, these molecules were demonstrated to be involved in pathogen recognition (ref trapping, Mon   et al 2010 PlosNTD), pathogen resistance (PNAS) and immune memory (Pinaud et al.) in *Biomphalaria* snails. Galectins, for their part, have been shown to be able to process multimerisation with activation of bactericidal activity (Galectin-1 dimers Blazevits et al 2016, Cytotoxicity and glycan-binding profile, Kawsar et al, 2011) that indicate a putative dual role of this family of molecule and make more complex the process of immune response during homologous immune challenge with both receptors or effectors with dual-functions. Other immune effectors like lbp/bpi or mytimacin actually described against bacterial pathogen could be discussed as a control of opportunistic Arasu, Kumaresan et al. 2017 or microbiota regulators s previously described in the plasmodium/mosquito interaction (Rodrigues, Brayner et al. 2010).

Concerning the downregulation of immune-related transcripts for one given parasite (clusters 8-10), we can show a balance between variants or members of these families that were activated (see up regulated cluster) and others that were down regulated (clusters 8-10). We could hypothesize that this complex process of recognition in *Biomphalaria* snails required the activation of solely several members of a given family of receptors while others were repressed as a trade-off to permit the expression of the receptors that will be necessary for an efficient acquired protection following the challenge. This would be necessary for the activation of a specific immune pathway against a given pathogen. Down-regulation of thioester-containing protein in the homologous challenge, for which the cellular immune response shift to a humoral one's, is very interesting. This vertebrate complement-like protein has been discussed to act as opsonin with implication from promoting phagocytosis, or lysis and even melanisation process in Invertebrates (Le Clec'h, Anderson et al. 2016). This typical component of cellular immune response in arthropod (Blandin and Levashina 2004, Buresova, Hajdusek et al. 2011, Volohonsky, Hopp et al. 2017) may act during primary cellular immune response and encapsulation of parasite. However, following the challenge, a humoral/plasmatic clearance was exclusively demonstrated, the down-regulation of TEP proteins appeared relevant in this context.

Vaccination as proof of immune memory?

Vaccination means immunization. Immunization means that pathogen extracts, when injected, must bring protection following a secondary challenge with the competent pathogen. Our injections were realized using native extractions that conserved a complex molecular solution that can include proteins, salts, ions, hormones, nucleic acid, etc. Unfortunately, we cannot be sure with these kind of immunization, which

components of this global parasite extract were the more relevant to explain the immunization and the acquisition of resistance. Injections of extraction buffer were used as control and permit to test at the same time the effect of needle injection (involving tissue wounding). No protection was observed following these control injections, and this despite that melanisation pathway was previously described as way of resistance in insect (Christensen, Li et al. 2005). All the machinery was moreover described in *Biomphalaria* (Le Clec'h, Anderson et al. 2016). Indeed, implication of this type of inflammatory pathway seems not to be sufficient to activate the acquisition of resistance. Extract of hepatopancreas from pool of naïve snails do not induce protection against *Schistosoma*, too. Non-self-recognition in *Biomphalaria* of a proteic extract from other individuals seems not to be sufficient to activate a non-specific cross immune response against a *Schistosoma* infection. In the case of vaccination, an antigenicity is transferred toward immunisation as mimicry of "natural infection" and launch an efficient immune response with a putative interaction protein/protein as expected in parasite antigen/immune receptor or ligand/TLR response. Unfortunately, even sympatric Sp1 or all extracts pooled all together can't transfer a total 100% protection as shown following homologous secondary infection (Figure 3). If vaccination experiments may be supported by a high specificity in recognition for the *Biomphalaria* immune system, they not succeeded to reach a perfect immune protection as expected following natural infection. Altogether, these results suggest rather that vaccination, as tested, not succeed to propose all the antigenicity needed in the snail to launch acquisition of immune memory, or rather that acquired resistance is depend of other factor that antigenicity recognition. Quantitative rt-PCR indicate that vaccination can mimic response of natural infection for some members while other one were regulated in an opposed manner comparing

natural infection and vaccination (Supplementary 3). Native extract can be improved by using Bge-cell line co-cultured parasites with longer maturation and putative expression of new antigens potentially required for the acquisition of resistance (Vermeire, Boyle et al. 2004).

Cell-free hemolymph transfer

Plasmatic compartment transfers induced a high protection in naïve snail, only when this plasma is recovered 1 day after the immune challenge. This result suggests that secretion of immune factors into the plasma early after the challenge is determinant in the pathogen recognition and killing observed for the immune shift phenotype. Nonetheless, molecules that transfer these capabilities to generate immune response are so far not known. Moreover, while natural infection induce a 100% resistance to another infection, vaccination and cell-transfer only reach 51% of protection. That suggest other source of support is needed to reach a 100% resistance response. Parasite development may be one source of continual immune regulators liberation (salts, ions, hormones, nucleic acid, (Lodes and Yoshino 1990, Yoshino, Brown et al. 2014, Nowacki, Swain et al. 2015)) to explain that acquired resistance is not supported only Sp1 antigenic recognition but complex parasitic factor detection.

In addition, few toxic molecules have been described in the *Biomphalaria plasma* to explain a humoral phenotype of clearance but the biomphalysin (Galinier, Portela et al. 2013). This beta-pore forming toxin may be transferred during vaccination and may stay toxic 15 days after injection to protect half of the tested snails. More study have now to be done to assess the support of plasma toxicity in *Biomphalaria* : do the biomphalysin are the only members of humoral cytotoxic agent in the plasma of challenged snails?

***In-vitro* toxicity and specificity of primed plasma**

Toxicity assay show for the first time the implication of humoral factors to explain specificity of innate immune memory in invertebrate. Homologous response, that means that the hemolymph was recovered from snail infected and challenged with the same strain of parasite, display always the higher *in-vitro* toxicity despite the origin of the strain used. That means that molecules are produced and secreted in the circulating plasmatic compartment to specifically bind and killed different set of pathogens. These results suggest that innate immune memory in *Biomphalaria* and its genotype-dependant immune memory is supported by complex repertoires of circulating sensors secreted during immune challenge humoral response to specifically recognised and killed yet encountered parasites.

Transcriptomic programming?

As a molecular support of immune acquired resistance, the transcriptionally part of genome have to be consider as the proof of which weapons are selected by snails to respond against *Schistosoma* re-infection. Previous RNAseq experiment (Pinaud, Portela et al. 2016) described a shift in transcript expression with challenge-specific clusters of differentially represented transcripts. Other invertebrate organism display the same pattern of innate immune memory response (Greenwood, Milutinovic et al. 2017). All transcripts considered remain specific for the challenged conditions. The symmetry between the up and down regulation of transcripts (ie, in the number of up and down regulated transcripts) described in Figure 2b. is pretty interesting because that show a complex programming of transcription to answer to the immune challenge. Everything let imagine a complex programming of genes to be transcribed or not, of mRNAs to be stable or not, to be translated or not. This programming

should be supported by epigenetic remodelling (methylation pattern, histone marks, chromatin structure, miRNA, lncRNA).

Trained cells as a moving support of memory?

Cytometry results show no cell differentiation or modifications of cell morphological features. It seems that the acquisition of the innate immune memory was not associated with the acquisition of new hemocyte subpopulation but rather by a transcriptional reprogramming of the yet existing hemocyte populations. If an epigenetic support must exist (REF) it must be important to consider ability for hemocyte to be reprogrammed in snails following primary infection. This phenotype has to be supported by a cocktail of molecules like cytokines, hormones or enzymes that may circulate in snail plasmatic compartment and spread resistance information to other naive cells (REF). Cell phenotype will be change also from their role into immune encapsulation to a new role in humoral factor release in plasma.

Combinatory repertoire for quite infinite immune sensing?

Peptidoglycan recognition proteins family, FREPs, C-type lectins, macrophage mannose receptor, TLRs, selectin or galectin are all members of different potential immune receptor superfamilies. They disposed of domains able to directly bound pathogens, carbohydrate recognition domain (CRD) or immunoglobulin (Ig) for the FREPs, leucine-rich repeat (LRR) for TLR or carbohydrate-binding protein domain for C-type lectin. Lot of these members have been already described in the interaction between *Biomphalaria* and *Schistosoma* in immune protean complexes. FREPs or c-type lectin for example, have be characterised in proteomic interactome (Moné, Yoshino) as well as in transcriptomic response (Hannington, Pinaud). Here we reveal

for the first time the complexity of putative immune receptors at the transcriptomic scale to insure immune specificity and so, their putative role at the proteomic scale could be proposed. These putative immune receptors have to be produced as extracellular or humoral receptors to be able to interact altogether. These results suggest the possibility that immune receptor in *Biomphalaria* may by associate each other to work in coherent concert of multimerisation to increase the snail recognition capabilities (Innate sensors, Medzhitov, **A peptidoglycan recognition protein in innate immunity conserved from insects to humans**, 10.1073/pnas.0404952102 10.1074/jbc.M111.264374 10.1111/imm.12460 10.1073/pnas.0710092105). Regulation of the good set of receptors and effectors allow these different actors to interact each other to create a specific shape for the immune complex and so display in immune challenge, a potential combinatory-repertoire of immune receptors far highly diversify and competent than the one observed for primary homologous or secondary heterologous infection (Schulenburg, Boehnisch et al. 2007). This combinatory-dependant expression of specificity must explain the faster/better sensing of *Schistosoma* in the secondary challenge when exposed to homologous parasite and their following total clearance, compared to the partial response displayed in heterologous one.

This is partly confirmed by the cytotoxic assays that show a better response against homologous parasites and suggest that this combination of receptors can circulate in the snail plasma and led to the observed phenotype of specific toxicity against a remembered target-pathogen. Altogether, these results suggest that putative immune receptor and effectors repertoires are required to acquire an efficient immune memory in the *Biomphalaria* snail. Question of how host select and remember the

good set of immune receptors and effectors to express them later following a potential secondary challenge will deserve further investigation.

Trained immunity in *Biomphalaria* instead of priming

These observations delayed to propose hypothesis of radiation evolution for the immune system from the single humoral immune response (bacterian toxin) or cellular innate immune response (increase phagocytosis) to the adaptive vertebrate B-cells immune response (clonal selection and proliferation).

Until recently, the effectiveness of the adaptive immune response in vertebrates may have masked the trained innate immune response observed for example in monocyte-to-macrophage response against *Candida albicans* (Netea, etc). Hypothesis of constant evolution selection with only a trained immune response in the r ecological strategy organisms to a trained and adaptive immune response in the K strategy organisms should be advanced. However, trained innate immune response implies an epigenetic support (chromatic structure, DNA methylation, histone marks or variants, etc) available in specialised cells to store and propagate specific information about pathogens.

Biomphalaria possesses two clearly different phenotypes against *Schistosoma* infection, an immune cell encapsulation during the first encounter with a *Schistosoma* parasite and then a humoral immune response following a secondary encounter. We propose in this study to join both phenotypes to propose a unified *Biomphalaria* immune memory schema of response: (i) primary encounter with a parasite and the encapsulation by hemocytes conduct to spark acquisition of resistance in host, (ii) immune shift support a new type of immune response from cellular to humoral and may be supported by a molecular reprogramming (epigenetic, transcriptomic and,

metabolomic/proteomic) , (iii) diffusion of circulation activators like cytokine (MIF?, REF or IL), hormones, salt or nucleic acids (lncRNA, miRNA) should support cell to cell communications and the spreading of resistance phenotype in whole snail, (iv) during secondary challenge, a release of diversified set of immune receptors and effectors (Biomphalysin) allow to kill more efficiently homologous parasite rather than heterologous by a humoral immune response. Different sets of immune receptors and effectors leads to differentiate an homologous to an heterologous immune challenge and permit to explain the genotype-dependant immune memory phenotype yet described.

Innate immune memory in *Biomphalaria* must be compared to trained immunity of innate cells in vertebrate for many point of view. Our results suggest once again that *Biomphalaria* acquired immune resistance must be supported by a trained immunity of the hémocytes to prepare the shift response. If confirming by further analyses, these results will suggest that *Biomphalaria*, as invertebrate can use this trained immunity shared from invertebrate to vertebrate and reveal a putative conservation of this mechanism across living for pathogen defence.

This theory needs now proteome interactome analysis to study the dynamic of response that is required to finally activate the competent immune complex. The mechanisms underlying cell molecular reprogramming (epigenetic or not) of immune-specialised cells during the immune shift needs more targeted studies to explore chromatin structure (MNase-seq, ATACseq, Histone marks), DNA methylation, small RNA and even metabolomics pathway to confirm or infirm this regulation processes similar to trained immunity. Experiments about specificity concerning invertebrate innate immune memory will contribute to improve knowledge of immune sensors in

invertebrates and in-fine paved the way to a better understanding of the evolution of innate immunity processes in invertebrates and vertebrates.

Acknowledgements and funding information:

We thank Ms. Anne Rognon for technical useful discussions concerning animal experimental protocol design.

This work was supported by the French National Agency for Research (ANR) through a project Invimory grant [ANR-13-JSV7-0009] to BG.

References

- Arts, R. J., L. A. Joosten and M. G. Netea (2016). "Immunometabolic circuits in trained immunity." *Semin Immunol* **28**(5): 425-430.
- Blandin, S. and E. A. Levashina (2004). "Thioester-containing proteins and insect immunity." *Mol Immunol* **40**(12): 903-908.
- Brown, T. and M. Rodriguez-Lanetty (2015). "Defending against pathogens - immunological priming and its molecular basis in a sea anemone, cnidarian." *Sci Rep* **5**: 17425.
- Buresova, V., O. Hajdusek, Z. Franta, G. Loosova, L. Grunclova, E. A. Levashina and P. Kopacek (2011). "Functional genomics of tick thioester-containing proteins reveal the ancient origin of the complement system." *J Innate Immun* **3**(6): 623-630.
- Calil, I. P. and E. P. Fontes (2016). "Plant immunity against viruses: antiviral immune receptors in focus." *Ann Bot*.
- Christensen, B. M., J. Li, C. C. Chen and A. J. Nappi (2005). "Melanization immune responses in mosquito vectors." *Trends Parasitol* **21**(4): 192-199.
- Cirimotich, C. M., Y. Dong, A. M. Clayton, S. L. Sandiford, J. A. Souza-Neto, M. Mulenga and G. Dimopoulos (2011). "Natural microbe-mediated refractoriness to Plasmodium infection in Anopheles gambiae." *Science* **332**(6031): 855-858.
- Cooper, M. D. and M. N. Alder (2006). "The evolution of adaptive immune systems." *Cell* **124**(4): 815-822.
- Dangl, J. L., D. M. Horvath and B. J. Staskawicz (2013). "Pivoting the plant immune system from dissection to deployment." *Science* **341**(6147): 746-751.
- Dhar, P., G. Z. Ng, E. M. Dunne and P. Sutton (2017). "Mucin 1 protects against severe Streptococcus pneumoniae infection." *Virulence*: 1-12.
- Dheilly, N. M., D. Duval, G. Mouahid, R. Emans, J. F. Allienne, R. Galinier, C. Genthon, E. Dubois, L. Du Pasquier, C. M. Adema, C. Grunau, G. Mitta and B. Gourbal (2015). "A family of variable immunoglobulin and lectin domain containing molecules in the snail Biomphalaria glabrata." *Dev Comp Immunol* **48**(1): 234-243.
- Du Pasquier, L. (2004). "Speculations on the origin of the vertebrate immune system." *Immunol Lett* **92**(1-2): 3-9.

Eason, D. D., J. P. Cannon, R. N. Haire, J. P. Rast, D. A. Ostrov and G. W. Litman (2004). "Mechanisms of antigen receptor evolution." Semin Immunol **16**(4): 215-226.

Eisen, M. B., P. T. Spellman, P. O. Brown and D. Botstein (1998). "Cluster analysis and display of genome-wide expression patterns." Proc Natl Acad Sci U S A **95**(25): 14863-14868.

Galinier, R., J. Portela, Y. Mone, J. F. Allienne, H. Henri, S. Delbecq, G. Mitta, B. Gourbal and D. Duval (2013). "Biomphalysin, a new beta pore-forming toxin involved in *Biomphalaria glabrata* immune defense against *Schistosoma mansoni*." PLoS Pathog **9**(3): e1003216.

Greenwood, J. M., B. Milutinovic, R. Peuss, S. Behrens, D. Esser, P. Rosenstiel, H. Schulenburg and J. Kurtz (2017). "Oral immune priming with *Bacillus thuringiensis* induces a shift in the gene expression of *Tribolium castaneum* larvae." BMC Genomics **18**(1): 329.

Haine, E. R., Y. Moret, M. T. Siva-Jothy and J. Rolff (2008). "Antimicrobial defense and persistent infection in insects." Science **322**(5905): 1257-1259.

Hargreaves, D. C. and R. Medzhitov (2005). "Innate sensors of microbial infection." J Clin Immunol **25**(6): 503-510.

Jorge Contreras-Garduno, Humberto Lanz-Mendoza, Bernardo Franco, Adriana Nava, Mario Pedraza-Reyes and J. Canales-Lazcano (2016). "Insect immune priming: ecology and experimental evidences." Ecological Entomology(41): 351-366.

King, C. H., K. Dickman and D. J. Tisch (2005). "Reassessment of the cost of chronic helminthic infection: a meta-analysis of disability-related outcomes in endemic schistosomiasis." Lancet **365**(9470): 1561-1569.

Kurtz, J. and K. Franz (2003). "Innate defence: evidence for memory in invertebrate immunity." Nature **425**(6953): 37-38.

Le Clec'h, W., T. J. Anderson and F. D. Chevalier (2016). "Characterization of hemolymph phenoloxidase activity in two *Biomphalaria* snail species and impact of *Schistosoma mansoni* infection." Parasit Vectors **9**: 32.

Lodes, M. J. and T. P. Yoshino (1990). "The effect of schistosome excretory-secretory products on *Biomphalaria glabrata* hemocyte motility." J Invertebr Pathol **56**(1): 75-85.

Marraffini, L. A. and E. J. Sontheimer (2008). "CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA." Science **322**(5909): 1843-1845.

Milutinovic, B. and J. Kurtz (2016). "Immune memory in invertebrates." Semin Immunol **28**(4): 328-342.

Moret, Y. and P. Schmid-Hempel (2000). "Survival for immunity: the price of immune system activation for bumblebee workers." Science **290**(5494): 1166-1168.

Moret, Y. and P. Schmid-Hempel (2001). "Immune defence in bumble-bee offspring." Nature **414**(6863): 506.

Netea, M. G., J. Quintin and J. W. van der Meer (2011). "Trained immunity: a memory for innate host defense." Cell Host Microbe **9**(5): 355-361.

Netea, M. G. and J. W. van der Meer (2017). "Trained Immunity: An Ancient Way of Remembering." Cell Host Microbe **21**(3): 297-300.

Nish, S. and R. Medzhitov (2011). "Host defense pathways: role of redundancy and compensation in infectious disease phenotypes." Immunity **34**(5): 629-636.

Nowacki, F. C., M. T. Swain, O. I. Klychnikov, U. Niazi, A. Ivens, J. F. Quintana, P. J. Hensbergen, C. H. Hokke, A. H. Buck and K. F. Hoffmann (2015). "Protein and small non-coding RNA-enriched extracellular vesicles are released by the pathogenic blood fluke *Schistosoma mansoni*." J Extracell Vesicles **4**: 28665.

Pham, L. N., M. S. Dionne, M. Shirasu-Hiza and D. S. Schneider (2007). "A specific primed immune response in *Drosophila* is dependent on phagocytes." PLoS Pathog **3**(3): e26.

Pinaud, S., J. Portela, D. Duval, F. C. Nowacki, M. A. Olive, J. F. Allienne, R. Galinier, N. M. Dheilly, S. Kieffer-Jaquinod, G. Mitta, A. Theron and B. Gourbal (2016). "A Shift from Cellular to Humoral Responses Contributes to Innate Immune Memory in the Vector Snail *Biomphalaria glabrata*." *PLoS Pathog* **12**(1): e1005361.

Portela, J., D. Duval, A. Rognon, R. Galinier, J. Boissier, C. Coustau, G. Mitta, A. Theron and B. Gourbal (2013). "Evidence for specific genotype-dependent immune priming in the lophotrochozoan *Biomphalaria glabrata* snail." *J Innate Immun* **5**(3): 261-276.

Rodrigues, J., F. A. Brayner, L. C. Alves, R. Dixit and C. Barillas-Mury (2010). "Hemocyte differentiation mediates innate immune memory in *Anopheles gambiae* mosquitoes." *Science* **329**(5997): 1353-1355.

Roger, E., G. Mitta, Y. Mone, A. Bouchut, A. Rognon, C. Grunau, J. Boissier, A. Theron and B. E. Gourbal (2008). "Molecular determinants of compatibility polymorphism in the *Biomphalaria glabrata*/*Schistosoma mansoni* model: new candidates identified by a global comparative proteomics approach." *Mol Biochem Parasitol* **157**(2): 205-216.

Roth, O. and J. Kurtz (2009). "Phagocytosis mediates specificity in the immune defence of an invertebrate, the woodlouse *Porcellio scaber* (Crustacea: Isopoda)." *Dev Comp Immunol* **33**(11): 1151-1155.

Schulenburg, H., C. Boehnisch and N. K. Michiels (2007). "How do invertebrates generate a highly specific innate immune response?" *Mol Immunol* **44**(13): 3338-3344.

Theron, A., J. R. Pages and A. Rognon (1997). "*Schistosoma mansoni*: distribution patterns of miracidia among *Biomphalaria glabrata* snail as related to host susceptibility and sporocyst regulatory processes." *Exp Parasitol* **85**(1): 1-9.

Vermeire, J. J., J. P. Boyle and T. P. Yoshino (2004). "Differential gene expression and the effects of *Biomphalaria glabrata* embryonic (Bge) cell factors during larval *Schistosoma mansoni* development." *Mol Biochem Parasitol* **135**(1): 153-157.

Volohonsky, G., A. K. Hopp, M. Saenger, J. Soichot, H. Scholze, J. Boch, S. A. Blandin and E. Marois (2017). "Transgenic Expression of the Anti-parasitic Factor TEP1 in the Malaria Mosquito *Anopheles gambiae*." *PLoS Pathog* **13**(1): e1006113.

Witteveldt, J., C. C. Cifuentes, J. M. Vlak and M. C. van Hulst (2004). "Protection of *Penaeus monodon* against white spot syndrome virus by oral vaccination." *J Virol* **78**(4): 2057-2061.

