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Aspects fonctionnel et évolutif de l'immunité mémoire chez les invertébrés : l'escargot vecteur de la Bilharziose intestinale *Biomphalaria glabrata* comme nouvel organisme modèle ?

Silvain Pinaud

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Délivré par
UNIVERSITE DE PERPIGNAN VIA DOMITIA

Préparée au sein de l'école doctorale
Énergie Environnement ED305
Et de l'unité de recherche
**Laboratoire Interactions Hôtes-Pathogènes et
Environnements (IHPE)**
UPVD – CNRS – IFREMER - UM

Spécialité :
Biologie

Présentée par
Silvain PINAUD

**ASPECTS FONCTIONNEL ET EVOLUTIF DE L'IMMUNITE
MEMOIRE CHEZ LES INVERTEBRES : L'ESCARGOT
VECTEUR DE LA BILHARZIOSE INTESTINALE
BIOMPHALARIA GLABRATA COMME NOUVEL
ORGANISME MODELE ?**

Soutenue le 19 Octobre 2017, devant le jury composé de

M. Dominique FERRANDON , DR, Strasbourg	Rapporteur
M. Jean-Michel DREZEN , DR, Tours	Rapporteur
M. Yannick MORET , DR, Dijon	Examineur
M. David DUNEAU , PhD, Toulouse	Examineur
M. Jose-Juan LOPEZ-RUBIO , CR, Montpellier	Examineur
M. Jean-Marc DERAGON , PR, Perpignan	Examineur
M. David DUVAL , MCF, Perpignan	Directeur de Thèse
M. Benjamin GOUBAL , MCF, Perpignan	Directeur de Thèse

Aspects fonctionnel et évolutif de l'immunité mémoire chez les invertébrés : l'escargot vecteur de la Bilharziose intestinale *Biomphalaria glabrata* comme nouvel organisme modèle ?



Sous la supervision de

Benjamin GOURBAL

David DUVAL

UNIVERSITÉ
PERPIGNAN
VIA
DOMITIA



IHPE

Interactions-Hôtes-Pathogènes-Environnements



WHO



La simplicité est la sophistication suprême.

Léonard de Vinci (1452-1519)

Merci!

Benjamin Gourbal & David Duval

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Allienne

Cécile, Julien KS, Julien P, Ronaldo, Annia, Damien, Nath, Max

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Anaïs

Papa, Maman, Élo, Énora, Auré, Delph, Loane, Nathan

Thierry, Gisèle, Jean, Marie-Hélène, Heidi

TOUTE la famille PINAUD, MASSOT, PORTET et FAUP, présente et partie

Mes amis, pas toujours près mais toujours prêts

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Suite page 325

Aspects fonctionnel et évolutif de l'immunité mémoire chez les invertébrés : l'escargot vecteur de la bilharziose intestinale *Biomphalaria glabrata* comme nouvel organisme modèle ?

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L'escargot vecteur de la bilharziose intestinale *Biomphalaria glabrata*, dorénavant un modèle d'étude de l'évolution de l'immunité mémoire chez les invertébrés.

Prologue

« Votre temps est limité, ne le gâchez pas en menant une existence qui n'est pas la vôtre. Ne soyez pas prisonnier des dogmes qui obligent à vivre en obéissant à la pensée d'autrui. Ne laissez pas le brouhaha extérieur étouffer votre voix intérieure. Ayez le courage de suivre votre cœur et votre intuition. L'un et l'autre savent ce que vous voulez réellement devenir. Le reste est secondaire. » Steve Jobs

L'escargot vecteur de la bilharziose intestinale *Biomphalaria glabrata* est dorénavant un modèle d'étude de l'évolution de l'immunité mémoire chez les invertébrés. Ce titre à la fois long et précis demande une grande quantité de concepts. Ces prochaines pages auront pour rôle d'expliquer en quoi les 5 dernières années de travail, depuis mon Master I jusqu'à la rédaction de cette thèse, les expériences, résultats, discussions et théories réfléchis, réalisés et imaginés m'ont amené à penser que *Biomphalaria* peut être considéré aujourd'hui en 2017, comme un très bon modèle pour étudier l'immunité, innée, mémoire, chez les invertébrés.

Ce prologue vise à présenter le cadre général de la recherche du groupe.