Yoshino, T. P., M. Brown, X. J. Wu, C. J. Jackson, R. Ocadiz-Ruiz, I. W. Chalmers, M. Kolb, C. H. Hokke and K. F. Hoffmann (2014). "Excreted/secreted *Schistosoma mansoni* venom allergen-like 9 (SmVAL9) modulates host extracellular matrix remodelling gene expression." *Int J Parasitol* **44**(8): 551-563.

Yoshino, T. P., X. J. Wu, H. Liu, L. A. Gonzalez, A. M. Deelder and C. H. Hokke (2012). "Glycotope sharing between snail hemolymph and larval schistosomes: larval transformation products alter shared glycan patterns of plasma proteins." *PLoS Negl Trop Dis* **6**(3): e1569.

Zhang, W., J. Zhu, X. Song, Z. Xu, X. Xue, X. Chen, X. Yang, Y. Li, X. Dong, S. Zhou, W. Li, Y. Qian, F. Liu and C. Su (2015). "An association of Aquaporin-4 with the immunoregulation of liver pathology in mice infected with *Schistosoma japonicum*." *Parasit Vectors* **8**: 37.

Zhu, N., X. Feng, C. He, H. Gao, L. Yang, Q. Ma, L. Guo, Y. Qiao, H. Yang and T. Ma (2011). "Defective macrophage function in aquaporin-3 deficiency." *FASEB J* **25**(12): 4233-4239.

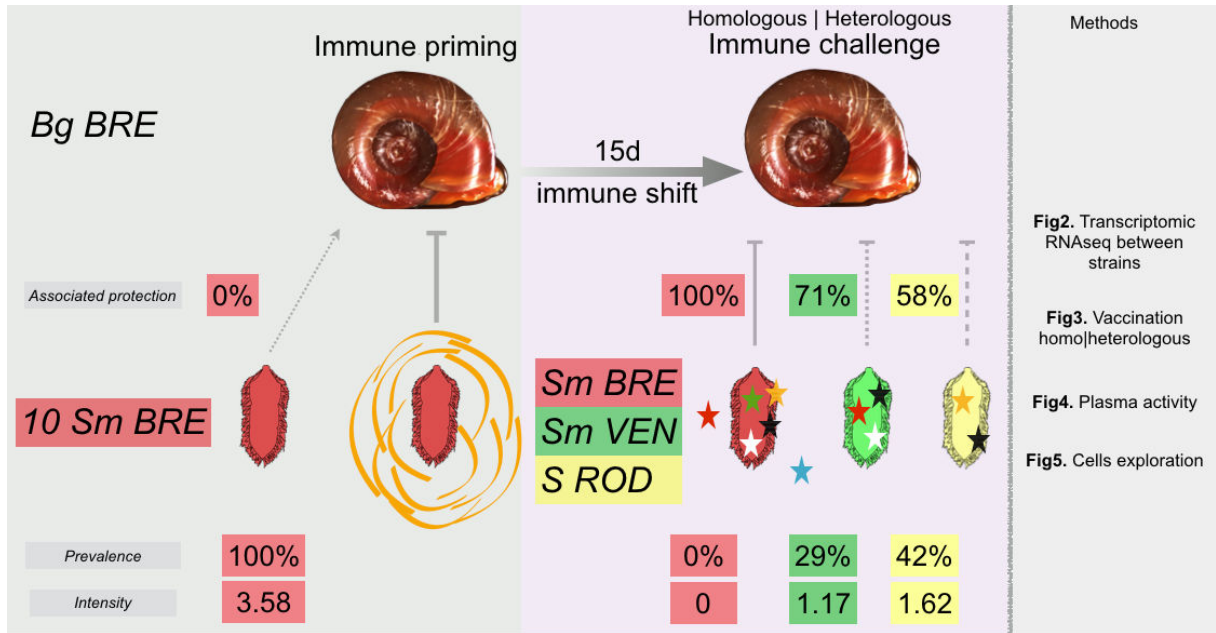


Figure 1. Overview of different analyses

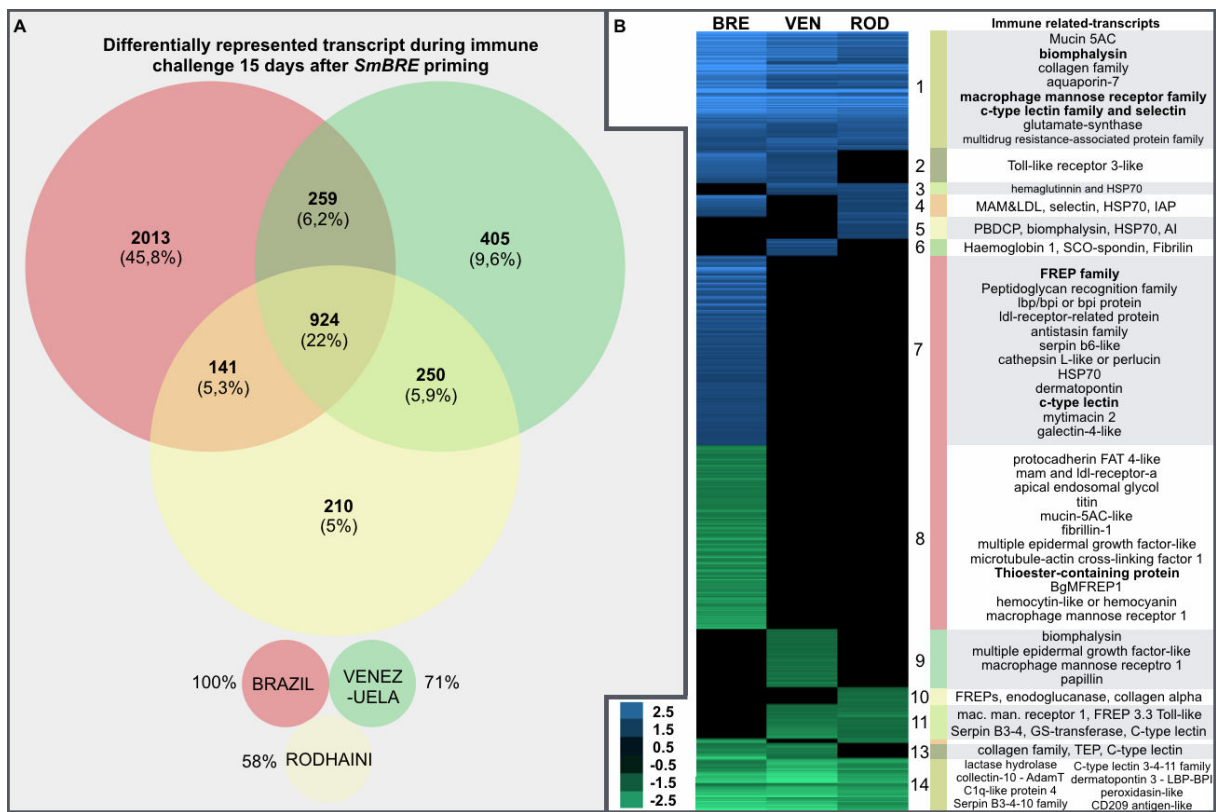


Figure 2 Transcriptomic analyses. A. Venn diagram of differentially represented transcripts depending of strain used for immune challenge. B.

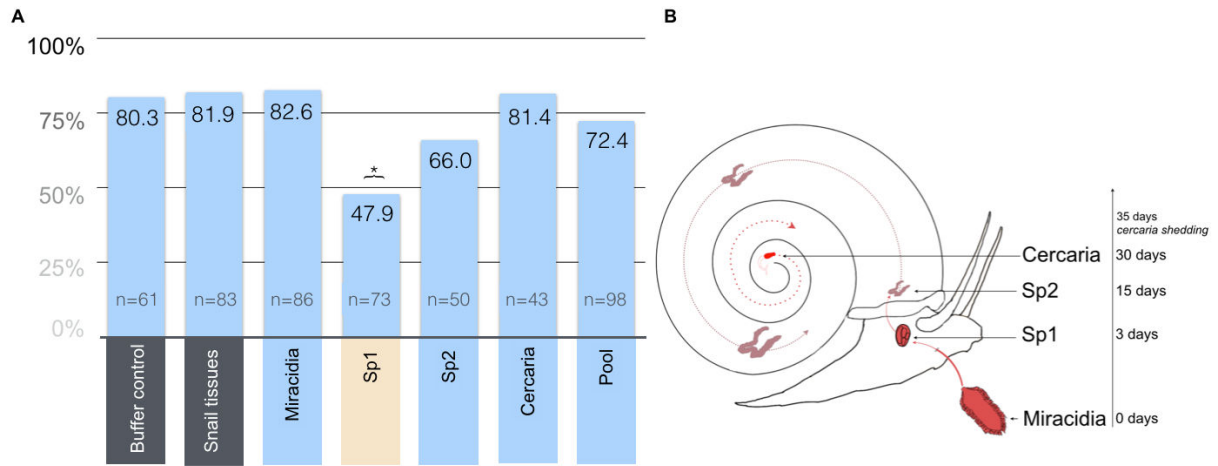


Figure 3. Vaccination A. Developmental stage of parasite used for immunisation B. Dynamic of development of parasite into the snail

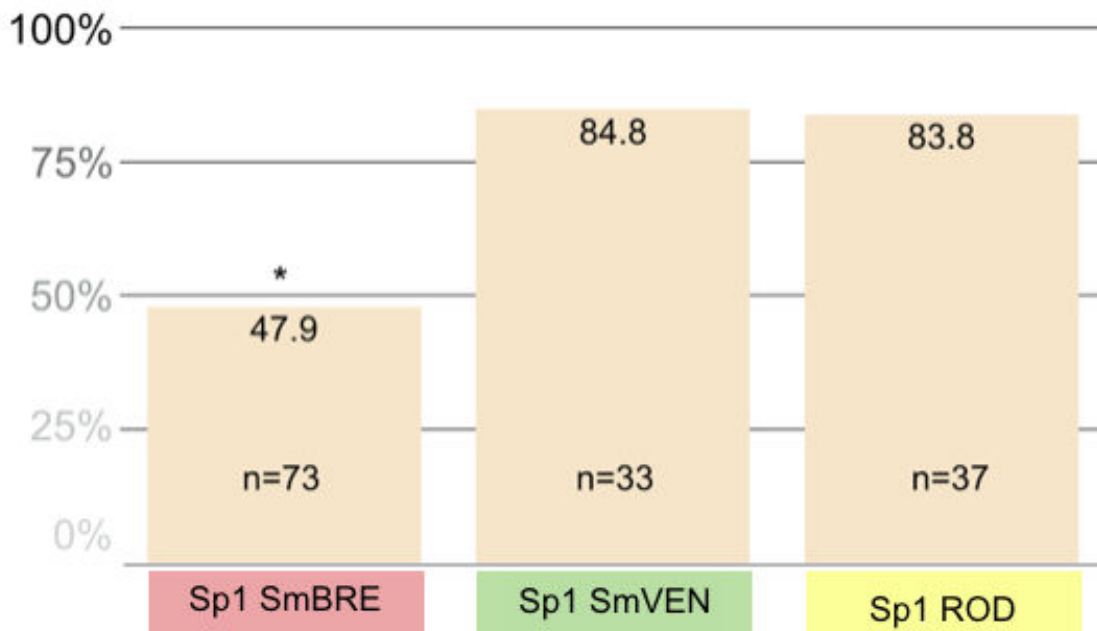


Figure 4 Heterologous vaccination with Sp1 extract

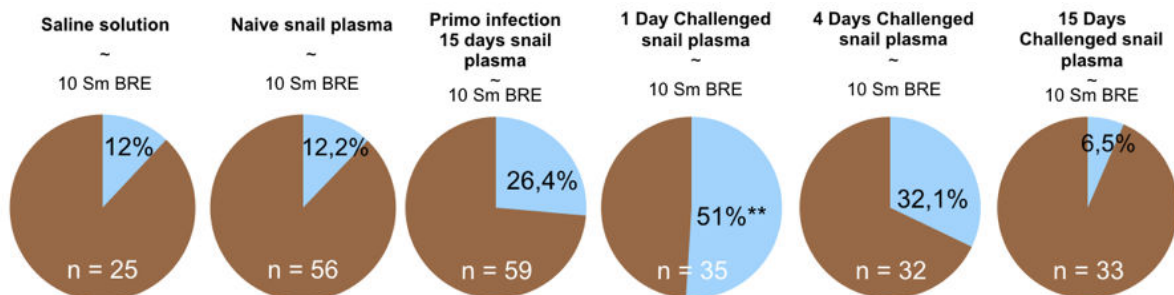


Figure 5. Hemolymph transfer

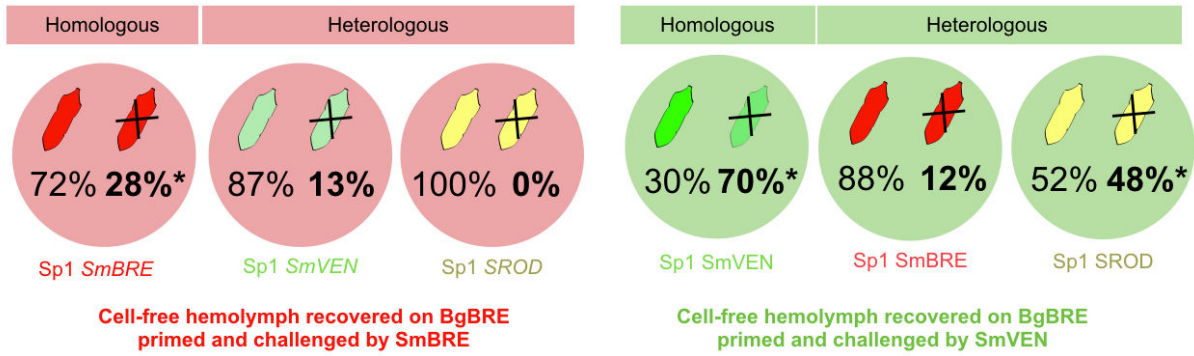
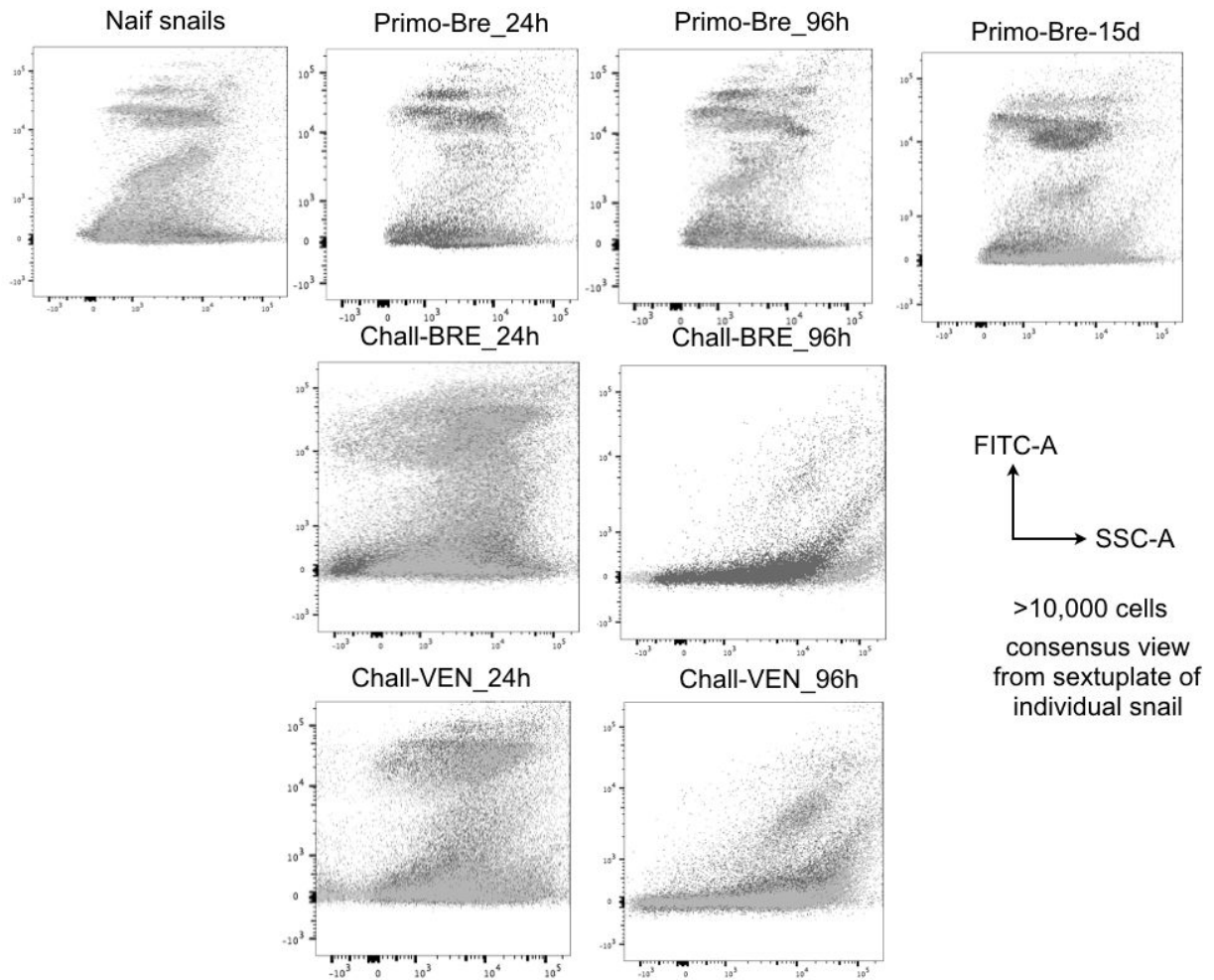
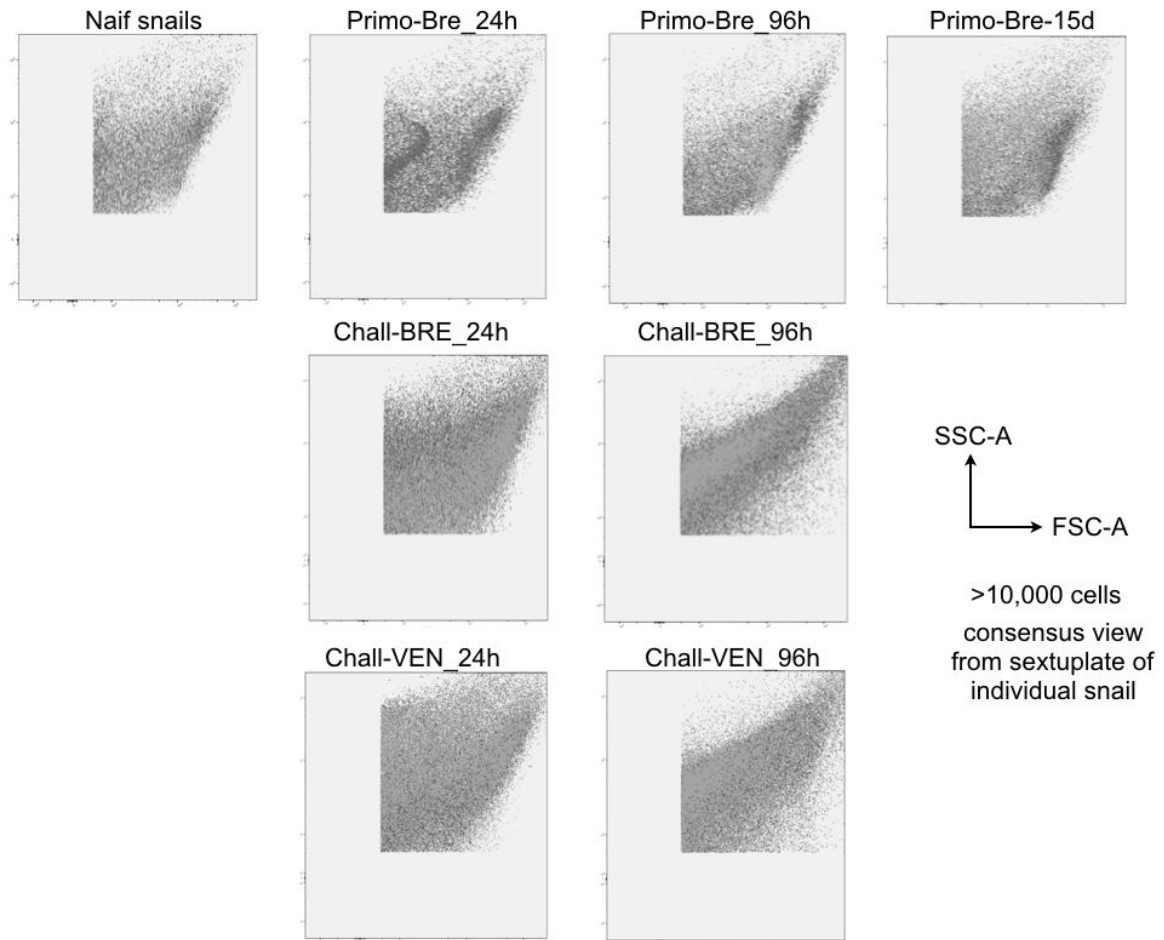


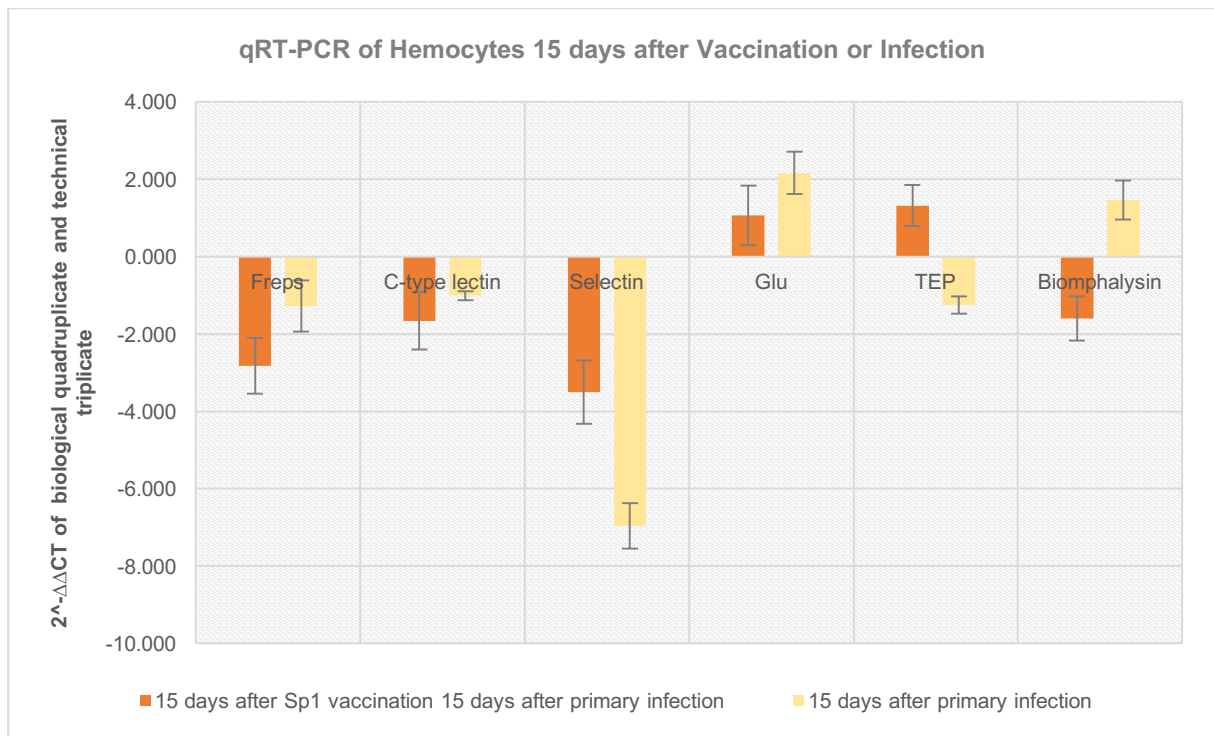
Figure 6. Specificity of *in-vitro* toxicity Red-left part present homologous SmBRE experiment while the green-right part the SmVEN homologous experiment. All tests were realised on BgBRE.