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« L'escargot vecteur de la bilharziose intestinale *Biomphalaria glabrata*... »



Figure 1. Cycle de vie du parasite trématode *Schistosoma mansoni* au sein d'un hôte définitif humain puis d'un hôte intermédiaire mollusque *Biomphalaria glabrata*

Figure 1. Le cycle de vie du parasite trématode *Schistosoma mansoni* est hétéroxène. Il nécessite deux hôtes, un hôte définitif vertébré (tel que l'homme) et un hôte intermédiaire invertébré (*Biomphalaria* sp). Un couple de parasites (la femelle vit associée au mâle dans le canal gynécophore de ce dernier) vit dans les mésentères entourant le système digestif. Les œufs doivent gagner le milieu extérieur en traversant différents téguments intestinaux jusqu'à la lumière du tube digestif pour la bilharziose intestinale à *Schistosoma mansoni*. Une fois dans l'environnement extérieur, si les œufs du parasite gagnent l'eau douce, un choc osmotique va provoquer l'éclosion de l'œuf et la libération d'une larve nageante nommée miracidium. Cette forme parasitaire devra trouver un escargot *Biomphalaria glabrata* et essayer de l'infester. S'il y parvient, il pénétrera dans les tissus en perdant ses plaques ciliées qui lui permettaient de nager. Ce miracidium va alors se transformer en Sporocyste primaire (Sp1). Ce Sp1 donnera alors par multiplication asexuée, un troisième stade de développement parasitaire, le sporocyste secondaire (Sp2). Ces derniers, vermiformes, vont alors pouvoir migrer dans les tissus du mollusque pour se rendre à l'interface entre l'hépatopancréas et l'ovotestis, se trouvant dans la partie centrale de la spire de l'escargot. Ces Sp2 vont alors donner un dernier stade de développement appelé cercaire. À partir d'un seul miracidium puis un seul Sp1, plusieurs centaines de Sp2 vont être produits pour des milliers de cercaires. Ces cercaires devront également quitter le mollusque pour regagner le milieu aquatique, pour ce faire elles effectuent une migration inverse à celle des Sp2 pour se retrouver dans le manteau d'où elles sortiront de manière active. Une fois ces dernières dans l'eau, elles seront capables de nager afin de trouver un nouvel hôte définitif vertébré (l'homme). La cercaire peut alors digérer la peau et rejoindre le système circulant. Les différentes cercaires, mâle ou femelle vont alors pouvoir se développer puis se transformer d'abord par une étape intermédiaire la schistosomule, puis vont atteindre le stade adulte, former des couples, et achèveront alors leur maturation sexuelle et pourront commencer à pondre des œufs. Le cycle sera ainsi bouclé.

On considère vecteur « tout objet, plante ou animal qui peut être contaminé par un agent pathogène et être utile à sa transmission » (Institute of Medicine (US) Forum on Microbial Threats 2008). Dans le cadre de la bilharziose intestinale, l'agent pathogène est un vers trématode de la famille des Schistosomatidae, *Schistosoma mansoni* nommé d'après le zooligiste Allemand Weinland en 1858. Ce vers gonochorique possède une vie exclusivement parasitaire nécessitant deux hôtes intermédiaires : un hôte vertébré et un hôte intermédiaire mollusque. Une des particularités de ces parasites repose sur la très grande spécificité d'association, notamment avec leurs hôtes intermédiaires. Le fait d'avoir un spectre d'hôte extrêmement restreint implique directement des pressions de sélection importantes chez les deux protagonistes afin d'entretenir ce couple hôte/parasite. Actuellement entre 252 et 340 millions de personnes sont infectées par toutes les espèces de schistosomes (GBD 2015 Disease and Injury Incidence and Prevalence Collaborators) pour laquelle *Schistosoma haematobium* est le plus problématique (bilharziose urogénitale). Les données épidémiologiques estiment à 0.1 % la mortalité associée (>200 000 morts annuels), affectant davantage les enfants des zones intertropicales africaines. Cette parasitose affecte significativement l'économie des zones touchées en induisant des pertes de productivité entre autres par la diminution de l'endurance (-20 %), la capacité à faire des exercices physiques (-60 %) ou la productivité au travail (-3 à 33 %) (King, Dickman et al. 2005). Des cas de bilharzioses non-endémiques ont été décrits en Corse récemment (Boissier, Grech-Angelini et al. 2016) suggérant que la multiplication des échanges d'hommes et de marchandises autour du globe, associée au réchauffement climatique pourraient redéfinir les limites des zones de transmissions endémiques de nombreuses maladies tropicales jusqu'ici négligées (Kincaid-Smith, Rey et al. 2017).