Supplementary 1. Cytometry essay in fluorescent-granularity



Supplementary 2. Cytometry assay in Size-granularity



Supplementary 3. Q-rtpPCR assay on priming candidate between natural infection and vaccination

Publication : Clearance of schistosome parasites by resistant genotypes at a single gene region in *Biomphalaria glabrata* snails involves cellular components of the hemolymph.

ALLAN Euan R.O., GOURBAL Benjamin, DORES Camila B., PORTET Anaïs, BAYNE Christopher J. and BLOUIN Michael S.

*Article en soumis dans International Journal for Parasitology

Ma participation à ces travaux :

Suite à la découverte d'une variation allélique dans une région de gène unique, le complexe de résistance Guadeloupe (GRC), entre des mollusques résistants et sensibles, une étude histologique fut menée pour mettre en évidence la réponse immunitaire de *B. glabrata* au cours d'une infection par *S. mansoni*. Dans ce cadre, j'ai participé à la réalisation des lames histologiques, plus particulière à leur coloration. Cette approche a permis de mettre en évidence une encapsulation des parasites dans les premières 24-48h chez des mollusques résistants. Par ailleurs cette résistance pouvait être transférée aux mollusques sensibles via l'hémolymphe. Cette GRC semble donc aider à la coordination de la réponse immunitaire cellulaire par médiation des hémocytes lors d'une infection par *S. mansoni*.



Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Clearance of schistosome parasites by resistant genotypes at a single genomic region in *Biomphalaria glabrata* snails involves cellular components of the hemolymph

Euan R.O. Allan^{a,*}, Benjamin Gourbal^b, Camila B. Dores^c, Anais Portet^b, Christopher J. Bayne^a, Michael S. Blouin^a

^a Department of Integrative Biology, College of Science, Oregon State University, Corvallis, OR, USA

^b Université Perpignan Via Domitia, Interactions Hôtes Pathogènes Environnements UMR 5244, CNRS, IFREMER, Univ. Montpellier, F-66860 Perpignan, France

^c Department of Biomedical Sciences, Oregon State University, Corvallis, OR, USA

ARTICLE INFO

Article history:

Received 12 July 2017

Received in revised form 23 August 2017

Accepted 31 August 2017

Available online xxxx

Keywords:

Schistosomiasis

Biomphalaria glabrata

Histology

Hemocyte

Resistance

Hemolymph transfer

Plasma transfer

ABSTRACT

Schistosomiasis is one of the most detrimental neglected tropical diseases. Controlling the spread of this parasitic illness requires effective sanitation, access to chemotherapeutic drugs, and control over populations of the freshwater snails, such as *Biomphalaria glabrata*, that are essential intermediate hosts for schistosomes. Effectively controlling this disease, while minimising ecological implications of such control, will require an extensive understanding of the immunological interactions between schistosomes and their molluscan intermediate hosts. Here we histologically characterise the clearance of schistosome larvae by snails that exhibit allelic variation at a single genomic region, the Guadeloupe resistance complex. We show that snails with a resistant Guadeloupe resistance complex genotype clear schistosomes within the first 24–48 h, and that this resistance can be transferred to susceptible snails via whole hemolymph but not cell-free plasma. These findings imply that Guadeloupe resistance complex-coded proteins help to coordinate hemocyte-mediated immune responses to schistosome infections in Guadeloupean snails.

© 2017 Australian Society for Parasitology. Published by Elsevier Ltd. All rights reserved.

1. Introduction

Schistosomiasis is a devastating and neglected tropical illness that is responsible for hundreds of thousands of deaths each year, and afflicts over 250 million people in the developing world (WHO, 2012, 2016). This disease continues to persist despite mass chemotherapeutic drug administration by the World Health Organization (WHO), primarily because there are no effective vaccines targeting schistosomes, sanitation is lacking in some developing regions, and other mammals can act as reservoir hosts and perpetuate the disease. Schistosomes require an intermediate snail host for their larval miracidial stage to develop, and regions that are unable to control these snails exhibit exacerbations in schistosomiasis cases (Sokolow et al., 2016). Presently, the only areas where schistosomes have been completely eliminated are regions where these intermediate snail hosts are extirpated, which makes them an essential target for disease control (Sokolow et al., 2016).

In the New World, miracidia from *Schistosoma mansoni* infect the aquatic snail *Biomphalaria glabrata*, which goes on to shed cercariae capable of causing human disease. This snail species has innate immune defenses that can both specifically and non-specifically target schistosomes for destruction (Coustau et al., 2015). The first line of defense against miracidial penetration is the integument of the snail. The integument provides a physical barrier of connective tissue and ciliated microvillus cells that protect from invading pathogens (Adlard, 2003). Miracidia must break through this barrier before they can infect any given individual. Successful miracidia, and the sporocysts that consequently develop, are then exposed to potentially degenerating humoral factors and attacked by motile hemocytes in the hemolymph and other tissues (Hanington et al., 2010; Loker, 2010). When schistosomes are recognised by hemocytes in resistant snails, they are encapsulated and exposed to anti-microbial effectors including reactive oxygen species which destroy the schistosome (Bender et al., 2005; Loker, 2010). Miracidia and sporocysts have numerous ways of evading destruction by humoral factors and hemocytes. Notable evasion tactics involve obscuring sporocyst detection post-integument penetration (Peterson et al., 2009), and the

* Corresponding author.

E-mail address: euanoallan@gmail.com (E.R.O. Allan).

production of oxidative scavengers to protect them from hemocyte-mediated damage if they are detected (Mourao Mde et al., 2009; Mone et al., 2011). Exposed snails that are able to recognise and efficiently degrade invading schistosomes can escape infection, and subsequently avoid transmitting the disease to humans (Portela et al., 2013; Pinaud et al., 2016).

Resistance of Guadeloupean *B. glabrata* (BgGUA) to Guadeloupean *S. mansoni* (SmGUA) has been shown to be strongly correlated with allelic variation in the Guadeloupe resistance complex (GRC), but not with mRNA expression levels of these genes (Tennesen et al., 2015b). The GRC is a gene region which contains novel genes with vast amino acid sequence differences and putative immune recognition functions. *Grctm6* is a novel GRC-encoded protein that has been shown to affect the number of cercariae shed per infected snail and is expressed in hemocytes (Allan et al., 2017b). Understanding the kinetics and biology of this new class of genes in schistosome defense is essential before they might be used in alternative schistosome control. Although these studies have highlighted the importance of the GRC during schistosome infection, little is known about the actual mechanisms by which snails bearing resistant genotypes differ from those bearing susceptible genotypes. Determining when resistant genotypes clear parasitic larvae, and whether cellular or humoral factors are involved in clearance, is essential to understanding the mechanistic role of the genes in the GRC.

Histological differences between highly resistant and highly susceptible strains of snails have been characterised extensively, but these studies did not describe the genes that may cause the differences between those strains (Richards and Minchella, 1987; Borges et al., 1998; Loker, 2010; Nacif-Pimenta et al., 2012). Similarly, both cellular and humoral factors have been shown to transfer resistance from highly resistant to highly susceptible strains, but again no study has linked this transfer to allelic variation in specific genes (Sullivan et al., 1995, 2004; Vasquez and Sullivan, 2001a,b,c; Pinaud et al., 2016). In the present study, we report that snails bearing resistant GRC haplotypes have fewer unencapsulated sporocysts than snails bearing susceptible haplotypes as early as 24 h p.i., despite having equivalent integument structure and susceptibility to penetration by miracidia. We also show that resistance can be transferred from resistant GRC genotypes to susceptible GRC genotypes via injections of the resistant haplotype's hemolymph but not cell-free plasma. These findings demonstrate that GRC-mediated resistance to schistosome exposure occurs during the early stages of infection and is likely cell-mediated.

2. Materials and methods

2.1. Maintenance, propagation, infection and inbred line derivation of *B. glabrata* and *S. mansoni*

Biomphalaria glabrata (BgGUA) and *S. mansoni* (SmGUA) were collected in 2005 from Dans Fond (Theron et al., 2008) on the island of Guadeloupe, and maintained as previously described (Theron et al., 2014; Tennesen et al., 2015b). The SmGUA strain of *S. mansoni* was cycled through BgGUA and hamsters, and parasites were isolated from hamster livers or from shedding snails. BgGUA snails were segregated based on their genotype at the GRC locus, and separated into nine independently derived inbred lines as previously described: three RR (resistant lines R^1 , R^2 , R^3), three S1S1 (susceptible lines $S1^1$, $S1^2$, $S1^3$), and three S2S2 (susceptible lines $S2^1$, $S2^2$, $S2^3$) (Tennesen et al., 2015b; Allan et al., 2017b). To produce these lines, independent founder snails, homozygous at the GRC locus, were allowed to self-fertilise for three generations. For all additional experiments, equal numbers of snails pooled from each of the three lines within a GRC genotype

were used (RR, S1S1, S2S2 or RR, SS; where SS is pooled S1S1 and S2S2). Experiments were performed on size/age matched (~7 mm, ~7 weeks old) cohorts of snails which were housed identically. This research adheres to Public Health Service Domestic Assurance for humane care and use of laboratory animals (PHS Animal Welfare Assurance Number A3229-01), as Animal Care and Use Proposal 4360, and was approved by the Oregon State University Institutional Animal Care and Use Committee, USA.

2.2. Confirmation of infection phenotypes in BgGUA lines

In order to verify that the inbred lines of BgGUA that were to be used for functional work behaved phenotypically like outbred snails of the same genotype, we examined the infection phenotype of all inbred lines using the same exposure protocol that was used on outbred BgGUA (Tennesen et al., 2015b). Parasite exposures of BgGUA with SmGUA were carried out as previously described (Tennesen et al., 2015b; Allan et al., 2017b). Snails were incubated in 2 ml of dechlorinated water in individual wells of a 24 well plate containing 20 miracidia for 24 h, and then transferred into tanks containing 10–15 snails each to be monitored for infection. Three independent lines each of RR (R^1 , R^2 , R^3), S1S1 ($S1^1$, $S1^2$, $S1^3$), and S2S2 ($S2^1$, $S2^2$, $S2^3$) snails were used to verify the susceptibility of these lines (Fig. 1). Infections were done on a minimum of two separate occasions with the total number of snails that were exposed exceeding 27 for any given line or treatment ($n = 32 S1^1$, $32 S1^2$, $28 S1^3$, $35 S2^1$, $33 S2^2$, $27 S2^3$, $55 R^1$, $31 R^2$, $42 R^3$). Exposed snails were scored for cercarial shedding by placing individual snails in 2 ml of dechlorinated water in a 24 well plate for 3 h, and scored as either infected or uninfected. This was done once per week between weeks 5–10 post exposure as previously described (Hanington et al., 2010, 2012; Pila et al., 2016b; Allan et al., 2017b).

2.3. Histological analysis

We histologically examined exposed snails of each genotype to determine the extent of early sporocyst development at different time points. Additionally, we examined the histological structure of the integument to ascertain if any difference in this outer barrier could explain differences in the infection phenotype of the GRC genotypes. For histological analysis of infected snails, BgGUA that were exposed to 20 miracidia were collected after 24 h, 48 h, or 10 days, and removed from their shells and fixed as previously described, with some modifications (Pinaud et al., 2016). In brief,

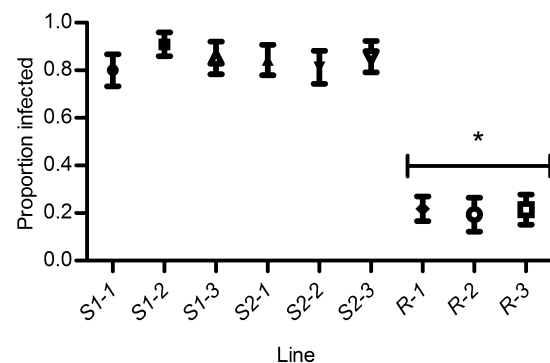


Fig. 1. Resistance phenotypes are conserved among inbred Guadeloupean *Biomphalaria glabrata* (BgGUA) lines. The susceptibility of nine independent homozygous BgGUA lines (3 × S1S1, S2S2 and RR) after exposure to 20 Guadeloupean *Schistosoma mansoni* (SmGUA) miracidia ($n = 32 S1^1$, $32 S1^2$, $28 S1^3$, $35 S2^1$, $33 S2^2$, $27 S2^3$, $55 R^1$, $31 R^2$, $42 R^3$). Data are presented as the proportion of infected snails (\pm the S.E. of proportions). Significant differences ($P < 0.05$, Z score of proportion) are denoted by asterisks (*).

two each of R^1 , R^2 , $S1^1$, $S1^2$, $S2^1$, $S2^2$ BgGUA ($n = 4$ RR and 8 SS) were dissected out of their shells and fixed in Bouin's solution (75% saturated aqueous picric acid, 20% formaldehyde, 5% acetic acid; pH 1.2–1.6). After a 48 h fixation, the snails underwent consecutive 12 h washes in 95% ethanol for 36 h, and were dehydrated using consecutive 12 h washes in butanol for 36 h prior to sectioning. Fixed whole BgGUA were embedded in paraffin, underwent transverse ($3\ \mu\text{m}$ thick) sectioning (RHEM platform, Montpellier, France), and were stained with azocarmine G and Heidenhain's azan (Pinaud et al., 2016). Approximately 300 sections were stained and analysed per snail, and all sporocysts in these sections were counted. Sections underwent the following: (i) re-hydration (toluene 95, 70, 30% ethanol and distilled water); (ii) coloration (azocarmine G, 70% ethanol/1% aniline, 1% acetic alcohol, distilled water, 5% phosphotungstic acid, distilled water, Heidenhain's azan) and (iii) dehydration (95% ethanol, absolute ethanol, toluene). The preparations were then mounted with Entellan (Sigma Life Science, St. Louis Missouri, USA) and examined. All embedding, staining and histological analysis of exposed snails were completed by the group of Dr. Gourbal at the University of Perpignan, France.

For analysis of the integument of BgGUA, snails were removed from their shells and fixed in Bouin's solution as described above. Fixed snails were placed in histology cassettes and sections underwent routine diagnostic H&E staining as previously described (Allan et al., 2014, 2017a). Slides were evaluated microscopically using a Nikon Eclipse 50i, and measurements were performed using the Nikon NIS-Elements Microscope Imaging Software. Integument thickness was measured on three distinct regions of the pedal sole from each snail. Four snails of each RR, S1S1, and S2S2 were evaluated by a veterinary pathologist (Dr. Dore) at the Veterinary Diagnostic Laboratory at Oregon State University.

2.4. Enumeration of miracidia during exposure

We wanted to know whether miracidia penetrate the three genotypes with equal frequency. Therefore, we quantified the minimum number of miracidia that did not penetrate during exposure by counting the number of miracidia remaining in an exposure well after snails were removed following 2 h exposures (Theron et al., 1997). Miracidia were enumerated under a dissecting scope ($n = 12$ RR, 12 S1S1, 12 S2S2).

2.5. Hemolymph and cell-free hemolymph (plasma) transfer

To examine if resistance could be transferred via the direct addition of cells or endogenous molecules, S2S2 or RR hemolymph was transferred to S1S1 snails (thus avoiding injection of any genotype's own hemolymph into itself) 24 h before exposure to 20 miracidia. Two independent experimental exposures were conducted. Exposures were performed as described above with the following modifications. Hemolymph was collected from R^1 , R^2 , R^3 , $S2^1$, $S2^2$ and $S2^3$ BgGUA snails (~ 10 snails per line) and pooled by genotype prior to injection (providing pooled RR hemolymph batches consisting of hemolymph from R^1 , R^2 , and R^3 ; and pooled S2S2 batches of hemolymph consisting of hemolymph from $S2^1$, $S2^2$ and $S2^3$). The number of cells in the hemolymph of the three genotypes of BgGUA was determined using a haemocytometer and did not differ between groups. A small volume of sterile snail saline (SSS: 5 mM HEPES, 3.7 mM NaOH, 36 mM NaCl, 2 mM KCl, 2 mM MgCl₂, 4 mM CaCl₂, pH 7.8) was added to ensure equal cell numbers if any discrepancy was observed on a given experimental day. Pooled RR or S2S2 hemolymph was immediately injected into S1S1 ($S1^1$, $S1^2$, $S1^3$) snails (~ 15 snails/line, 20 μl /snail). Concurrently, S1S1 ($S1^1$, $S1^2$, $S1^3$) snails (~ 15 snails/line, 20 μl /snail) were also injected with filter sterilised SSS as sham controls (Adema et al., 1993; Pila

et al., 2016a). As a third treatment, RR hemolymph was also left for 15 min and centrifuged for 15 min at 2000g (for cell-free plasma collection) and injected into S1S1 ($S1^1$, $S1^2$, $S1^3$) snails (~ 15 snails/line, 20 μl /snail on one occasion) (Pila et al., 2016b; Pinaud et al., 2016; Allan et al., 2017b). Snails that survived past week 2 for cercarial scoring exceeded 31 per group and were scored on week 5 ($n = 69$ S1S1 with SSS sham, $n = 67$ S1S1 with S2S2 hemolymph, $n = 31$ S1S1 with RR plasma, $n = 74$ S1S1 with RR hemolymph). Hemocyte only preparations were attempted, but cell viability and morphology were unacceptable for transfer after isolation. These observations are consistent with previous findings that isolation and resuspension of hemocytes can negatively affect their viability (Dr. Christopher Bayne, personal observation).

2.6. Statistical analysis

Statistical analyses were carried using GraphPad Prism software (La Jolla, CA, USA) (Allan and Yates, 2015). A one-way ANOVA (or unpaired Student's *t*-test) with a Tukey post-test was used for comparing means ($P < 0.05$). Before reanalysis, a natural log transformation (ln) was performed if data did not have equal variances (Bartlett's or *F*-test) (Allan and Habibi, 2012). Proportions were compared by calculating the *Z* score (standard score) of the population (Allan et al., 2017b).

3. Results

3.1. Resistance phenotypes are conserved among inbred lines of BgGUA

In the outbred BgGUA population the *R* allele confers an eight-fold decrease in the odds of infection (Tennessen et al., 2015b). Here we verified that inbred lines derived from that outbred population behaved similarly. All three lines homozygous for the resistant allele (RR) became infected $\sim 20\%$ of the time post-exposure, while all six families homozygous for one of the two susceptible GRC alleles (S1S1, S2S2) were infected at a rate of $\sim 80\%$ (Fig. 1) (Tennessen et al., 2015b; Allan et al., 2017b). These lines were then used for histological and mechanistic interrogation of these genotypes.

3.2. Despite equivalent integument anatomy and miracidial penetration, resistant BgGUA have fewer free sporocysts after exposure

The integument is one of the first lines of defense during pathogenic exposure. Therefore, we considered the possibility that GRC-encoded products could influence integument structure such that SmGUA larvae were less able to penetrate the integument of RR lines than SS lines. For this reason we compared, at the histological level, the integuments of SS and RR snails. Additionally, by counting miracidia left behind when snails were removed after 2 h of exposure, we collected independent data indicative of miracidial penetration of snail skin. At a histological level, the structure, cellular composition and thickness (all determined by a veterinary pathologist) of the integument did not vary among GRC genotypes (Fig. 2A, B). Additionally, the number of miracidia unable to gain access (< 1 on average) to the snail after 2 h did not differ between resistant and susceptible snails, suggesting that equal numbers of miracidia penetrate these snails during an exposure (Fig. 2C). Therefore, we have no reason to believe that miracidia differentially penetrate the three genotypes.

We histologically examined snails 24 h, 48 h, and 10 days post-exposure. We found no sporocysts in RR snails 10 days after exposure, so accordingly no additional analysis was conducted. After as little as 24 h, sporocysts in RR snails were clearly in the late stages of destruction, while the majority of sporocysts in SS snails were

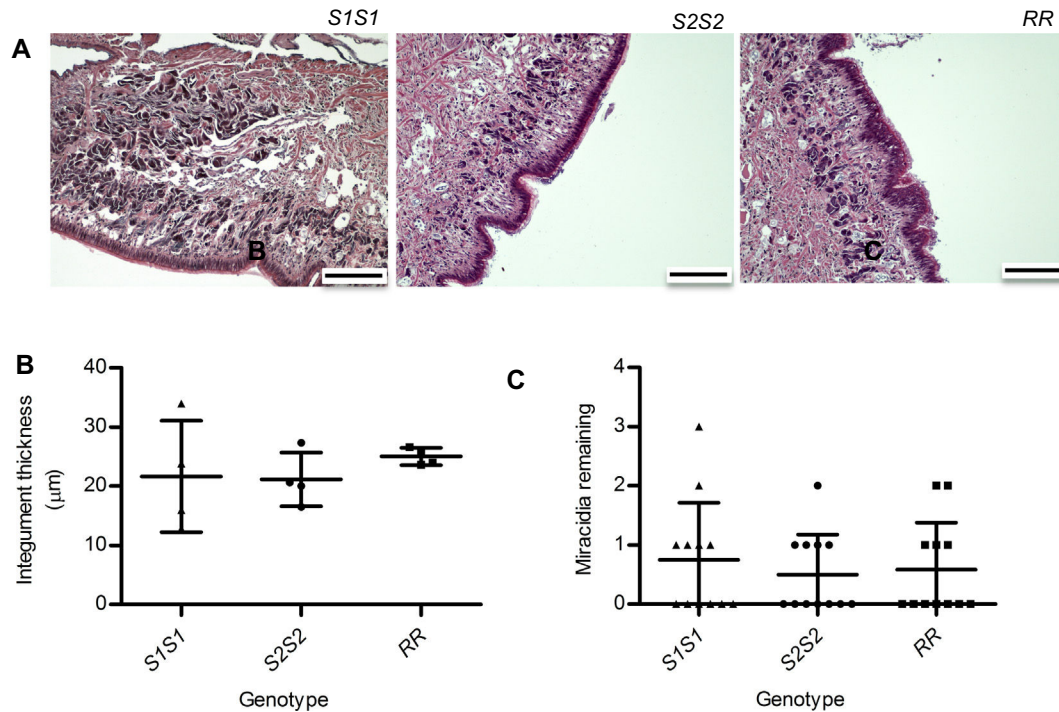


Fig. 2. Resistant genotypes do not have modified integument anatomy or thickness, nor do they prevent *Schistosoma mansoni* miracidial penetration in Guadeloupean *Biomphalaria glabrata* (BgGUA). (A) The head-foot integument architecture of S1S1, S2S2, and RR BgGUA (scale bar = 100 µm). (B) The thickness of the integument of the head-foot of S1S1, S2S2, and RR BgGUA ($n = 4$). (C) The number of miracidia remaining in a well 2 h post-BgGUA exposure to 20 miracidia ($n = 12$). Data presented as means (\pm S.D.). No significant differences ($P > 0.05$, ANOVA) were observed.

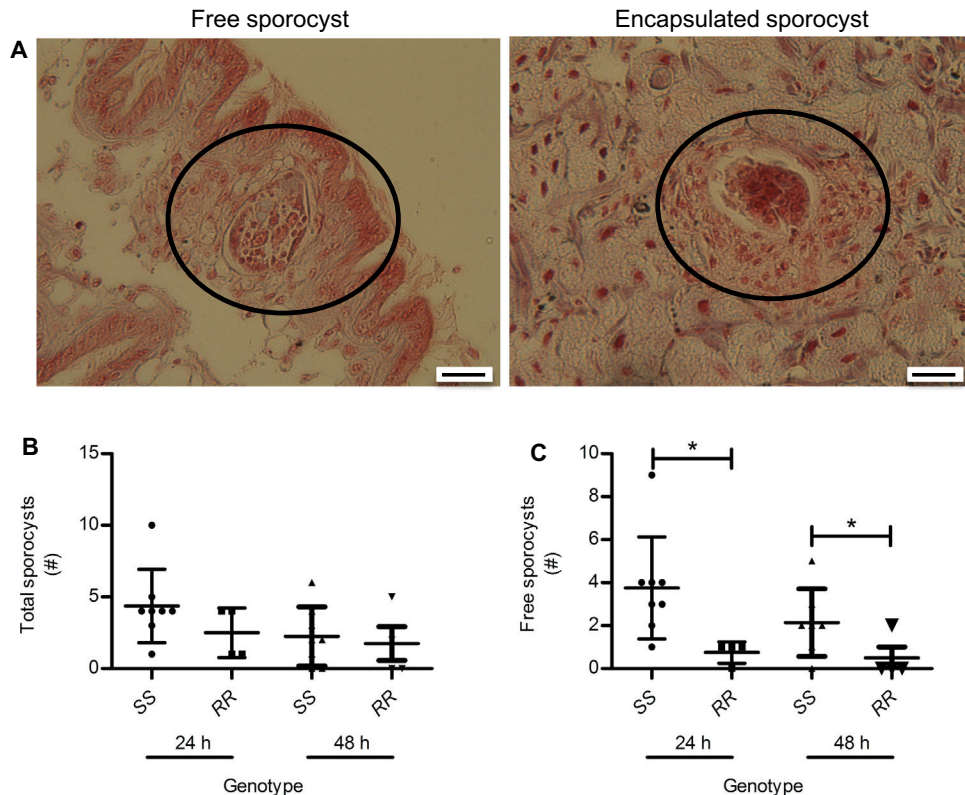


Fig. 3. Resistant Guadeloupean *Biomphalaria glabrata* (BgGUA) have fewer free sporocysts, and destroy schistosomes during the initial stages of infection. (A) Free and encapsulated sporocysts in BgGUA 24 h post-exposure (scale bar = 200 µm). (B) Total number and (C) number of free (unencapsulated/undegenerated) sporocysts found in individual SS and RR BgGUA 24 h and 48 h post-exposure. Data presented as means (\pm S.D.). Significant differences ($P < 0.05$, student's t -test) are denoted by asterisks (*).

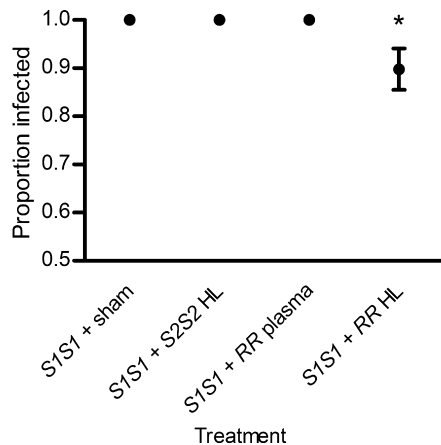


Fig. 4. Transfer of resistant (*RR*) hemolymph, but not cell-free plasma or sensitive (*SS*) hemolymph, confers resistance to Guadeloupean *Schistosoma mansoni* (*SmGUA*). The susceptibility of homozygous *S1S1* snails after exposure to 20 miracidia and transfer treatments. Groups of snails were treated with sham (*SSS*), *S2S2* hemolymph (*HL*), *RR* plasma, or *RR* hemolymph (*HL*) 24 h pre-exposure ($n = 69$ *S1S1* + sham, $n = 67$ *S1S1* + *S2S2* *HL*, $n = 31$ *S1S1* + *RR* plasma, $n = 74$ *S1S1* + *RR* *HL*). Data are presented as the proportion of infected snails (\pm the S.E. of proportions), and significant differences ($P < 0.05$, Z score of proportion) are denoted by an asterisk (*).

not evidently under immune attack (Fig. 3A). Indeed, the number of ‘free’ (not degenerating, encapsulated or under any identifiable suppression by the immune system) sporocysts was four-fold higher in *SS* snails (Fig. 3C). However, the total number of sporocysts identified at 24 and 48 h did not differ between *RR* and *SS* snails (Fig. 3B), an observation that is consistent with the conclusion that miracidia penetrate all three genotypes equally well (Figs. 2 and 3).

3.3. Schistosome resistance is conferred to *SS* snails after transfer of *RR* hemolymph

The integument is anatomically similar in *RR* and *SS* snails and equal numbers of parasites gain access to each genotype; yet *RR* snails rapidly clear schistosomes whereas *SS* snails generally fail to do so. Therefore, we sought to determine whether the protective factor is carried in the hemolymph and, if so, whether it is humoral or cellular. We therefore attempted to transfer resistance to *SS* snails via *RR* hemolymph as it likely contains mobile hemocytes and humoral factors that may recognise and coordinate the initial innate internal defense response against schistosomes. *RR* hemolymph conferred a statistically significant $\sim 10\%$ increase in resistance of *S1S1* snails, while *RR* cell-free plasma or *S2S2* hemolymph had no effect on susceptibility (Fig. 4). Therefore, cells in *RR* hemolymph were able to significantly improve the resistance of susceptible snails. Interestingly, the susceptibility of *SS* snails increased to 100% during the transfer treatment, probably owing to stress, mechanical damage or the immunological consequences of performing injections just 24 h before exposure. Attempts to transfer isolated hemocytes were unsuccessful because the majority of isolated hemocytes did not survive isolation, resuspension and passage through a transfer needle.

4. Discussion

Understanding the immunological interactions between *B. glabrata* and schistosomes is believed to be essential to the future control of schistosomiasis (Pearce and MacDonald, 2002; Mourao Mde et al., 2009; Stefanic et al., 2010; Bhardwaj et al., 2011; Valentim et al., 2013; Theron et al., 2014; Coustau et al., 2015; Guidi et al.,

2015; Tennessen et al., 2015a,b; Pila et al., 2016b; Pinaud et al., 2016; Sokolow et al., 2016; Allan et al., 2017b). Compatible snail species evade schistosome infection via complex physical and immunological mechanisms often involving parasite recognition (Bender et al., 2005; Goodall et al., 2006; Hanington et al., 2010; Loker, 2010; Moremen et al., 2012; Theron et al., 2014; Coustau et al., 2015; Tennessen et al., 2015b; Pinaud et al., 2016; Allan et al., 2017b). The mechanistic bases of these interactions, and their importance to the spread of schistosomiasis by snails, are still under intensive scrutiny.

The GRC region has been shown to have a strong allelic correlation with the resistance of *BgGUA* to *SmGUA* (Tennessen et al., 2015b). Proteins in the GRC have been suggested to have an immune recognition function due to their putative structure (Tennessen et al., 2015b; Allan et al., 2017b). Furthermore, at least one protein in this region has been shown to be integral to the suppression of schistosomes in these snails and is expressed in the hemolymph (Allan et al., 2017b). Determining how the GRC influences the progression of schistosome infection is an integral part of understanding how these genes may allow some snails to be highly resistant to infection. To facilitate the functional study of resistant and susceptible individuals, homozygous *BgGUA* lines were developed based on their genotype at the GRC. These lines maintained the resistance phenotypes that were previously observed in the outbred population, allowing for more extensive functional assessments of infection (Tennessen et al., 2015b; Allan et al., 2017b).

In the present study, we determined that individual snails with resistant GRC genotypes destroy more compatible sporocysts in the initial days following infection than susceptible individuals, despite apparently having the same initial schistosome burden. We went on to show that some of this resistance can be transferred to susceptible snails by injecting them with resistant hemolymph, and that hemocytes are probably essential for this defensive priming. Therefore, GRC proteins are likely involved in the initial response of hemocytes to schistosome infection.

After the shell, the molluscan integument is the first line of defense against most pathogens and parasites. This structure consists of an epithelium and subepithelium, which predominantly consist of ciliated cuboidal cells and secretory cells, respectively (Adlard, 2003). These cells comprise a physical barrier to invaders, as well as a motile and secretory network that can damage or remove pathogens. Although the integument has never been implicated in differences among snail strains in susceptibility to schistosomes, we decided to check for variation among genotypes in this structure to rule out the possibility that differences between GRC genotypes in thickness or structure could be causing fewer miracidia to penetrate *RR* snails.

Additionally, given that there were few developing sporocysts (~ 1 – 4 sporocysts in 300 sections/snail) found per snail, this examination also allowed us to determine if the first physical barrier the miracidia encounter differed between genotypes and could have been partially responsible for so few sporocysts. The integuments of *RR* and *SS* *BgGUA* were indistinguishable with regards to cell structure, cell composition and thickness at a histological level. Accordingly, the parasitic burden was equivalent across *RR* and *SS* snails, suggesting that schistosome resistance is probably due to an immune response to sporocysts after the miracidia have penetrated the snail. It is possible that more subtle differences in the integument, or in the kinetics of schistosome penetration between genotypes, could be differentiated by electron microscopy or immunohistochemistry. However, we found similar numbers of total schistosome larvae in *RR* and *SS* snails at 24 and 48 h post-exposure and similar numbers of miracidia left behind after 2 h exposures. So, it is unlikely that such subtle differences in the integument influenced the overall ability of *SmGUA* to penetrate

the integument of BgGUA. The number of parasites found in the snails and remaining in the wells does not account for all of the parasites from the exposure. It is possible that some of these parasites were attached to the shell, or caught in the mucus of the snail and lost from our analysis. Additional microscopic methods could be used in future studies to ascertain where some of these parasites end up, and the roles that the mucus and shell may play in infection.

The developmental capacity of miracidia has been shown to be proportional to the number of miracidia that penetrate the host (Theron et al., 1997). In general, sporocysts that do not survive have been shown to be encapsulated within the first 48 h, and the immune response to these sporocysts, mediated by hemocytes and humoral factors, has been shown to be faster and more extensive in more resistant strains of *B. glabrata* (Theron et al., 1997; Pereira et al., 2008). Parasite larvae which are not destroyed can be found developing weeks after an exposure in individuals that become infected, but prior to cercarial shedding. Indeed, we found that RR individuals had fewer ‘free’ (not encapsulated by hemocytes or degenerated by humoral factors) sporocysts despite having the same initial miracidial burden after exposure. RR snails were able to clear parasite larvae such that that no histological evidence of exposure was evident after 10 days in any of the RR snails that we examined.

Given that the transfer of cell-free hemolymph has been shown to confer resistance during the initial phases of infection in other strains of *B. glabrata* (Pereira et al., 2008; Pinaud et al., 2016), we aimed to determine if the transfer of different hemolymph treatments could improve the resistance of SS individuals. The transfer procedure itself increased the infection outcome to 100% in control SS snails, likely due to stress, mechanical damage, hemolymph dilution, induction of an unintended immune response, or a combination of these factors. Despite this, transferred S2S2 hemolymph and RR plasma (cell-free hemolymph including humoral factors) did not alter the resistance of S1S1 snails, but RR hemolymph conferred a 10% increase in resistance. This result implies that proteins encoded by genes in the GRC region are most likely present in RR hemocytes (as RR plasma alone did not confer resistance), and are partially responsible for the resistance phenotypes we observed and the efficient clearance of schistosome larvae by RR snails, as previously hypothesised (Tennessen et al., 2015b; Allan et al., 2017b). These results also confirm that humoral factors in RR BgGUA are probably not responsible for their greater resistance to schistosomes, but we cannot eliminate the possibility that they may work synergistically with hemocytes because we were unable to transfer a pure hemocyte preparation.

In summary, we found that schistosomes are equally likely to penetrate the three BgGUA genotypes, but that RR snails clear schistosomes much more effectively and rapidly – most within 48 h. This difference between GUA region genotypes is similar to what has been observed in comparisons between highly differentiated lines of snails that differ in their susceptibility to particular strains of *S. mansoni* (Richards and Minchella, 1987; Theron et al., 1997; Borges et al., 1998; Loker, 2010; Nacif-Pimenta et al., 2012). However, we believe this is the first time such variation can be ascribed to variation in particular genes. We postulate that proteins encoded in the GRC region are acting via hemocytes in the hemolymph because hemolymph from RR snails was able to transfer some resistance to SS individuals, while cell-free plasma was not. Previous work showed that proteins coded for by the GRC regions have structures reminiscent of immune recognition proteins and are expressed in hemolymph (Tennessen et al., 2015b; Allan et al., 2017b). These previous observations and our results here are all consistent with the hypothesis that proteins encoded in the GRC region are involved in pathogen recognition by hemocytes.

Acknowledgements

We would like to thank Jacob Tennessen, Stephanie Bollmann, Leeah Whittier, Ryan Wilson and Ekaterina Peremyslova for their technical support. This work was supported by funding from the National Institutes of Health, USA (AI109134 and AI111201) obtained by MB, and the French National Agency for Research (ANR) through a project Invimory grant (ANR-13-JSV7-0009) obtained by BG.

References

- Adema, C.M., van Deutekom-Mulder, E.C., van der Knaap, W.P., Sminia, T., 1993. NADPH-oxidase activity: the probable source of reactive oxygen intermediate generation in hemocytes of the gastropod *Lymnaea stagnalis*. *J. Leukoc. Biol.* 54, 379–383.
- Aldard, R.D., 2003. *Histology and Anatomy-pathology of Molluscs: A Guide for Diagnosticians*. European Community Reference Laboratory, Ifremer, La Tremblade, France.
- Allan, E.R., Habibi, H.R., 2012. Direct effects of triiodothyronine on production of anterior pituitary hormones and gonadal steroids in goldfish. *Mol. Reprod. Dev.* 79, 592–602.
- Allan, E.R., Taylor, P., Balce, D.R., Pirzadeh, P., McKenna, N.T., Renaux, B., Warren, A.L., Jirik, F.R., Yates, R.M., 2014. NADPH oxidase modifies patterns of MHC class II-restricted epitopic repertoires through redox control of antigen processing. *J. Immunol.* 192, 4989–5001.
- Allan, E.R., Yates, R.M., 2015. Redundancy between cysteine cathepsins in murine experimental autoimmune encephalomyelitis. *PLoS One* 10, e0128945.
- Allan, E.R.O., Campden, R.L., Ewanchuk, B.W., Taylor, P., Balce, D.R., McKenna, N.T., Greene, C.J., Warren, A.L., Reinheckel, T., Yates, R.M., 2017a. A role for cathepsin Z in neuroinflammation provides mechanistic support for an epigenetic risk factor in multiple sclerosis. *J. Neuroinflammation* 14, 103.
- Allan, E.R.O., Tennessen, J.A., Bollmann, S.R., Hanington, P.C., Bayne, C.J., Blouin, M.S., 2017b. Schistosome infectivity in the snail, *Biomphalaria glabrata*, is partially dependent on the expression of Grctm6, a Guadeloupe Resistance Complex protein. *PLoS Negl. Trop. Dis.* 11.
- Bender, R.C., Broderick, E.J., Goodall, C.P., Bayne, C.J., 2005. Respiratory burst of *Biomphalaria glabrata* hemocytes: *Schistosoma mansoni*-resistant snails produce more extracellular H₂O₂ than susceptible snails. *J. Parasitol.* 91, 275–279.
- Bhardwaj, R., Krautz-Peterson, G., Skelly, P.J., 2011. Using RNA interference in *Schistosoma mansoni*. *Methods Mol. Biol.* 764, 223–239.
- Borges, C.M., de Souza, C.P., Andrade, Z.A., 1998. Histopathologic features associated with susceptibility and resistance of *Biomphalaria* snails to infection with *Schistosoma mansoni*. *Mem. Inst. Oswaldo Cruz* 93 (Suppl 1), 117–121.
- Coustau, C., Gourbal, B., Duval, D., Yoshino, T.P., Adema, C.M., Mitta, G., 2015. Advances in gastropod immunity from the study of the interaction between the snail *Biomphalaria glabrata* and its parasites: a review of research progress over the last decade. *Fish Shellfish Immunol.* 46, 5–16.
- Goodall, C.P., Bender, R.C., Brooks, J.K., Bayne, C.J., 2006. *Biomphalaria glabrata* cytosolic copper/zinc superoxide dismutase (SOD1) gene: association of SOD1 alleles with resistance/susceptibility to *Schistosoma mansoni*. *Mol. Biochem. Parasitol.* 147, 207–210.
- Guidi, A., Mansour, N.R., Paveley, R.A., Carruthers, I.M., Besnard, J., Hopkins, A.L., Gilbert, I.H., Bickle, Q.D., 2015. Application of RNAi to genomic drug target validation in schistosomes. *PLoS Negl. Trop. Dis.* 9, e0003801.
- Hanington, P.C., Forsy, M.A., Dragoo, J.W., Zhang, S.M., Adema, C.M., Loker, E.S., 2010. Role for a somatically diversified lectin in resistance of an invertebrate to parasite infection. *Proc. Natl. Acad. Sci. U.S.A.* 107, 21087–21092.
- Hanington, P.C., Forsy, M.A., Loker, E.S., 2012. A somatically diversified defense factor, FREP3, is a determinant of snail resistance to schistosome infection. *PLoS Negl. Trop. Dis.* 6, e1591.
- Loker, E.S., 2010. *Gastropod Immunobiology*. *Adv. Exp. Med. Biol.* 708, 17–43.
- Mone, Y., Ribou, A.C., Cosseau, C., Duval, D., Theron, A., Mitta, G., Gourbal, B., 2011. An example of molecular co-evolution: reactive oxygen species (ROS) and ROS scavenger levels in *Schistosoma mansoni*/*Biomphalaria glabrata* interactions. *Int. J. Parasitol.* 41, 721–730.
- Moremen, K.W., Tiemeyer, M., Nairn, A.V., 2012. Vertebrate protein glycosylation: diversity, synthesis and function. *Nat. Rev. Mol. Cell Biol.* 13, 448–462.
- Mourao Mde, M., Dinguirard, N., Franco, G.R., Yoshino, T.P., 2009. Role of the endogenous antioxidant system in the protection of *Schistosoma mansoni* primary sporocysts against exogenous oxidative stress. *PLoS Negl. Trop. Dis.* 3, e550.
- Nacif-Pimenta, R., de Mattos, A.C., Orfano Ada, S., Barbosa, L., Pimenta, P.F., Coelho, P.M., 2012. *Schistosoma mansoni* in susceptible and resistant snail strains *Biomphalaria tenagophila*: in vivo tissue response and in vitro hemocyte interactions. *PLoS One* 7, e45637.
- Pearce, E.J., MacDonald, A.S., 2002. The immunobiology of schistosomiasis. *Nat. Rev. Immunol.* 2, 499–511.
- Pereira, C.A.J., Martins-Souza, R.L., Correa, A., Coelho, P.M.Z., Negrao-Correa, D., 2008. Participation of cell-free haemolymph of *Biomphalaria tenagophila* in the defence mechanism against *Schistosoma mansoni* sporocysts. *Parasite Immunol.* 30, 610–619.

- Peterson, N.A., Hokke, C.H., Deelder, A.M., Yoshino, T.P., 2009. Glycotope analysis in miracidia and primary sporocysts of *Schistosoma mansoni*: differential expression during the miracidium-to-sporocyst transformation. *Int. J. Parasitol.* 39, 1331–1344.
- Pila, E.A., Gordy, M.A., Phillips, V.K., Kabore, A.L., Rudko, S.P., Hanington, P.C., 2016a. Endogenous growth factor stimulation of hemocyte proliferation induces resistance to *Schistosoma mansoni* challenge in the snail host. *Proc. Natl. Acad. Sci. U.S.A.* 113, 5305–5310.
- Pila, E.A., Tarrabain, M., Kabore, A.L., Hanington, P.C., 2016b. A novel toll-like receptor (TLR) influences compatibility between the gastropod *Biomphalaria glabrata*, and the digenean trematode *Schistosoma mansoni*. *PLoS Pathog.* 12, e1005513.
- Pinaud, S., Portela, J., Duval, D., Nowacki, F.C., Olive, M.A., Allienne, J.F., Galinier, R., Dheilly, N.M., Kieffer-Jaquino, S., Mitta, G., Theron, A., Gourbal, B., 2016. A shift from cellular to humoral responses contributes to innate immune memory in the vector snail *Biomphalaria glabrata*. *PLoS Pathog.* 12, e1005361.
- Portela, J., Duval, D., Rognon, A., Galinier, R., Boissier, J., Coustau, C., Mitta, G., Theron, A., Gourbal, B., 2013. Evidence for specific genotype-dependent immune priming in the lophotrochozoan *Biomphalaria glabrata* snail. *J. Innate Immun.* 5, 261–276.
- Richards, C.S., Minchella, D.J., 1987. Transient non-susceptibility to *Schistosoma mansoni* associated with atrial amoebocytic accumulations in the snail host *Biomphalaria glabrata*. *Parasitology* 95 (Pt 3), 499–505.
- Sokolow, S.H., Wood, C.L., Jones, I.J., Swartz, S.J., Lopez, M., Hsieh, M.H., Lafferty, K.D., Kuris, A.M., Rickards, C., De Leo, G.A., 2016. Global assessment of schistosomiasis control over the past century shows targeting the snail intermediate host works best. *PLoS Negl. Trop. Dis.* 10, e0004794.
- Stefanic, S., Dvorak, J., Horn, M., Braschi, S., Sojka, D., Ruelas, D.S., Suzuki, B., Lim, K. C., Hopkins, S.D., McKerrow, J.H., Caffrey, C.R., 2010. RNA interference in *Schistosoma mansoni* schistosomula: selectivity, sensitivity and operation for larger-scale screening. *PLoS Negl. Trop. Dis.* 4, e850.
- Sullivan, J.T., Pikiros, S.S., Alonzo, A.Q., 2004. Mitotic responses to extracts of miracidia and cercariae of *Schistosoma mansoni* in the amebocyte-producing organ of the snail intermediate host *Biomphalaria glabrata*. *J. Parasitol.* 90, 92–96.
- Sullivan, J.T., Spence, J.V., Nunez, J.K., 1995. Killing of *Schistosoma mansoni* sporocysts in *Biomphalaria glabrata* implanted with amoebocyte-producing organ allografts from resistant snails. *J. Parasitol.* 81, 829–833.
- Tennessen, J.A., Bonner, K.M., Bollmann, S.R., Johnstun, J.A., Yeh, J.Y., Marine, M., Tavalire, H.F., Bayne, C.J., Blouin, M.S., 2015a. Genome-wide scan and test of candidate genes in the Snail *Biomphalaria glabrata* reveal new locus influencing resistance to *Schistosoma mansoni*. *PLoS Negl. Trop. Dis.* 9, e0004077.
- Tennessen, J.A., Theron, A., Marine, M., Yeh, J.Y., Rognon, A., Blouin, M.S., 2015b. Hyperdiverse gene cluster in snail host conveys resistance to human schistosome parasites. *PLoS Genet.* 11, e1005067.
- Theron, A., Coustau, C., Rognon, A., Gourbiere, S., Blouin, M.S., 2008. Effects of laboratory culture on compatibility between snails and schistosomes. *Parasitology* 135, 1179–1188.
- Theron, A., Pages, J.R., Rognon, A., 1997. *Schistosoma mansoni*: distribution patterns of miracidia among *Biomphalaria glabrata* snail as related to host susceptibility and sporocyst regulatory processes. *Exp. Parasitol.* 85, 1–9.
- Theron, A., Rognon, A., Gourbal, B., Mitta, G., 2014. Multi-parasite host susceptibility and multi-host parasite infectivity: a new approach of the *Biomphalaria glabrata*/*Schistosoma mansoni* compatibility polymorphism. *Infect. Genet. Evol.* 26, 80–88.
- Valentim, C.L., Cioli, D., Chevalier, F.D., Cao, X., Taylor, A.B., Holloway, S.P., Pica-Mattoccia, L., Guidi, A., Basso, A., Tsai, I.J., Berriman, M., Carvalho-Queiroz, C., Almeida, M., Aguilar, H., Frantz, D.E., Hart, P.J., LoVerde, P.T., Anderson, T.J., 2013. Genetic and molecular basis of drug resistance and species-specific drug action in schistosome parasites. *Science* 342, 1385–1389.
- Vasquez, R.E., Sullivan, J.T., 2001a. Effect of miracidial dose on adoptively transferred resistance to *Schistosoma mansoni* in the snail intermediate host, *Biomphalaria glabrata*. *J. Parasitol.* 87, 460–462.
- Vasquez, R.E., Sullivan, J.T., 2001b. Further characterization of passively transferred resistance to *Schistosoma mansoni* in the snail intermediate host *Biomphalaria glabrata*. *J. Parasitol.* 87, 1360–1365.
- Vasquez, R.E., Sullivan, J.T., 2001c. Hematopoietic tissue allografts in *Biomphalaria glabrata* (Mollusca: Pulmonata) induce humoral immunity to *Schistosoma mansoni*. *Dev. Comp. Immunol.* 25, 561–564.
- WHO, 2012. Schistosomiasis: population requiring preventive chemotherapy and number of people treated in 2010. *Wkly. Epidemiol. Rec.* 87, 37–44.
- WHO, 2016. Schistosomiasis: number of people treated worldwide in 2014. *Wkly. Epidemiol. Rec.* 91, 53–60.

Remerciements

Remerciements

Nous arrivons à la fin de ce mémoire de thèse, il est tant pour moi de remercier toutes les personnes qui ont partagé mon quotidien pendant ces 5 dernières années et qui m'ont permis d'arriver où je suis aujourd'hui.

Pour commencer, je voudrais dans un premier temps remercier les membres de mon jury de thèse : M^{me} Christine Braquart-Varnier, M^{me} Elisabeth Huguet, M. Franck Prugnotte et enfin M. Guillaume Mitta, merci à tous d'avoir accepté d'évaluer ce travail de thèse. Christine, merci pour votre participation et le soutien que vous m'avez apporté au long de ma thèse à l'occasion des comités de suivi de thèse. Je tenais particulièrement à ce que vous fassiez partie de mon jury de thèse pour clôturer cette histoire. Elisabeth, merci d'avoir chamboulé tout votre emploi du temps pour moi. Franck, un retour aux sources me semble t'il, merci d'avoir réussi à prendre du temps pour être le rapporteur de cette thèse. Et enfin Guillaume, merci tout d'abord de m'avoir accueilli au laboratoire, merci aussi pour ces échanges durant ces trois années et finalement merci d'avoir accepté de faire partie de mon jury de thèse. Il me semblait tout naturel qu'il en soit ainsi étant donné que j'ai suivi les pas de la découverte du polymorphisme de compatibilité, j'espère que les travaux menés pendant ces trois ans n'auront fait que rendre ce sujet encore plus passionnant.

Je voudrais ensuite remercier mes chefs, Benjamin et Richard !! Un grand merci pour tout ce que vous m'avez apporté et tout ce que nous avons pu partager ensemble !

Benjamin, depuis 5 ans, vous m'avez fait confiance ! J'ai grandi grâce à vous, et même été capable d'écrire une thèse ! Vous avez été (je sais bien l'écrire maintenant :-)) pour moi plus qu'un encadrant toujours là quand on a besoin. Les choses me tiennent parfois un peu trop à coeur :-)) mais vous avez toujours été là pour trouver les mots dans les moments positifs et ceux un peu moins ! Merci pour tout chef !

Richard, nous allons essayer de rester sérieux :-), je ne retiens qu'une chose LA phrase « tes westerns sont presque plus beau que le mien :-)) » haha ! Bien entendu, je plaisante, nous avons partagé beaucoup ... des western ^^... mais pas que ! En attendant le papier TEP, c'est un peu notre bébé ! Finalement, je n'aurais jamais été assez reconnaissante de la polo qui roule en toute circonstance et ne qui perd pas son embrayage à chaque petit trottoir :-))

David, mon non-chef, mais c'est tout comme, un grand merci !

David, nous avons partagé tant de choses ! Je ne sais pas si j'aimais la biologie cellulaire avant mais maintenant c'est une certitude j'adore ! Toutes les journées entières passées dans le froid du confocal ou dans la Cl direction le FACS, ont été des moments importants pour moi ! Nous partageons le même goût, je pense donc il vaut mieux un bon verre de vin que de long discours ! J'espère tout de même que nous allons ENFIN pouvoir déboucher la bouteille qui fermente depuis 3 longues années dans l'armoire... :-))

Je trouve très compliqué de mettre des mots sur la grande gratitude que j'ai envers vous trois ! À jamais je resterais l'enfant de l'axe 2 ! Merci à vous trois pour toutes ces choses que vous m'avez apporté !

Remerciements

Un grand merci à tout le laboratoire IHPE, merci pour cet accueil pendant ces 5 années. Chaque personne contribue à la réussite des étudiants qui passent entre les murs de ce labo ! Merci à tous pour ces moments de partage, tant scientifique et non-scientifique !

Christoph, le chef d'équipe ! Parfois compliqué d'allier manips et réunions d'équipes, mais ça a toujours été un lieu de partage et d'échange qui m'ont fait grandir scientifiquement !

Diane, je te laisse l'entière exclusivité du bruit « talonnesque » dans les couloirs :-) ! Pour ce qui est des ordres de missions, j'ai laissé tomber l'espoir de les faire sans fautes ...

Anne, finis mes innombrables venues dans le bureau à vous embêter ! Que la force de la fête de la science soit avec toi !! Cette année, on viendra te voir ... mais en touristes :-)

Cristian ! Que dire... tu es génial ! Tu es toujours partant pour te mêler aux « jeunes », c'est toujours un plaisir de passer des moments avec toi ! Maintenant les tomates-mozza sont que pour toi, on se battra plus chez Marie-Blachère :-) !

Jérôme, les pauses repas conviviales, c'est hyper important au labo ! Les grandes discussions qui émergent de nos gamelles sont des vrais moments de partage qui sont essentiels lors de journées difficiles !

Nat, mon amour des JMJ ... :-) bon, j'arrête, je vais l'avoir dans la tête pour la journée :-) ! Bon bien je t'annonce officiellement que je n'ai PLUS besoin de mollusque... oui oui promis cette fois-ci ! Tu vas te retrouver avec des milliers de mollusques qui ne trouverons plus une seconde famille d'accueil dans mes bacs « oui oui donne, je m'en servirais » :-) !

Anne, finie les longuuuuueees journées de manips avec la playlist de RTL2 qui revient toutes les deux heures et nos longues discussions sur les vacances ou les groupes de rock :-) ! Tu nous l'as bien vendu la Guadeloupe, mais on aura jamais trouvé le temps d'y aller pendant ces trois ans :-) !

Olivier, nous n'aurons partagé qu'une année ensemble et pourtant, tu nous auras bien fait rêver avec ton expérience en Terre Adélie ! Par contre le sommet du Canigou, ce sera pour une prochaine fois :-)

Jeff, finis le partage du renouvellement du papier de paillasse :-) Le petit lutin (comme dirait Guillaume) essentiel au bon fonctionnement de tout le labo !

Eve, on partage déjà notre département d'origine :-) mais pas que ! Dès le master, tu as été là pour m'apprendre mes premières lignes de codes (et les seules) ! Mais nous avons atteint des sommets pendant notre super MOOC de R qui était à la fois très drôle et très studieux aussi et surtout que nous avons eu !

Annia, j'espère qu'un jour, nous viendrons dans ta maison sur la plage :-) en attendant, nous avons un jolie avocat qui nous rappelle ton passage !

Damien, tu as vu, j'y suis arrivé à écrire ma thèse et 3 papier pour hier ... bon ok pour août, c'est pas si mal déjà :-)

Kelly, le Pharo, c'est plutôt pas mal quand même ! Par contre définitivement le tour du lac on y arrivera pas ! De plus, je pense que tu as laissé ton empreinte sûr (ton ex) mon bureau ... tu verras le bazar depuis que je rédige ... je te jure MON bureau est véritablement en bordel :-) !

Ronaldo, le labo est aussi fait pour des rencontres internationales ! À mon grand désespoir, tous les Ronaldo ne sont pas des champions de foot, d'autres sont plutôt biologico-muscicien c'est pas mal non plus :-)

Bon courage pour la traduction, mais je suis sûre que Cristian sera là pour t'aider :-)

Bon vent à toi !

Remerciements

Les filles, Camille, Manon ! Prenez soin de Julien et Damien ne les faites pas trop souffrir, je compte sur vous, ils sont fragiles :-)

Bien sûr, je ne pouvais pas remercier le labo sans oublier les anciens !

Tout d'abord André ! Merci pour ces quelques moments partagés ensemble ! Je n'oublierai jamais notre séance photo pour essayer de partager un instant vos mythiques missions sur le terrain, en Guadeloupe ... mais nous, seulement dans le patio !

Marion, Sarah, David, Rodolpho, trois ans, c'est long, on en rencontre des gens vous étiez « les vieux » et nous « les bébés » quand vous êtes tous partis ! Vous nous avez appris la vie entre thésards du fond du labo qui finissent tard le soir, alors merci à vous !

L'IHPE c'est aussi Montpellier, nous nous rencontrons pour parler science, pour jouer au ping-pong ou danser de la salsa, mais c'est toujours très enrichissant de grandir dans un laboratoire qui présente des axes de recherches si hétéroclites ! Bien sûr, je ne peux pas parler de Montpellier sans parler de Guillaume ! Merci pour l'initiation au FACS et SURTOUT pour la journée à passer des tubes, des tubes, des tubes ... enfin tu as compris :-)

Max nous nous sommes vraiment rencontré à la semaine d'inté, haaaaaa cette semaine d'inté que du bonheur, de la rigolade et des parties de ping-pong ... le reste c'était en option !

Aude, nouvelle venue parmi nous, mais nous avons eu le temps de partager pas mal de choses, t'inquiète, je ne t'abandonne pas tout de suite pour le pilates ! À nous le niveau 3 ? :-)

Julien P., je ne sais pas où te placer, labo pas labo tout est la question :-), tu coups, tu es au labo, mais juste après ! Tu nous à fait partager tes manips de thèse et donné le goût du labo, tu n'y es pas pour rien si on s'est autant acharné à reste ... et tu en sais quelque chose toi aussi tu t'accroches :-) ! Alors merci pour cette découverte qui fut le début de l'aventure !

Merci les amis ! Le laboratoire peut aussi être le lieu de début de nouvelles amitiés ! Je pense que pour ma part, c'est le cas ! Cécile, Guillaume, Julien sans nos parcours croisés au laboratoire jamais nous serions rencontré, quel dommage ! Je ne vous oublierai pas, vous avez été pour moi la bouteille d'oxygène qui permet de surmonter les moments difficiles ! Tant de choses partagés ensemble ! Du reste, c'était TELLLLLLLLLLEEEEEEMMMMMMENT bien :-) !

Julien, ça va sans dire que notre meilleur moment de thèse restera à jamais l'expérimentation animale ! J'ai appris quelque chose de très important pendant ces 10 jours, d'abord un café et seulement après, je peux commencer à te parler :-) ! Nous te laissons maître du bureau !

Guillaume, le code c'est pas le code ?! Rien que pour toi, il faudrait trois pages entières de remerciements ! Tu m'as rassuré dans mes moments de doutes, en tant que vieux :-) ! Tu étais là prêt à me sauver la vie après ma bouchée de cacahuète ! Les bières à la sortie du boulot beaucoup trop tard, nous pouvions toujours les partager avec toi ! Bref, tu as été essentiel dans ma thèse et vous avez laissé un grand vide avec Cécile quand vous êtes partie !

Cécile ! Je m'étais promis de ne plus jamais avoir d'amiEs, mais tu m'as fait me trahir ! Ces soirées passées tout les 4 étaient des purs moments de bonheur ! Mais nous avons bien géré la distance ! Du reste, c'est chouette de t'avoir comme amie :-) !!

Les amis en dehors du labo, c'est aussi essentiel ! Je sais que vous ne lirez jamais ma thèse et surtout vous n'arriverez jamais jusqu'au bout mais je devais vous remercier dans ce mémoire, car vous avez

Remerciements

contribué à mon bien-être mental et donc aussi à la réussite de ce travail ! Alors merci à vous, même si parfois, on vous gave à dire que nous sommes débordés et fatigués, vous êtes toujours là pour nous ! Que ce soit pour boire un verre, aller à la plage (où venir vous chercher de retour de plage sur la voie rapide :-)), faire d'innombrable barbecue, faire des soirées jeux jusqu'à l'aube, et même vous occuper de Pêche et des plantes lorsque nous avons besoin d'aller se ressourcer ! Alors un grand merci à vous Bruno et Odessa !

Alex, depuis que nous nous sommes rencontrés sur les bancs de la Ll, nous sommes devenus de vrais amis, que la distance n'a pas séparé ! Nous vivons à quelques choses prêt les mêmes aventures, toi à Montpellier, nous à Perpignan ! Mais tu ne dis jamais non à passer une soirée avec nous lors de nos réunions délocalisées à Montpellier !

Finalement Ju & Silvère, depuis le lycée, on ne se quitte plus ! Uniquement, la distance géographique nous sépare, lorsque nous traversons chacun la moitié de la France pour se retrouver, c'est comme si nous nous étions jamais quittés !!!

Finalement merci à ma famille ! Qu'est-ce que nous serions sans vous ! Vous vous êtes accrochés pour toujours nous soutenir et comprendre ce que nous faisons et faire le dos rond des longues semaines sans que nous nous voyions ! Tant de choses seraient à dire, mais tout cela sera simplement regrouper sous grand MERCI !

Maman, Papa, Mamé, Mamie, Papé, Papo, Lélé, Jean, Heidi, Maman Jean, Lolie, Guy, Élo, Auré, Delph, Loane, Enora, Nathan MERCI !

Je finirais juste par toi, sans qui je ne serais jamais arrivée où je suis aujourd'hui, sans qui je n'aurais même imaginé faire une thèse ! Nous sommes complices tant dans la vie qu'au laboratoire et c'est tous les jours un vrai bonheur ! Merci pour tout !

Bibliographie

Bibliographie

- Abdelrhman KFA, Bacci G, Marras B, Nistri A, Schintu M, Ugolini A, Mengoni A. 2017. Exploring the bacterial gut microbiota of supralittoral talitrid amphipods. *Research in Microbiology* 168:74-84.
- Abnave P, Muracciole X, Ghigo E. 2017. Macrophages in Invertebrates: From Insects and Crustaceans to Marine Bivalves. *Macrophages* 62:147-158.
- Adema CM, Hertel La, Miller RD, Loker ES. 1997. A family of fibrinogen-related proteins that precipitates parasite-derived molecules is produced by an invertebrate after infection. *Proceedings of the National Academy of Sciences of the United States of America* 94:8691-8696.
- Adema CM, Hillier LW, Jones CS, Loker ES, Knight M, Minx P, Oliveira G, Raghavan N, Shedlock A, do Amaral LR, et al. 2017. Whole genome analysis of a schistosomiasis-transmitting freshwater snail. *Nature Communications* 8:15451.
- Adema CM, Loker ES. 2015. Digenean-gastropod host associations inform on aspects of specific immunity in snails. *Developmental and comparative immunology* 48:275-283.
- Agaisse H, Perrimon N. 2004. The roles of JAK/STAT signaling in *Drosophila* immune responses. *Immunological Reviews* 198:72-82.
- Agatsuma T, Iwagami M, Liu CX, Rajapakse RPVJ, Mondal MMH, Kitikoon V, Ambu S, Agatsuma Y, Blair D, Higuchi T. 2002. Affinities between Asian non-human *Schistosoma* species, the *S. indicum* group, and the African human schistosomes. *Journal of Helminthology* 76:7-19.
- Apte SS, Parks WC. 2015. Metalloproteinases: A parade of functions in matrix biology and an outlook for the future. *Matrix Biology* 44:1-6.
- Arefin B, Kunc M, Krautz R, Theopold U. 2017. The Immune Phenotype of Three *Drosophila* Leukemia Models. *G3 & Genes|Genomes|Genetics*:g3.117.039487-g039483.039117.039487.
- Armitage SAO, Kurtz J, Brites D, Dong Y, Du Pasquier L, Wang H-C. 2017. Dscam1 in Pancrustacean Immunity: Current Status and a Look to the Future. *Frontiers in Immunology* 8:662.
- Armitage SAO, Peuß R, Kurtz J. 2015. Dscam and pancrustacean immune memory - A review of the evidence. *Developmental and Comparative Immunology* 48:315-323.
- Attwood SW, Upatham ES, Meng XH, Qiu DC, Southgate VR. 2003. The phylogeography of Asian *Schistosoma* (Trematoda: Schistosomatidae). *Parasitology* 125:99-112.
- Bae YS, Choi MK, Lee W-J. 2010. Dual oxidase in mucosal immunity and host & microbe homeostasis. *Trends in Immunology* 31:278-287.
- Bandoni SM, Mulvey M, Loker ES. 1995. Phylogenetic analysis of eleven species of *Biomphalaria* Preston, 1910 (Gastropoda: Planorbidae) based on comparisons of allozymes. *Biological Journal of the Linnean Society* 54:1-27.
- Baron OL, van West P, Industri B, Ponchet M, Dubreuil G, Gourbal B, Reichhart J-M, Coustau C. 2013. Parental Transfer of the Antimicrobial Protein LBP/BPI Protects *Biomphalaria glabrata* Eggs against Oomycete Infections. *PLoS Pathogens* 9:e1003792-e1003792.

Bibliographie

- Basch PF. 1975. An interpretation of snail-trematode infection rates: Specificity based on concordance of compatible phenotypes. *International Journal for Parasitology* 5:449-452.
- Basch PF. 1976. PARASITOLOGICAL Intermediate Host Specificity in *Schistosoma mansoni*. *Experimental Parasitology* 169:150-169.
- Bataillon T, Dillmann C, Gaba S, Goldringer I, Kaltz O, Mery F, Nidelet T, Schneider DS, Spor A. 2010. Évolution Expérimentale. In: Thomas F, Lefèvre T, Raymond M, editors. *Biologie Évolutive: de boeck*. p. 617-646.
- Bathige SDNK, Umasuthan N, Whang I, Lim BS, Won SH, Lee J. 2014. Antibacterial activity and immune responses of a molluscan macrophage expressed gene-1 from disk abalone, *Haliotis discus discus*. *Fish and Shellfish Immunology* 39:263-272.
- Bayne CJ, Hahn UK, Bender RC. 2001. Mechanisms of molluscan host resistance and of parasite strategies for survival. *Parasitology* 123:159-167.
- Bender RC, Bayne CJ. 1996. Purification and characterization of a tetrameric alpha-macroglobulin proteinase inhibitor from the gastropod mollusc *Biomphalaria glabrata*. *Biochemical Journal* 316:893-900.
- Beneden Mv. 1878. *Les commensaux et les parasites dans le règne animal*. Paris: G. Baillière.
- Bergquist R, Utzinger J, Keiser J. 2017. Controlling schistosomiasis with praziquantel: How much longer without a viable alternative? *Infectious Diseases of Poverty* 6:74.
- Bettarel Y, Bouvier T, Nguyen HK, Thu PT. 2015. The versatile nature of coral-associated viruses. *Environmental Microbiology* 17:3433-3439.
- Blacher E, Levy M, Tatirovsky E, Elinav E. 2017. Microbiome-Modulated Metabolites at the Interface of Host Immunity. *The Journal of Immunology* 198:572.
- Blandin S, Levashina Ea. 2004. Thioester-containing proteins and insect immunity. *Molecular Immunology* 40:903-908.
- Blandin SA, Marois E, Levashina EA. 2008. Antimalarial Responses in *Anopheles gambiae*: From a Complement-like Protein to a Complement-like Pathway. *Cell Host & Microbe* 3:364-374.
- Bou Aoun R, Hetru C, Troxler L, Doucet D, Ferrandon D, Matt N. 2010. Analysis of Thioester-Containing Proteins during the Innate Immune Response of *Drosophila melanogaster*. *Journal of Innate Immunity* 3:52-64.
- Bouchut A, Roger E, Coustau C, Gourbal B, Mitta G. 2006. Compatibility in the *Biomphalaria glabrata*/*Echinostoma caproni* model: Potential involvement of adhesion genes. *International Journal for Parasitology* 36:175-184.
- Braquart-Varnier C, Altinli M, Pigeault R, Chevalier FD, Grève P, Bouchon D, Sicard M. 2015. The Mutualistic Side of *Wolbachia*-Isopod Interactions: *Wolbachia* Mediated Protection Against Pathogenic Intracellular Bacteria. *Frontiers in Microbiology* 6:1388.
- Braquart-Varnier C, Lachat M, Herbinière J, Johnson M, Caubet Y, Bouchon D, Sicard M. 2008. *Wolbachia* Mediate Variation of Host Immunocompetence. *PLoS ONE* 3:e3286.

Bibliographie

- Brites D, McTaggart S, Morris K, Anderson J, Thomas K, Colson I, Fabbro T, Little TJ, Ebert D, Du Pasquier L. 2008. The Dscam homologue of the crustacean *Daphnia* is diversified by alternative splicing like in insects. *Molecular Biology and Evolution* 25:1429-1439.
- Brockhurst MA, Buckling A, Poullain V, Hochberg ME. 2007. THE IMPACT OF MIGRATION FROM PARASITE-FREE PATCHES ON ANTAGONISTIC HOST-PARASITE COEVOLUTION. *Evolution* 61:1238-1243.
- Brockton V, Henson JH, Raftos DA, Majeske AJ, Kim Y-O, Smith LC. 2008. Localization and diversity of 185/333 proteins from the purple sea urchin – unexpected protein-size range and protein expression in a new coelomocyte type. *Journal of Cell Science* 121:339.
- Broderick NA, Buchon N, Lemaitre B. 2014. Microbiota-Induced Changes in *Drosophila melanogaster* Host Gene Expression and Gut Morphology Microbiota-Induced Changes in *Drosophila melanogaster* Host Gene. 5:1-13.
- Brummel T, Ching A, Seroude L, Simon AF, Benzer S. 2004. *Drosophila* lifespan enhancement by exogenous bacteria. *Proceedings of the National Academy of Sciences* 101:12974-12979.
- Buchmann K. 2014. Evolution of innate immunity: Clues from invertebrates via fish to mammals. *Frontiers in Immunology* 5:1-8.
- Buchon N, Broderick NA, Chakrabarti S, Lee W-j, Buchon N, Broderick NA, Chakrabarti S, Lemaitre B. 2009a. activity through multiple pathways in *Drosophila* Invasive and indigenous microbiota impact intestinal stem cell activity through multiple pathways in *Drosophila*. 2333-2344.
- Buchon N, Broderick NA, Chakrabarti S, Lee W-j, Buchon N, Broderick NA, Chakrabarti S, Lemaitre B. 2009b. Invasive and indigenous microbiota impact intestinal stem cell activity through multiple pathways in *Drosophila*. *Genes & Development* 23:2333-2344.
- Buchon N, Broderick NA, Lemaitre B. 2013. Gut homeostasis in a microbial world: insights from *Drosophila melanogaster*. *Nat Rev Micro* 11:615-626.
- Buchon N, Silverman N, Cherry S. 2014. Immunity in *Drosophila melanogaster* - from microbial recognition to whole-organism physiology. *Nature Reviews Immunology* 14:796-810.
- Buckley KM, Munshaw S, Kepler TB, Smith LC. 2008. The 185/333 Gene Family Is a Rapidly Diversifying Host-Defense Gene Cluster in the Purple Sea Urchin *Strongylocentrotus purpuratus*. *Journal of Molecular Biology* 379:912-928.
- Buresova V, Hajdusek O, Franta Z, Loosova G, Grunclova L, Levashina EA, Kopáček P. 2011. Functional Genomics of Tick Thioester-Containing Proteins Reveal the Ancient Origin of the Complement System. *Journal of Innate Immunity* 3:623-630.
- Campbell G, Jones CS, Lockyer aE, Hughes S, Brown D, Noble LR, Rollinson D. 2000. Molecular evidence supports an african affinity of the neotropical freshwater gastropod, *Biomphalaria glabrata*, say 1818, an intermediate host for *Schistosoma mansoni*. *Proceedings. Biological sciences / The Royal Society* 267:2351-2358.
- Canesi L, Scarpato A, Betti M, Ciacci C, Pruzzo C, Gallo G. 2002. Bacterial killing by mytilus hemocyte monolayers as a model for investigating the signaling pathways involved in mussel immune defence. *Marine Environmental Research* 54:547-551.

Bibliographie

- Carissimo G, Pondeville E, McFarlane M, Dietrich I, Mitri C, Bischoff E, Antoniewski C, Bourgouin C, Failloux A-B, Kohl A, et al. 2015. Antiviral immunity of *Anopheles gambiae* is highly compartmentalized, with distinct roles for RNA interference and gut microbiota. *Proceedings of the National Academy of Sciences* 112:E176-E185.
- Cavalcanti MGS, Filho FC, Mendonça AMB, Duarte GR, Barbosa CCGS, De Castro CMMB, Alves LC, Brayner FA. 2012. Morphological characterization of hemocytes from *Biomphalaria glabrata* and *Biomphalaria straminea*. *Micron* (Oxford, England : 1993) 43:285-291.
- Cerenius L, Söderhäll K. 2004. The prophenoloxidase-activating system in invertebrates. *Immunological Reviews* 198:116-126.
- Chandel K, Mendki MJ, Parikh RY, Kulkarni G, Tikar SN, Sukumaran D, Prakash S, Parashar BD, Shouche YS, Veer V. 2013. Midgut microbial community of *Culex quinquefasciatus* mosquito populations from India. *PLoS ONE* 8.
- Chandler JA, Lang J, Bhatnagar S, Eisen JA, Kopp A. 2011. Bacterial communities of diverse *Drosophila* species: Ecological context of a host-microbe model system. *PLoS Genetics* 7.
- Charrier M, Fonty G, Gaillard-Martinie B, Ainouche K, Andant G. 2006. Isolation and characterization of cultivable fermentive bacteria from the intestine of two edible snails, *Helix pomatia* and *Cornu aspersum* (Gastropoda: Pulmonata). *Biological Research* 39:669-681.
- Chaston JM, Dobson AJ, Newell PD, Douglas AE. 2016. Host Genetic Control of the Microbiota Mediates the *Drosophila* Nutritional Phenotype. *John. Applied and Environmental Microbiology* 82:671-679.
- Chevalier F, Herbinière-Gaboreau J, Bertaux J, Raimond M, Morel F, Bouchon D, Grève P, Braquart-Varnier C. 2011. The immune cellular effectors of terrestrial isopod *Armadillidium vulgare*: Meeting with their invaders, *Wolbachia*. *PLoS ONE* 6.
- Chevignon G, Thézé J, Cambier S, Poulain J, Da Silva C, Bézier A, Musset K, Moreau SJM, Drezen J-M, Hugué E. 2014. Functional Annotation of *Cotesia congregata* Bracovirus: Identification of Viral Genes Expressed in Parasitized Host Immune Tissues. *Journal of Virology* 88:8795-8812.
- Chou PH, Chang HS, Chen IT, Lin HY, Chen YM, Yang HL, Wang KCHC. 2009. The putative invertebrate adaptive immune protein *Litopenaeus vannamei* Dscam (LvDscam) is the first reported Dscam to lack a transmembrane domain and cytoplasmic tail. *Developmental and Comparative Immunology* 33:1258-1267.
- Christiaansen A, Varga SM, Spencer JV. 2015. Viral manipulation of the host immune response. *Current opinion in immunology* 36:54-60.
- Christophides GK, Zdobnov E, Barillas-Mury C, Birney E, Blandin S, Blass C, Brey PT, Collins FH, Danielli A, Dimopoulos G, et al. 2002. Immunity-related genes and gene families in *Anopheles gambiae*: A comparative genomic analysis (supplementary material). *Science* (New York, N.Y.) 298:159-165.
- Cirimotich CM, Dong Y, Clayton AM, Sandiford SL, Souza-Neto JA, Mulenga M, Dimopoulos G. 2011. Natural microbe - mediated refractoriness to *Plasmodium* infection in *Anopheles gambiae*. *Science* 332:855-858.

Bibliographie

- Cirimotich CM, Dong Y, Garver LS, Sim S. 2010. Mosquito immune defenses against Plasmodium infection. *Dev Comp Immunol* 34:387-395.
- Clark RI, Salazar A, Yamada R, Fitz-gibbon S, Alcaraz J, Rana A, Rera M, Pellegrini M, William W, Walker DW. 2015. Distinct shifts in microbiota composition during Drosophila aging impair intestinal function and drive mortality. *Cell Rep.* 12:1656-1667.
- Clayton AM, Dong Y, George D. 2014. The Anopheles innate immune system in the defense against malaria infection. *Journal of Innate Immunity* 6:169-181.
- Combes C. 2000. Pressions sélectives dans les systèmes parasites-hôtes. 194:19-23.
- Corby-Harris V, Pontaroli AC, Shimkets LJ, Bennetzen JL, Habel KE, Promislow DEL. 2007. Geographical distribution and diversity of bacteria associated with natural populations of Drosophila melanogaster. *Applied and Environmental Microbiology* 73:3470-3479.
- Coustau C, Gourbal B, Duval D, Yoshino TP, Adema CM, Mitta G. 2015. Advances in gastropod immunity from the study of the interaction between the snail Biomphalaria glabrata and its parasites: A review of research progress over the last decade. *Fish & Shellfish Immunology*.
- Crellen T, Allan F, David S, Durrant C, Huckvale T, Holroyd N, Emery AM, Rollinson D, Aanensen DM, Berriman M, et al. 2016. Whole genome resequencing of the human parasite Schistosoma mansoni reveals population history and effects of selection. *Scientific reports* 6:20954-20954.
- Dai L-S, Sun Y, Sun Y-X, Zhu B-J, Liu C-L. 2016. Characterization and function of a cathepsin B in red crayfish (Procambarus clarkii) following lipopolysaccharide challenge. *Fish & Shellfish Immunology* 56:162-168.
- Danovaro R, Armeni M, Corinaldesi C, Mei ML. 2003. Viruses and marine pollution. *Marine Pollution Bulletin* 46:301-304.
- David MR, Santos LMBD, Vicente ACP, Maciel-de-Freitas R. 2016. Effects of environment, dietary regime and ageing on the dengue vector microbiota: Evidence of a core microbiota throughout Aedes aegypti lifespan. *Memorias do Instituto Oswaldo Cruz* 111:577-587.
- De Moraes Mourão M, Dinguirard N, Franco GR, Yoshino TP. 2009. Phenotypic screen of early-developing larvae of the blood fluke, Schistosoma mansoni, using RNA interference. *PLoS Neglected Tropical Diseases* 3.
- DeJong RJ, Morgan Ja, Paraense WL, Pointier JP, Amarista M, Ayeh-Kumi PF, Babiker a, Barbosa CS, Brémond P, Pedro Canese a, et al. 2001. Evolutionary relationships and biogeography of Biomphalaria (Gastropoda: Planorbidae) with implications regarding its role as host of the human bloodfluke, Schistosoma mansoni. *Molecular biology and evolution* 18:2225-2239.
- Deleury E, Dubreuil G, Elangovan N, Wajnberg E, Reichhart JM, Gourbal B, Duval D, Baron OL, Gouzy J, Coustau C. 2012. Specific versus non-specific immune responses in an invertebrate species evidenced by a comparative de novo sequencing study. *PLoS ONE* 7.
- Dennison NJ, BenMarzouk-Hidalgo OJ, Dimopoulos G. 2016. MicroRNA-regulation of Anopheles gambiae immunity to Plasmodium falciparum infection and midgut microbiota. *Dev Comp Immunol.* 28:1304-1314.
- Dheilly NM, Duval D, Mouahid G, Emans R, Allienne J-F, Galinier R, Genthon C, Dubois E, Du Pasquier L, Adema CM, et al. 2015. A family of variable immunoglobulin and lectin domain

Bibliographie

- containing molecules in the snail *Biomphalaria glabrata*. *Developmental & Comparative Immunology* 48:234-243.
- Dheilly NM, Maure F, Ravallec M, Galinier R, Doyon J, Duval D, Leger L, Volkoff A-N, Missé D, Nidelet S, et al. 2015. Who is the puppet master? Replication of a parasitic wasp-associated virus correlates with host behaviour manipulation. *Proceedings of the Royal Society B: Biological Sciences* 282:20142773.
- Dheilly NM, Poulin R, Thomas F, Hadziavdic K, Lekang K, Lanzen A, Jonassen I, Thompson EM, Troedsson C, Sansone CL, et al. 2015. Biological warfare: Microorganisms as drivers of host-parasite interactions. *PLoS ONE* 9:1-7.
- Dimopoulos G, Christophides GK, Meister S, Schultz J, White KP, Barillas-Mury C, Kafatos FC. 2002. Genome expression analysis of *Anopheles gambiae*: Responses to injury, bacterial challenge, and malaria infection. *Proceedings of the National Academy of Sciences* 99:8814-8819.
- Dimopoulos G, Richman A, Müller H-M, Kafatos FC. 1997. Molecular immune responses of the mosquito *Anopheles gambiae* to bacteria and malaria parasites. *Proceedings of the National Academy of Sciences of the United States of America* 94:11508-11513.
- Dinparast Djadid N, Jazayeri H, Raz A, Favia G, Ricci I, Zakeri S. 2011. Identification of the midgut microbiota of *An. stephensi* and *c. maculipennis* for their application as a paratransgenic tool against malaria. *PLoS ONE* 6:6-12.
- Dittmer J, Lesobre J, Moumen B, Bouchon D. 2016. Host origin and tissue-microhabitat shaping the microbiota of the terrestrial isopod *Armadillidium vulgare*. *Microbiology Ecology*:1-40.
- Dong Y, Manfredini F, Dimopoulos G. 2009. Implication of the mosquito midgut microbiota in the defense against malaria parasites. *PLoS Pathogens* 5.
- Dong Y, Taylor HE, Dimopoulos G. 2006. AgDscam, a hypervariable immunoglobulin domain-containing receptor of the *Anopheles gambiae* innate immune system. *PLoS Biology* 4:1137-1146.
- Drezen J-M, Chevignon G, Louis F, Huguet E. 2014. Origin and evolution of symbiotic viruses associated with parasitoid wasps. *Current Opinion in Insect Science* 6:35-43.
- Du X, Poltorak A, Wei Y, Beutler B. 2000. Three novel mammalian toll-like receptors: gene structure, expression, and evolution. *European cytokine network* 11:362-371.
- Ducklow HW, Boyle PJ, Mangel PW, Strong C, Mitchell R. 1979. Bacterial flora of the schistosome vector snail *Biomphalaria glabrata*. *Applied and Environmental Microbiology* 38:667-672.
- Dziarski R, Gupta D. 2006. The peptidoglycan recognition proteins (PGRPs). *Genome Biology* 7:232-232.
- Ebert D. 1998. Experimental Evolution of Parasites. *Science* 282:1432.
- Ebert D, Joachim Carius H, Little T, Decaestecker E. 2004. The Evolution of Virulence When Parasites Cause Host Castration and Gigantism. *The American Naturalist* 164:S19-S32.
- Ehrlich PR, Raven PH. 1964. BUTTERFLIES AND PLANTS: A STUDY IN COEVOLUTION. *Evolution* 18:586-608.

Bibliographie

- Elrod-Erickson M, Mishra S, Schneider D. 2010. Interactions between the cellular and humoral immune responses in *Drosophila*. *Current Biology* 10:781-784.
- Fallon PG, Doenhoff MJ. 1994. Drug-Resistant Schistosomiasis: Resistance to Praziquantel and Oxamniquine Induced in *Schistosoma Mansoni* in Mice is Drug Specific. *The American Journal of Tropical Medicine and Hygiene* 51:83-88.
- Ferrandon D, Imler J-L, Hetru C, Hoffmann Ja. 2007. The *Drosophila* systemic immune response: sensing and signalling during bacterial and fungal infections. *Nature Reviews Immunology* 7:862-874.
- Ferrandon D, Imler JL, Hoffmann JA. 2004. Sensing infection in *Drosophila*: Toll and beyond. *Seminars in Immunology* 16:43-53.
- Foley E, O'Farrell PH. 2003. Nitric oxide contributes to induction of innate immune responses to gram-negative bacteria in *Drosophila*. *Genes & Development* 17:115-125.
- Fraiture M, Baxter RHG, Steinert S, Chelliah Y, Frolet C, Quispe-Tintaya W, Hoffmann Ja, Blandin Sa, Levashina Ea. 2009. Two Mosquito LRR Proteins Function as Complement Control Factors in the TEP1-Mediated Killing of *Plasmodium*. *Cell Host & Microbe* 5:273-284.
- Futo M, Armitage SAO, Kurtz J. 2016. Microbiota plays a role in oral immune priming in *Tribolium castaneum*. *Frontiers in Microbiology* 6:1-10.
- Galinier R, Portela J, Moné Y, Allienne JF, Henri H, Delbecq S, Mitta G, Gourbal B, Duval D. 2013. Biomphalysin, a New β Pore-forming Toxin Involved in *Biomphalaria glabrata* Immune Defense against *Schistosoma mansoni*. *PLoS Pathogens* 9:e1003216-e1003216.
- Galinier R, Roger E, Moné Y, Duval D, Portet A, Pinaud S, Chaparro C, Grunau C, Genthon C, Dubois E, et al. 2017. A multistrain approach to studying the mechanisms underlying compatibility in the interaction between *Biomphalaria glabrata* and *Schistosoma mansoni*. *PLOS Neglected Tropical Diseases* 11:e0005398.
- Galinier R, Tetreau G, Portet A, Pinaud S, Duval D, Gourbal B. 2017. First characterization of viruses from freshwater snails of the genus *Biomphalaria*, the intermediate host of the parasite *Schistosoma mansoni*. *Acta Tropica* 167:196-203.
- Garcia JS, Maldonado Junior A, Bidau CJ, Corrêa LdR, Lanfredi RM, Coelho PMZ. 2010. The effect of early infection with *Echinostoma paraensei* on the interaction of *Schistosoma mansoni* with *Biomphalaria glabrata* and *Biomphalaria tenagophila*. *Memórias do Instituto Oswaldo Cruz* 105:499-503.
- Garver LS, Bahia AC, Das S, Souza-Neto JA, Shiao J, Dong Y, Dimopoulos G. 2012. Anopheles Imd pathway factors and effectors in infection intensity-dependent anti-*Plasmodium* action. *PLoS Pathogens* 8:7-9.
- Gasmi L, Boulain H, Gauthier J, Hua-Van A, Musset K, Jakubowska AK, Aury J-M, Volkoff A-N, Huguet E, Herrero S, et al. 2015. Recurrent Domestication by Lepidoptera of Genes from Their Parasites Mediated by Bracoviruses. *PLoS Genetics* 11:e1005470.
- Gavery MR, Roberts SB. 2014. A context dependent role for DNA methylation in bivalves. *Briefings in Functional Genomics* 13:217-222.

Bibliographie

- Gendrin M, Turlure F, Rodgers FH, Cohuet A, Morlais I, Christophides GK. 2017. The peptidoglycan recognition proteins PGRPLA and PGRPLB regulate Anopheles immunity to bacteria and affect infection by Plasmodium. *Journal of Innate Immunity*, in revision:1-10.
- Gimonneau G, Tchioffo MT, Abate L, Boissière A, Awono-ambéné PH, Nsango SE, Christen R, Morlais I. 2014. Composition of Anopheles coluzzii and Anopheles gambiae microbiota from larval to adult stages. *Infection, Genetics and Evolution* 28:715-724.
- Gonzalez-Ceron L, Santillan F, Rodriguez MH, Mendez D, Hernandez-Avila JE. 2003. Bacteria in midguts of field-collected Anopheles albimanus block Plasmodium vivax sporogonic development. *J Med Entomol* 40:371-374.
- Gordy MA, Pila EA, Hanington PC. 2015. The role of fibrinogen-related proteins in the gastropod immune response. *Fish & Shellfish Immunology* 46:39-49.
- Greischar MA, Koskella B. 2007. A synthesis of experimental work on parasite local adaptation. *Ecology Letters* 10:418-434.
- Guillou F, Roger E, Moné Y, Rognon A, Grunau C, Théron A, Mitta G, Coustau C, Gourbal BEF. 2007. Excretory–secretory proteome of larval Schistosoma mansoni and Echinostoma caproni, two parasites of Biomphalaria glabrata. *Molecular and Biochemical Parasitology* 155:45-56.
- Habtewold T, Groom Z, Christophides GK. 2017. Immune resistance and tolerance strategies in malaria vector and non-vector mosquitoes. *Parasites & Vectors* 10:186-186.
- Hahn UK, Bender RC, Bayne CJ. 2001. Involvement of nitric oxide in killing of Schistosoma mansoni sporocysts by hemocytes from resistant Biomphalaria glabrata. *The Journal of parasitology* 87:778-785.
- Hahn UK, Bender RC, Bayne CJ. 2000. Production of reactive oxygen species by hemocytes of Biomphalaria glabrata: carbohydrate-specific stimulation. *Developmental and Comparative Immunology* 24:531-541.
- Hanington PC, Forsy MA, Loker ES. 2012. A somatically diversified defense factor, FREP3, is a determinant of snail resistance to schistosome infection. *PLoS Neglected Tropical Diseases* 6.
- Hanington PC, Lun CM, Adema CM, Loker ES. 2010. Time series analysis of the transcriptional responses of Biomphalaria glabrata throughout the course of intramolluscan development of Schistosoma mansoni and Echinostoma paraensei. *International Journal for Parasitology* 40:819-831.
- Hann TN, Chiang YA, Yeh YC, Wang HC. 2015. Reprint of "Review of Dscam-mediated immunity in shrimp and other arthropods". *Developmental and Comparative Immunology* 48:306-314.
- Harris KR. 1975. The Fine Structure of Encapsulation in Biomphalaria Glabrata. *Annals of the New York Academy of Sciences* 266:446-464.
- Harris KR, Cheng TC. 1974. THE ENCAPSULATION PROCESS IN BIOMPHALARZA GLABRATA INFECTED WITH THE METASPOGONIDIA STRONGYLUS A - T - E - S - S : LIGHT MICROSCOPY.
- Hathaway JJM, Adema CM, Stout BA, Mobarak CD, Loker ES. 2010. Identification of protein components of egg masses indicates parental investment in immunoprotection of offspring by

Bibliographie

- Biomphalaria glabrata (Gastropoda, Mollusca). Developmental and Comparative Immunology 34:425-435.
- Hattori D, Demir E, Kim HW, Viragh E, Zipursky SL, Dickson BJ. 2007. Dscam diversity is essential for neuronal wiring and self-recognition. Nature 449:223-227.
- Henry E, Fung N, Liu J, Drakakaki G, Coaker G. 2015. Beyond Glycolysis: GAPDHs Are Multifunctional Enzymes Involved in Regulation of ROS, Autophagy, and Plant Immune Responses. PLoS Genetics 11:e1005199.
- Herniou EA, Huguet E, Thézé J, Bézier A, Periquet G, Drezen J-M. 2013. When parasitic wasps hijacked viruses: genomic and functional evolution of polydnaviruses. Philosophical Transactions of the Royal Society B: Biological Sciences 368:20130051.
- Hibino T, Loza-Coll M, Messier C, Majeske AJ, Cohen AH, Terwilliger DP, Buckley KM, Brockton V, Nair SV, Berney K, et al. 2006. The immune gene repertoire encoded in the purple sea urchin genome. Developmental Biology 300:349-365.
- Hillyer JF, Strand MR. 2014. Mosquito hemocyte-mediated immune responses. Current opinion in insect science 3:14-21.
- Hoffmann JA. 2003. The immune response of Drosophila. Nature 426:33-38.
- Hoffmann JA, Kafatos FC, Janeway CA, Ezekowitz RAB. 1999. Phylogenetic Perspectives in Innate Immunity. Science 284:1313.
- Hooper LV, Littman DR, Macpherson AJ. 2012. Interactions between the microbiota and the immune system. Science (New York, N.Y.) 336:1268-1273.
- Igartua C, Davenport ER, Gilad Y, Nicolae DL, Pinto J, Ober C. 2017. Host genetic variation in mucosal immunity pathways influences the upper airway microbiome. Microbiome 5:16.
- Ittiprasert W, Knight M. 2012. Reversing the Resistance Phenotype of the Biomphalaria glabrata Snail Host Schistosoma mansoni Infection by Temperature Modulation. PLoS Pathogens 8:e1002677-e1002677.
- Ittiprasert W, Miller A, Myers J, Nene V, El-Sayed NM, Knight M. 2010. Identification of immediate response genes dominantly expressed in juvenile resistant and susceptible Biomphalaria glabrata snails upon exposure to Schistosoma mansoni. Molecular and Biochemical Parasitology 169:27-39.
- Iturbe-Ormaetxe I, Walker T, O' Neill SL. 2011. Wolbachia and the biological control of mosquito-borne disease. EMBO Reports 12:508-518.
- Jacomin A-C, Fauvarque M-O, Taillebourg E. 2016. A functional endosomal pathway is necessary for lysosome biogenesis in Drosophila. BMC Cell Biology 17:36.
- Jaramillo-Gutierrez G, Rodrigues J, Ndikuyeze G, Povelones M, Molina-Cruz A, Barillas-Mury C. 2009. Mosquito immune responses and compatibility between Plasmodium parasites and anopheline mosquitoes. BMC Microbiology 9:154-154.
- Jauzein C, Donaghy L, Volety AK. 2013. Flow cytometric characterization of hemocytes of the sunray venus clam Macrocallista nimbosa and influence of salinity variation. Fish & Shellfish Immunology 35:716-724.

Bibliographie

- Jianyong L, Zhao X, Christensen BM. 1994. Dopachrome conversion activity in *Aedes aegypti*: Significance during melanotic encapsulation of parasites and cuticular tanning. *Insect Biochemistry and Molecular Biology* 24:1043-1049.
- Joshi D, Pan X, McFadden MJ, Bevins D, Liang X, Lu P, Thiem S, Xi Z. 2017. The Maternally Inheritable Wolbachia wAlbB Induces Refractoriness to *Plasmodium berghei* in *Anopheles stephensi*. *Frontiers in Microbiology* 8:366.
- Jourdane J, Mounkassa JB. 1986. Topographic shifting of primary sporocysts of *Schistosoma mansoni* in *Biomphalaria pfeifferi* as a result of coinfection with *Echinostoma caproni*. *Journal of invertebrate pathology* 48:269-274.
- Jupatanakul N, Sim S, Angler??-Rodr??guez YI, Souza-Neto J, Das S, Poti KE, Rossi SL, Bergren N, Vasilakis N, Dimopoulos G. 2017. Engineered *Aedes aegypti* JAK/STAT Pathway-Mediated Immunity to Dengue Virus. *PLoS Neglected Tropical Diseases* 11:1-24.
- Keebaugh ES, Schlenke TA. 2012. Adaptive evolution of a novel *Drosophila* lectin induced by parasitic wasp attack. *Molecular Biology and Evolution* 29:565-577.
- Kilpatrick DC. 2002. Animal lectins: a historical introduction and overview. *Biochimica et biophysica acta* 1572:187-197.
- King CH, Dickman K, Tisch DJ. 2005. Reassessment of the cost of chronic helminthic infection: a meta-analysis of disability-related outcomes in endemic schistosomiasis. *Lancet (London, England)* 365:1561-1569.
- King CL, Kumaraswami V, Poindexter RW, Kumari S, Jayaraman K, Alling DW, Ottesen EA, Nutman TB. 1992. Immunologic tolerance in lymphatic filariasis. Diminished parasite-specific T and B lymphocyte precursor frequency in the microfilaremic state. *Journal of Clinical Investigation* 89:1403-1410.
- Knight M, Elhelu O, Smith M, Haugen B, Miller A, Raghavan N, Wellman C, Cousin C, Dixon F, Mann V, et al. 2015. Susceptibility of Snails to Infection with Schistosomes is influenced by Temperature and Expression of Heat Shock Proteins. *Epidemiology (Sunnyvale, Calif.)* 5:189.
- Lagueux M, Perrodou E, Levashina EA, Capovilla M, Hoffmann JA. 2000. Constitutive expression of a complement-like protein in Toll and JAK gain-of-function mutants of *Drosophila*. *Proceedings of the National Academy of Sciences* 97:11427-11432.
- Langand AJ, Morand S. 1998. HERITABLE NON-SUSCEPTIBILITY IN AN ALLOPATRIC HOST-PARASITE SYSTEM: *BIOMPHALARIA GLABRATA CAPRONI* (MOLLUSCA)-*ECHINOSTOMA* (PLATYHELMINTHIGENEA). 84:739-742.
- Leclerc V, Pelte N, Chamy LE, Martinelli C, Ligoxygakis P, Hoffmann JA, Reichhart J-M. 2006. Prophenoloxidase activation is not required for survival to microbial infections in *Drosophila*. *EMBO Reports* 7:231-235.
- Lee MH, Osaki T, Lee JY, Baek MJ, Zhang R, Park JW, Kawabata S, Soderhall K, Lee BL. 2004. Peptidoglycan recognition proteins involved in 1,3- β -D-glucan-dependent prophenoloxidase activation system of insect. *J Biol Chem* 279.

Bibliographie

- Lemaitre B, Hoffmann J. 2007. The Host Defense of *Drosophila melanogaster*. *Annual Review of Immunology* 25:697-743.
- Levashina EA, Moita LF, Blandin S, Vriend G, Lagueux M, Kafatos FC. 2001. Conserved Role of a Complement-like Protein in Phagocytosis Revealed by dsRNA Knockout in Cultured Cells of the Mosquito, *Anopheles gambiae*. *Cell* 104:709-718.
- Li C, Blencke H-M, Haug T, Jørgensen Ø, Stensvåg K. 2014. Expression of antimicrobial peptides in coelomocytes and embryos of the green sea urchin (*Strongylocentrotus droebachiensis*). *Developmental and comparative immunology* 43:106-113.
- Li C, Li H, Xiao B, Chen Y, Wang S, L K, Yin B, Li S, He J. 2017. Identification and functional analysis of a TEP gene from a crustacean reveals its transcriptional regulation mediated by NF- κ B and JNK pathways and its broad protective roles against multiple pathogens. *Developmental and Comparative Immunology* 70:45-58.
- Little TJ, Watt K, Ebert D. 2006. PARASITE-HOST SPECIFICITY: EXPERIMENTAL STUDIES ON THE BASIS OF PARASITE ADAPTATION. *Evolution* 60:31-38.
- Lockyer AE, Emery AM, Kane Ra, Walker AJ, Mayer CD, Mitta G, Coustau C, Adema CM, Hanelt B, Rollinson D, et al. 2012. Early differential gene expression in haemocytes from resistant and susceptible *Biomphalaria glabrata* strains in response to *Schistosoma mansoni*. *PloS one* 7:e51102-e51102.
- Lockyer AE, Jones CS, Noble LR, Rollinson D. 2004. Trematodes and snails: an intimate association. *Canadian Journal of Zoology* 82:251-269.
- Lockyer AE, Olson PD, Ostergaard P, Rollinson D, Johnston DA, Attwood SW, Southgate VR, Horak P, Snyder SD, Le TH, et al. 2003. The phylogeny of the Schistosomatidae based on three genes with emphasis on the interrelationships of *Schistosoma* Weinland, 1858. *Parasitology* 126:203-224.
- Loker ES, Adema CM, Zhang S-M, Kepler TB. 2004. Invertebrate immune systems – not homogeneous, not simple, not well understood. *Immunological Reviews* 198:10-24.
- Loker ES, Bayne CJ, Buckley PM, Kruse KT. 1982. Ultrastructure of encapsulation of *Schistosoma mansoni* mother sporocysts by hemocytes of juveniles of the 10-R2 strain of *Biomphalaria glabrata*. *The Journal of parasitology* 68:84-94.
- Loker ES, Couch L, Hertel LA. 1994. Elevated agglutination titres in plasma of *Biomphalaria glabrata* exposed to *Echinostoma paraensei*: characterization and functional relevance of a trematode-induced response. *Parasitology* 108 (Pt 1):17-26.
- Loker ES, Yui MA, Bayne CJ. 1984. *Schistosoma mansoni*: Agglutination of sporocysts, and formation of gels on miracidia transforming in plasma of *Biomphalaria glabrata*. *Experimental Parasitology* 58:56-62.
- Lowenberger CA, Kamal S, Chiles J, Paskewitz S, Bulet P, Hoffmann JA, Christensen BM. 1999. Mosquito-Plasmodium Interactions in Response to Immune Activation of the Vector. *Experimental Parasitology* 91:59-69.

Bibliographie

- Léonard PM, Adema CM, Zhang S-M, Loker ES. 2001. Structure of two FREP genes that combine IgSF and fibrinogen domains, with comments on diversity of the FREP gene family in the snail *Biomphalaria glabrata*. *Gene* 269:155-165.
- Maizels RM, McSorley HJ. 2016. Regulation of the host immune system by helminth parasites. *The Journal of Allergy and Clinical Immunology* 138:666-675.
- Marchalonis JJ, Schluter SF. 1989. Immunoproteins in evolution. *Developmental & Comparative Immunology* 13:285-301.
- Martinez J, Fleury F, Varaldi J. 2015. Competitive outcome of multiple infections in a behavior-manipulating virus/wasp interaction. *Ecology and Evolution* 5:5934-5945.
- Martinson VG, Douglas AE, Jaenike J. 2017. Community structure of the gut microbiota in sympatric species of wild *Drosophila*. *Ecology Letters* 20:629-639.
- Martínez VG, Moestrup SK, Holmskov U, Mollenhauer J, Lozano F. 2011. The Conserved Scavenger Receptor Cysteine-Rich Superfamily in Therapy and Diagnosis. *Pharmacological Reviews* 63:967.
- Matozzo V, Bailo L. 2015. A first insight into haemocytes of the smooth venus clam *Callista chione*. *Fish & Shellfish Immunology* 42:494-502.
- Minguez L, Buronfosse T, Beisel J-N, Giambérini L. 2012. Parasitism can be a confounding factor in assessing the response of zebra mussels to water contamination. *Environmental Pollution* 162:234-240.
- Minguez L, Molloy DP, Guérolde F, Giambérini L. 2011. Zebra mussel (*Dreissena polymorpha*) parasites: Potentially useful bioindicators of freshwater quality? *Water Research* 45:665-673.
- Mitta G, Adema CM, Gourbal B, Loker ES, Theron A. 2012. Compatibility polymorphism in snail/schistosome interactions: From field to theory to molecular mechanisms. *Developmental and Comparative Immunology* 37:1-8.
- Mitta G, Galinier R, Tisseyre P, Allienne JF, Girerd-Chambaz Y, Guillou F, Bouchut a, Coustau C. 2005. Gene discovery and expression analysis of immune-relevant genes from *Biomphalaria glabrata* hemocytes. *Developmental and comparative immunology* 29:393-407.
- Mitta G, Gourbal B, Grunau C, Knight M, Bridger JM, Théron A. 2017. Chapter Three – The Compatibility Between *Biomphalaria glabrata* Snails and *Schistosoma mansoni*: An Increasingly Complex Puzzle. *Advances in Parasitology* 97:111-145.
- Moné Y, Gourbal B, Duval D, du Pasquier L, Kieffer-Jaquinod S, Mitta G. 2010. A large repertoire of parasite epitopes matched by a large repertoire of host immune receptors in an invertebrate host/parasite model. *PLoS Neglected Tropical Diseases* 4.
- Moné Y, Ribou A-C, Cosseau C, Duval D, Théron A, Mitta G, Gourbal B. 2011. An example of molecular co-evolution: Reactive oxygen species (ROS) and ROS scavenger levels in *Schistosoma mansoni*/*Biomphalaria glabrata* interactions. *International Journal for Parasitology* 41:721-730.
- Moreau S, Huguet E, Drezen J-M. 2009. Polydnviruses as tools to deliver wasp virulence factors to impair lepidopteran host immunity. In: Oxford University Press. p. 137-158.

Bibliographie

- Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, Lu G, Pyke AT, Hedges LM, Rocha BC, Hall-Mendelin S, Day A, Riegler M, et al. 2009. A Wolbachia Symbiont in *Aedes aegypti* Limits Infection with Dengue, Chikungunya, and Plasmodium. *Cell* 139:1268-1278.
- Morgan JAT, Dejong RJ, Adeoye GO, Ansa EDO, Barbosa CS, Brémond P, Cesari IM, Charbonnel N, Corrêa LR, Coulibaly G, et al. 2005. Origin and diversification of the human parasite *Schistosoma mansoni*. *Molecular Ecology* 14:3889-3902.
- Mortimer NT, Goecks J, Kacsoh BZ, Mobley JA, Bowersock GJ, Taylor J, Schlenke TA. 2013. Parasitoid wasp venom SERCA regulates *Drosophila* calcium levels and inhibits cellular immunity. *Proceedings of the National Academy of Sciences of the United States of America* 110:9427-9432.
- Muñoz M, Cedeño R, Rodríguez J, Van Der Knaap WPW, Mialhe E, Bachère E. 2000. Measurement of reactive oxygen intermediate production in haemocytes of the penaeid shrimp, *Penaeus vannamei*. *Aquaculture* 191:89-107.
- Nakamoto M, Moy RH, Xu J, Bambina S, Yasunaga A, Shelly SS, Gold B, Cherry S. 2012. Virus recognition by Toll-7 activates antiviral autophagy in *Drosophila*. *Immunity* 36:658-667.
- Nappi AJ, Ottaviani E. 2000. Cytotoxicity and cytotoxic molecules in invertebrates. *BioEssays* 22:469-480.
- Neubauer EF, Poole AZ, Weis VM, Davy SK. 2016. The scavenger receptor repertoire in six cnidarian species and its putative role in cnidarian-dinoflagellate symbiosis. *PeerJ* 4:e2692-e2692.
- Nidelet T, Kaltz O. 2007. DIRECT AND CORRELATED RESPONSES TO SELECTION IN A HOST-PARASITE SYSTEM: TESTING FOR THE EMERGENCE OF GENOTYPE SPECIFICITY. *Evolution* 61:1803-1811.
- Osta MA, Christophides GK, Vlachou D, Kafatos FC. 2004. Innate immunity in the malaria vector *Anopheles gambiae*: comparative and functional genomics. *The Journal of experimental biology* 207:2551-2563.
- Pancer Z. 2000. Dynamic expression of multiple scavenger receptor cysteine-rich genes in coelomocytes of the purple sea urchin. *Proceedings of the National Academy of Sciences of the United States of America* 97:13156-13161.
- Pancer Z, Rast JP, Davidson EH. 1999. Origins of immunity: Transcription factors and homologues of effector genes of the vertebrate immune system expressed in sea urchin coelomocytes. *Immunogenetics* 49:773-786.
- Paraense WL, Correa LR. 1963. Variation in susceptibility of populations of *Australorbis glabratus* to a strain of *Schistosoma mansoni*. *Revista do Instituto de Medicina Tropical de Sao Paulo* 5:15-22.
- Pasquier LD. 2005. Insects Diversify One Molecule to Serve Two Systems. *Science* 3034:2004-2005.
- Pees B, Yang W, Z??rate-Potes A, Schulenburg H, Dierking K. 2016. High Innate Immune Specificity through Diversified C-Type Lectin-Like Domain Proteins in Invertebrates. *Journal of Innate Immunity* 8:129-142.

Bibliographie

- Perrin C, Lepesant JM, Roger E, Duval D, Fneich S, Thuillier V, Allienne J-F, Mitta G, Grunau C, Cosseau C. 2013. *Schistosoma mansoni* Mucin Gene (SmPoMuc) Expression: Epigenetic Control to Shape Adaptation to a New Host. *PLoS Pathogens* 9:e1003571-e1003571.
- Pierce HG. 1993. The nonmarine mollusks of the late Oligocene–early Miocene Cabbage Patch fauna of western Montana III. Aquatic mollusks and conclusions. *Journal of Paleontology* 67:980-993.
- Pila EA, Gordy MA, Phillips VK, Kabore AL, Rudko SP, Hanington PC. 2016. Endogenous growth factor stimulation of hemocyte proliferation induces resistance to *Schistosoma mansoni* challenge in the snail host. *Proceedings of the National Academy of Sciences*: 201521239-201521239.
- Pila EA, Tarrabain M, Kabore AL, Hanington PC. 2016. A Novel Toll-Like Receptor (TLR) Influences Compatibility between the Gastropod *Biomphalaria glabrata*, and the Digenean Trematode *Schistosoma mansoni*. *PLoS pathogens* 12:e1005513-e1005513.
- Pinaud S, Portela J, Duval D, Nowacki FC, Olive M-A, Allienne J-F, Galinier R, Dheilly NM, Kieffer-Jaquinod S, Mitta G, et al. 2016. A Shift from Cellular to Humoral Responses Contributes to Innate Immune Memory in the Vector Snail *Biomphalaria glabrata*. *PLOS Pathogens* 12:e1005361-e1005361.
- Pinsino A, Matranga V. 2015. Sea urchin immune cells as sentinels of environmental stress. *Developmental and Comparative Immunology* 49:198-205.
- Pompon J, Levashina EA. 2015. A New Role of the Mosquito Complement-like Cascade in Male Fertility in *Anopheles gambiae*. *PLoS Biology* 13:1-17.
- Portet A, Pinaud S, Tetreau G, Galinier R, Cosseau C, Duval D, Grunau C, Mitta G, Gourbal B. 2017. Integrated multi-omic analyses in *Biomphalaria-Schistosoma* dialogue reveal the immunobiological significance of FREP-SmPoMuc interaction. *Developmental & Comparative Immunology*.
- Pradeu T. 2016. Mutualistic viruses and the heteronomy of life. *Studies in History and Philosophy of Science Part C :Studies in History and Philosophy of Biological and Biomedical Sciences* 59:80-88.
- Pradeu T, Eric V. 2014. The discontinuity theory of immunity. *Sci Immunol* 1:1-9.
- Pumpuni CB, Beier MS, Nataro JP, Guers LD, Davis JR. 1993. *Plasmodium falciparum* : Inhibition of sporogonic development in *Anopheles stephensi* by Gram-negative bacteria. *Experimental parasitology*:195-199.
- Ramade F. 2003. *Éléments d'Écologie*: DUNOD.
- Ramirez JL, Garver LS, Brayner FA, Alves LC, Rodrigues J, Molina-Cruz A, Barillas-Mury C. 2014. The role of hemocytes in *Anopheles gambiae* antiplasmodial immunity. *Journal of Innate Immunity* 6:119-128.
- Ramirez JL, Souza-Neto J, Torres Cosme R, Rovira J, Ortiz A, Pascale JM, Dimopoulos G. 2012. Reciprocal Tripartite Interactions between the *Aedes aegypti* Midgut Microbiota, Innate Immune System and Dengue Virus Influences Vector Competence. *PLoS Neglected Tropical Diseases* 6:e1561.

Bibliographie

- Reynolds LA, Finlay BB, Maizels RM. 2015. Cohabitation in the Intestine: Interactions among Helminth Parasites, Bacterial Microbiota, and Host Immunity. *Journal of immunology (Baltimore, Md. : 1950)* 195:4059-4066.
- Richards CS, Shade PC. 1987. The genetic variation of compatibility in *Biomphalaria glabrata* and *Schistosoma mansoni*. *The Journal of parasitology* 73:1146-1151.
- Rodrigues J, Brayner FA, Alves LC, Dixit R, Barillas-mury C. 2010. Hemocyte Differentiation Mediates Innate Immune Memory in *Anopheles gambiae* Mosquitoes. *Science* 329:1353-1355.
- Roger E. 2008. Bases moléculaires du polymorphisme de compatibilité dans l'interaction *Schistosoma mansoni* / *Biomphalaria glabrata*. Thèse.
- Roger E, Gourbal B, Grunau C, Pierce RJ, Galinier R, Mitta G. 2008. Expression analysis of highly polymorphic mucin proteins (Sm PoMuc) from the parasite *Schistosoma mansoni*. *Molecular and Biochemical Parasitology* 157:217-227.
- Roger E, Grunau C, Pierce RJ, Hirai H, Gourbal B, Galinier R, Emans R, Cesari IM, Cosseau C, Mitta G. 2008. Controlled Chaos of Polymorphic Mucins in a Metazoan Parasite (*Schistosoma mansoni*) Interacting with Its Invertebrate Host (*Biomphalaria glabrata*). *PLoS Neglected Tropical Diseases* 2:e330-e330.
- Roger E, Mitta G, Moné Y, Bouchut A, Rognon A, Grunau C, Boissier J, Théron A, Gourbal BEF. 2008. Molecular determinants of compatibility polymorphism in the *Biomphalaria glabrata*/*Schistosoma mansoni* model: new candidates identified by a global comparative proteomics approach. *Molecular and biochemical parasitology* 157:205-216.
- Rosenberg E, Zilber-Rosenberg I. 2011. Symbiosis and development: The hologenome concept. *Birth Defects Research Part C - Embryo Today: Reviews* 93:56-66.
- Ryu J-H, Kim S-H, Lee H-Y, Bai JY, Nam Y-D, Bae J-W, Lee DG, Shin SC, Ha E-M, Lee W-J. 2008. Innate Immune Homeostasis by the Homeobox Gene *Caudal* and Commensal-Gut Mutualism in *Drosophila*. *Science* 319:777.
- Sandiford SL, Dong Y, Pike A, Blumberg BJ, Bahia AC, Dimopoulos G. 2015. Cytoplasmic Actin Is an Extracellular Insect Immune Factor which Is Secreted upon Immune Challenge and Mediates Phagocytosis and Direct Killing of Bacteria, and Is a Plasmodium Antagonist. *PLoS Pathogens* 11:e1004631.
- Sansone CL, Cohen J, Yasunaga A, Xu J, Osborn G, Subramanian H, Gold B, Buchon N, Cherry S. 2015. Microbiota-dependent priming of antiviral intestinal immunity in *Drosophila*. *Cell Host and Microbe* 18:571-581.
- Sarma JV, Ward PA. 2011. The Complement System. *Cell and tissue research* 343:227-235.
- Schmucker D, Clemens JC, Shu H, Worby CA, Xiao J, Muda M, Dixon JE, Zipursky SL. 2000. *Drosophila* Dscam Is an Axon Guidance Receptor Exhibiting Extraordinary Molecular Diversity. *Cell* 101:671-684.
- Schnitger AKD, Yassine H, Kafatos FC, Osta MA. 2009. Two C-type lectins cooperate to defend *Anopheles gambiae* against Gram-negative bacteria. *Journal of Biological Chemistry* 284:17616-17624.

Bibliographie

- Sekiguchi R, Fujito NT, Nonaka M. 2012. Evolution of the thioester-containing proteins (TEPs) of the arthropoda, revealed by molecular cloning of TEP genes from a spider, *Hasarius adansoni*. *Developmental and Comparative Immunology* 36:483-489.
- Sharon G, Segal D, Zilber-Rosenberg I, Rosenberg E. 2011. Symbiotic bacteria are responsible for diet-induced mating preference in *Drosophila melanogaster*, providing support for the hologenome concept of evolution. *Gut Microbes* 2:190-192.
- Shin SC, Kim SH, You H, Kim B, Kim AC, Lee KA, Yoon JH, Ryu JH, Lee WJ. 2011. *Drosophila* Microbiome Modulates Host Developmental and Metabolic Homeostasis via Insulin Signaling. *Science* 334:670-674.
- Shokal U, Kopydlowski H, Eleftherianos I. 2017. The distinct function of *Tep2* and *Tep6* in the immune defense of *Drosophila melanogaster* against the pathogen *Photorhabdus*. *Virulence* 5594:1-15.
- Silva TM, Melo ES, Lopes ACS, Veras DL, Duarte CR, Alves LC, Brayner FA. 2013. Characterization of the bacterial microbiota of *Biomphalaria glabrata* (Say, 1818) (Mollusca: Gastropoda) from Brazil. *Letters in applied microbiology* 57:19-25.
- Smith RC, Barillas-mury C, Jacobs-Lorena M. 2015. Hemocyte differentiation mediates the mosquito late-phase immune response against *Plasmodium* in *Anopheles gambiae*. *Proc Natl Acad Sci U S A* 112:E3412-3420.
- Soliman MFM. 2009. *Fasciola gigantica*: Cercarial shedding pattern from *Lymnaea natalensis* after long-term exposure to cadmium at different temperatures. *Experimental Parasitology* 121:307-311.
- Song YL, Hsieh YT. 1994. Immunostimulation of tiger shrimp (*Penaeus monodon*) hemocytes for generation of microbicidal substances: analysis of reactive oxygen species. *Developmental and comparative immunology* 18:201-209.
- Soria F, Sierra C, Bouquelet S, Brassart C, Agundis C, Zenteno E, Vazquez L. 2006. The effect of sugars and free amino acids from the freshwater prawn *Macrobrachium rosenbergii* hemolymph on lectin activity and on oxidative burst. *Comparative biochemistry and physiology. Toxicology & pharmacology : CBP* 142:212-219.
- Staubach F, Baines JF, Künzel S, Bik EM, Petrov DA. 2013. Host Species and Environmental Effects on Bacterial Communities Associated with *Drosophila* in the Laboratory and in the Natural Environment. *PLoS ONE* 8.
- Stockwell MP, Clulow J, Mahony MJ. 2012. Sodium Chloride Inhibits the Growth and Infective Capacity of the Amphibian Chytrid Fungus and Increases Host Survival Rates. *PLoS ONE* 7:e36942.
- Straif SC, Mbogo CNM, Toure AM, Walker ED, Kaufman M, Toure YT, Beier JC. 1998. Midgut Bacteria in *Anopheles gambiae* and *An. funestus* (Diptera: Culicidae) from Kenya and Mali. *Journal of Medical Entomology* 35:222-226.
- Strand MR, Burke GR. 2012. Polydnviruses as Symbionts and Gene Delivery Systems. *PLoS Pathogens* 8:e1002757.

Bibliographie

- Tang H, Kambris Z, Lemaitre B, Hashimoto C. 2008. A SERPIN THAT REGULATES IMMUNE MELANIZATION IN THE RESPIRATORY SYSTEM OF DROSOPHILA. *Developmental cell* 15:617-626.
- Taracena ML, Oliveira PL, Almendares O, Umaña C, Lowenberger C, Dotson EM, Paiva-Silva GO, Pennington PM. 2015. Genetically Modifying the Insect Gut Microbiota to Control Chagas Disease Vectors through Systemic RNAi. *PLoS Neglected Tropical Diseases* 9:1-14.
- Tauszig S, Jouanguy E, Hoffmann JA, Imler J-L. 2000. Toll-related receptors and the control of antimicrobial peptide expression in *Drosophila*. *Proceedings of the National Academy of Sciences* 97:10520-10525.
- Tennessen JA, Théron A, Marine M, Yeh J-Y, Rognon A, Blouin MS. 2015. Hyperdiverse Gene Cluster in Snail Host Conveys Resistance to Human Schistosome Parasites. *PLoS Genetics* 11:e1005067.
- Tentcheva D, Gauthier L, Zappulla N, Dainat B, Cousserans F, Colin ME, Bergoin M. 2004. Prevalence and Seasonal Variations of Six Bee Viruses in *Apis mellifera* L. and *Varroa destructor* Mite Populations in France. *Applied and Environmental Microbiology* 70:7185-7191.
- Terenius O, Lindh JM, Eriksson-Gonzales K, Bussière L, Laugen AT, Bergquist H, Titanji K, Faye I. 2012. Midgut bacterial dynamics in *Aedes aegypti*. *FEMS Microbiology Ecology* 80:556-565.
- Terwilliger DP, Buckley KM, Brockton V, Ritter NJ, Smith LC. 2007. Distinctive expression patterns of 185/333 genes in the purple sea urchin, *Strongylocentrotus purpuratus*: an unexpectedly diverse family of transcripts in response to LPS, β -1,3-glucan, and dsRNA. *BMC Molecular Biology* 8:16-16.
- Terwilliger DP, Buckley KM, Mehta D, Moorjani PG, Smith LC. 2006. Unexpected diversity displayed in cDNAs expressed by the immune cells of the purple sea urchin, *Strongylocentrotus purpuratus*. *Physiological Genomics* 26:134.
- Terwilliger DP, Clow LA, Gross PS, Smith LC. 2004. Constitutive expression and alternative splicing of the exons encoding SCRs in Sp152, the sea urchin homologue of complement factor B. Implications on the evolution of the Bf/C2 gene family. *Immunogenetics* 56:531-543.
- Theis KR, Dheilly NM, Klassen JL, Brucker RM, Baines JF, Bosch TCG, Cryan JF, Gilbert SF, Goodnight CJ, Lloyd EA, et al. 2016. Getting the Hologenome Concept Right: an Eco-Evolutionary Framework for Hosts and Their Microbiomes. *mSystems* 11:e00028-00016.
- Theron A, Coustau C, Rognon A, Gourbière S, Blouin MS. 2008. Effects of laboratory culture on compatibility between snails and schistosomes. *Parasitology* 135:1179-1188.
- Theron A, Rognon A, Gourbal B, Mitta G. 2014. Multi-parasite host susceptibility and multi-host parasite infectivity: A new approach of the *Biomphalaria glabrata*/*Schistosoma mansoni* compatibility polymorphism. *Infection, Genetics and Evolution* 26:80-88.
- Théron A, Pages J-R, Rognon A. 1997. *Schistosoma mansoni* : Distribution Patterns of Miracidia among *Biomphalaria glabrata* Snail as Related to Host Susceptibility and Sporocyst Regulatory Processes. 9:1-9.
- Théron A, Théron A, Coustau C. 2005. Are *Biomphalaria* snails resistant to *Schistosoma mansoni*? *Journal of Helminthology* 79:187-191.

Bibliographie

- Tirard C, Abbadie L, Laloï D, Koubbi P. 2016. *Écologie*: DUNOD.
- Turner MW. 1996. Mannose-binding lectin: the pluripotent molecule of the innate immune system. *Immunology Today* 17:532-540.
- Urbanová V, Šíma R, Šauman I, Hajdušek O, Kopáček P. 2015. Thioester-containing proteins of the tick *Ixodes ricinus*: Gene expression, response to microbial challenge and their role in phagocytosis of the yeast *Candida albicans*. *Developmental & Comparative Immunology* 48:55-64.
- Van Horn DJ, Garcia JR, Loker ES, Mitchell KR, Mkoji GM, Adema CM, Takacs-Vesbach CD. 2012. Complex intestinal bacterial communities in three species of planorbid snails. *Journal of Molluscan Studies* 78:74-80.
- van Lookeren Campagne M, Wiesmann C, Brown EJ. 2007. Macrophage complement receptors and pathogen clearance. *Cellular Microbiology* 9:2095-2102.
- Van Valen L. 1973. BODY SIZE AND NUMBERS OF PLANTS AND ANIMALS. *Evolution* 27:27-35.
- Van Valen L. 1974. Molecular evolution as predicted by natural selection. *Journal of molecular evolution* 3:89-101.
- Vanha-aho LM, Valanne S, Rämetsä M. 2016. Cytokines in *Drosophila* immunity. *Immunology Letters* 170:42-51.
- Vasta GR, Ahmed H, Blanchet MA, Fernández-Robledo JA, Amzel LM. 2012. Diversity in recognition of glycans by F-type lectins and galectins: molecular, structural, and biophysical aspects. *Annals of the New York Academy of Sciences* 1253:E14-E26.
- Vazquez L, Alpuche J, Maldonado G, Agundis C, Pereyra-Morales A, Zenteno E. 2009. Immunity mechanisms in crustaceans 15(3). *Innate Immunity* 15:179-188.
- Vitetta L, Coulson S, Linnane AW, Butt H. 2013. The Gastrointestinal Microbiome and Musculoskeletal Diseases: A Beneficial Role for Probiotics and Prebiotics. *Pathogens* 2:606-626.
- Vizioli J, Bulet P, Hoffmann JA, Kafatos FC, Müller H-M, Dimopoulos G. 2001. Gambicin: A novel immune responsive antimicrobial peptide from the malaria vector *Anopheles gambiae*. *Proceedings of the National Academy of Sciences of the United States of America* 98:12630-12635.
- Vlisidou I, Wood W. 2015. *Drosophila* blood cells and their role in immune responses. *FEBS Journal* 282:1368-1382.
- Volohonsky G, Hopp AK, Saenger M, Soichot J, Scholze H, Boch J, Blandin SA, Marois E. 2017. Transgenic Expression of the Anti-parasitic Factor TEP1 in the Malaria Mosquito *Anopheles gambiae*. *PLoS Pathogens* 13:1-26.
- Volohonsky G, Steinert S, Levashina EA. 2010. Focusing on complement in the antiparasitic defense of mosquitoes. *Trends in Parasitology* 26:1-3.
- Volz J, Müller H-M, Zdanowicz A, Kafatos FC, Osta MA. 2006. A genetic module regulates the melanization response of *Anopheles* to *Plasmodium*. *Cellular Microbiology* 8:1392-1405.
- Vuilleumier F, Andors A. 1993. *Biological relationships between Africa and South America*. New Haven: Yale University Press.

Bibliographie

- Wang XW, Wang JX. 2015. Crustacean hemolymph microbiota: Endemic, tightly controlled, and utilization expectable. *Molecular Immunology* 68:404-411.
- Wang XW, Xu JD, Zhao XF, Vasta GR, Wang JX. 2014. A shrimp C-type lectin inhibits proliferation of the hemolymph microbiota by maintaining the expression of antimicrobial peptides. *Journal of Biological Chemistry* 289:11779-11790.
- Watson FL. 2005. Extensive Diversity of Ig-Superfamily Proteins in the Immune System of Insects. *Science* 309:1874-1878.
- Webster JP, Davies CM. 2001. Coevolution and compatibility in the snail–schistosome system. *Parasitology* 123:41-56.
- Werren JH, Baldo L, Clark ME. 2008. Wolbachia: master manipulators of invertebrate biology. *Nat Rev Micro* 6:741-751.
- Wertheim B, Kraaijeveld AR, Schuster E, Blanc E, Hopkins M, Pletcher SD, Strand MR, Partridge L, Godfray HCJ. 2005. Genome-wide gene expression in response to parasitoid attack in *Drosophila*. *Genome Biology* 6:R94-R94.
- Wong ACN, Chaston JM, Douglas AE. 2013. The inconstant gut microbiota of *Drosophila* species revealed by 16S rRNA gene analysis. *The ISME Journal* 7:1922-1932.
- Wong ACN, Dobson AJ, Douglas AE. 2014. Gut microbiota dictates the metabolic response of *Drosophila* to diet. *Journal of Experimental Biology* 217:1894-1901.
- Woodruff DS, Mulvey M. 1997. Neotropical schistosomiasis: African affinities of the host snail *Biomphalaria glabrata* (Gastropoda: Planorbidae). *Biological Journal of the Linnean Society* 60:505-516.
- Wu C, Noonin C, Jiravanichpaisal P, Söderhäll I, Söderhäll K. 2012. An insect TEP in a crustacean is specific for cuticular tissues and involved in intestinal defense. *Insect Biochemistry and Molecular Biology* 42:71-80.
- Wu X-J, Dinguirard N, Sabat G, Lui H-d, Gonzalez L, Gehring M, Bickham-Wright U, Yoshino TP. 2017. Proteomic analysis of *Biomphalaria glabrata* plasma proteins with binding affinity to those expressed by early developing larval *Schistosoma mansoni*. *PLoS Pathogens* 13:e1006081.
- Xi Z, Ramirez JL, Dimopoulos G. 2008. The *Aedes aegypti* toll pathway controls dengue virus infection. *PLoS Pathogens* 4.
- Yadav KK, Bora A, Datta S, Chandel K, Gogoi HK, Prasad GBKS, Veer V. 2015. Molecular characterization of midgut microbiota of *Aedes albopictus* and *Aedes aegypti* from Arunachal Pradesh, India. *Parasites & Vectors* 8:641-641.
- Yassine H, Kamareddine L, Osta MA. 2012. The Mosquito Melanization Response Is Implicated in Defense against the Entomopathogenic Fungus *Beauveria bassiana*. *PLOS Pathogens* 8:e1003029.
- Yassine H, Osta MA. 2010. *Anopheles gambiae* innate immunity. *Cellular Microbiology* 12:1-9.
- Yoshino TP, Dinguirard N, Kunert J, Hokke CH. 2008. Molecular and functional characterization of a tandem-repeat galectin from the freshwater snail *Biomphalaria glabrata*, intermediate host of the human blood fluke *Schistosoma mansoni*. *Gene* 411:46-58.

Bibliographie

- Zelensky AN, Gready JE. 2005. The C-type lectin-like domain superfamily. *FEBS Journal* 272:6179-6217.
- Zhang H, Song L, Li C, Zhao J, Wang H, Gao Q, Xu W. 2007. Molecular cloning and characterization of a thioester-containing protein from Zhikong scallop *Chlamys farreri*. *Molecular Immunology* 44:3492-3500.
- Zhang S-M, Adema CM, Kepler TB, Loker ES. 2004. Diversification of Ig superfamily genes in an invertebrate. *Science (New York, N.Y.)* 305:251-254.
- Zhang S-M, Léonard PM, Adema CM, Loker ES. 2001. Parasite-responsive IgSF members in the snail *Biomphalaria glabrata*: characterization of novel genes with tandemly arranged IgSF domains and a fibrinogen domain. *Immunogenetics* 53:684-694.
- Zilber-Rosenberg I, Rosenberg E. 2008. Role of microorganisms in the evolution of animals and plants: The hologenome theory of evolution. *FEMS Microbiology Reviews* 32:723-735.
- Zimmer C. 2012. Introduction à l'évolution: De Boeck.
- Švec P, Devriese LA, Sedláček I, Baele M, Vancanneyt M, Haesebrouck F, Swings J, Doškař J. 2002. Characterization of yellow-pigmented and motile enterococci isolated from intestines of the garden snail *Helix aspersa*. *Journal of Applied Microbiology* 92:951-957.

Résumé : *Biomphalaria glabrata* est un mollusque d'eau douce, vivant en Amérique latine. Ce planorbe est principalement connu pour être l'hôte intermédiaire de *Schistosoma mansoni*, vers plat parasite responsable de la bilharziose intestinale, seconde endémie parasitaire humaine mondiale derrière le paludisme.

Dans ce contexte, il apparaît clairement qu'une meilleure compréhension de l'interaction entre le parasite et le mollusque, hôte intermédiaire, représente une voie de recherche prometteuse. La compréhension des interactions immunologiques entre l'escargot et le parasite ainsi que des mécanismes moléculaires par lesquels les deux partenaires interagissent apparaît comme un pré-requis à la découverte de nouvelles cibles ou de nouvelles stratégies afin de développer des moyens de lutte contre le pathogène.

Le projet de cette thèse s'inscrit dans cette optique et vise à une meilleure compréhension des interactions immunologiques entre le mollusque *Biomphalaria glabrata* et le trématode *Schistosoma mansoni*. Différents aspects de l'interaction entre *B.glabrata* et *S.mansoni* ont été explorés, des bases moléculaires et cellulaires à l'interaction tripartite entre l'immunité du mollusque, son microbiote et le pathogène. Dans un premier temps nous avons pu démontrer un gradient d'infectivité des parasites et de susceptibilité des mollusques de différentes provenances géographiques. De plus, l'interaction immunologique entre le mollusque et le parasite est supportée par une adaptation locale, à l'échelle moléculaire. Nous avons également pu montrer qu'une opsonine, la BgTEP, jouait un rôle clé dans l'interaction entre *B.glabrata* et ses différents pathogènes. Enfin, l'existence d'une véritable interaction tripartite entre la réponse immunitaire du mollusque, son microbiote et son parasite a pu être mise en évidence.

Mots clés: *B. glabrata*, *S. mansoni*, Immunité innée, Adaptation locale, BgTEP, Interaction tripartite, Microbiote

Abstract : *Biomphalaria glabrata* is tropical fresh water snail, living in Latin America. This planorbe is the intermediary host of *Schistosoma mansoni*, a trematode responsible for the intestinal Schistosomiasis, second worldwide human vector-borne disease after the malaria.

In this context, a better comprehension of the parasite/snail interaction is necessary and appears a promising research field. The understanding of immunological interaction between the host and the parasite and the molecular mechanisms used by the two partners appears like essential for the discovery of new targets and new strategies in order to develop means of struggle against the pathogen.

The aim of this thesis is to better understand the immunological interactions between the *B. glabrata* snail and *S. mansoni* trematode. Different aspects of the interaction between the snail and the parasite have been explored, from molecular and cellular bases to the tripartite interaction between the snail immunity, its microbiota and the pathogen. In a first step we have been able to demonstrate a gradient of parasite infectivities and snail susceptibilities from different geographical origins. Moreover, the immunological interaction between *B. glabrata* and *S. mansoni* is supported by local adaptation, at the molecular level. We were also able to show that an opsonin, the BgTEP, plays a key role in the interaction between *B. glabrata* and its various pathogens. Finally, the existence of a true tripartite interaction between the snail immune response, its microbiota and its parasite could be demonstrated.

Keywords: *B. glabrata*, *S. mansoni*, Innate Immunity, Local Adaptation, BgTEP, Tripartite interaction, Microbiota