Les Schistosomes vivent en couple dans les mésentères de l'hôte définitif, les œufs issus de leurs reproductions doivent perpétuer le cycle en s'échappant de leur hôte (Figure 1). Ces derniers sont pourvus d'un éperon, et, lorsqu'ils sont libérés dans le système circulant humain, doivent alors traverser l'endothélium vasculaire et les muqueuses intestinales ou vésicales pour gagner le milieu extérieur. Chez l'espèce *Schistosoma mansoni*, responsable de la Bilharziose intestinale, les œufs passent par le système intestinal pour gagner le milieu extérieur. L'inflammation due à l'évacuation des œufs provoque une hépato-splénomégalie (gonflement inflammatoire du foie et de la rate) et une immuno-suppression localisée chronique qui favorise l'apparition de nombreux cancers (foie, rate, tube digestif). Les œufs n'arrivant pas à traverser se retrouvent bloqués, soit dans le foie à cause de son rôle de filtre sanguin, soit dans la paroi du tube digestif. Ces derniers vont alors être calcifiés et provoquer en raison de l'encombrement mécanique dans les organes, douleurs, diarrhée, anémie et hématurie. À terme, l'altération générale des tissus provoque également l'apparition de cancer du tube digestif, du foie, et finalement la mort.

Évacués dans les selles et les fèces, les œufs gagnant l'eau vont alors libérer

une larve de type miracidium (Figure 1), une larve nageuse capable d'infester activement un second hôte intermédiaire intervenant dans ce cycle de vie complexe. Cet hôte et principal vecteur en Amérique latine est l'escargot d'eau douce : *Biomphalaria glabrata*. L'infection active par le miracidium est trans-tégumentaire par digestion des tissus. À la suite de sa pénétration dans l'escargot (Figure 1), le miracidium va perdre ses plaques ciliées qu'il utilisait pour son déplacement en milieu aquatique, et se transforme alors en un stade sporocyste primaire (Sp1). Ce stade est le premier stade à véritablement se développer dans le vecteur et former un amas cohérent de cellules indifférenciées capables après une dizaine de jours de générer le stade suivant de développement parasitaire au sein de l'escargot : le sporocyste secondaire (Sp2). Plusieurs dizaines de Sp2 vont donc émerger de chaque Sp1, puis grâce à leur structure vermiforme, vont se diriger vers l'apex du corps du mollusque, où se trouvent la glande digestive et la glande reproductrice (appelée également ovotestis). Les Sp2 finissent leur migration à l'interface entre ces deux organes. Une fois localisés au niveau de l'apex du mollusque, ces Sp2 vont pouvoir amorcer une multiplication asexuée intense et produire le dernier stade de développement parasitaire dans l'escargot : la cercaire. Chaque Sp2 est capable de produire plusieurs centaines de cercaires. Ces cercaires vont devoir effectuer la migration inverse des Sp2 pour quitter le mollusque vecteur et rejoindre à nouveau le milieu extérieur aquatique. Parce qu'à partir d'un seul miracidium infestant, il peut y avoir une production de milliers de cercaires pendant plusieurs semaines, on parle de multiplication parasitaire clonale au sein de l'hôte intermédiaire. Le développement du parasite dans l'escargot dure environ 30 à 35 jours. Cette larve nageante cercaire aura pour rôle de trouver un nouvel hôte définitif et de l'infester pour perpétuer le cycle de vie de l'espèce.

C'est de cette problématique épidémiologique que la thématique « Schistosomes » a débuté au laboratoire, il y a plus de 30 ans aujourd'hui. Aujourd'hui, cette recherche s'établit de manière beaucoup plus intégrative, de l'épidémiologie, des tests médicamenteux contre ce parasite, jusqu'à l'étude des interactions immunologiques entre ce parasite et ses hôtes, chez l'homme et chez l'escargot, son hôte définitif et son vecteur.

Biomphalaria se positionne aujourd'hui comme le second vecteur de la bilharziose à travers le monde. Son succès évolutif lui vaut d'être présent à la fois en Afrique et en Amérique dans de nombreuses zones endémiques de bilharziose intestinale et uro-génitale.

L'histoire évolutive de ce couple est par ailleurs très intéressante. En effet, le *Protobiomphalaria*, c'est-à-dire l'escargot évolutivement ancestral de *Biomphalaria*, proviendrait d'Amérique Latine, fort de son succès évolutif, il aurait profité de boues transportées par des oiseaux migrateurs et/ou des radeaux végétaux dérivants sur les océans pour finalement coloniser l'actuel continent Africain (1,8-3,6 Ma (Campbell, Jones et al. 2000)). De son côté, les *Protoschistosoma* sont issus d'Asie,

et vont profiter de conditions climatiques très proches entre le sud-est de l'Asie et jusqu'au sous-continent Indien pour coloniser l'Afrique (1.2-10 Ma (Morgan, Dejong et al. 2005)). *Schistosoma* se développe plutôt dans des escargots d'autres genres qui auraient pu être *Bulinus* ou *Limnea* en Afrique, ces derniers vont alors progressivement être remplacés par des escargots du genre *Biomphalaria* lors de l'arrivée de ces derniers en Afrique. Après quelques millions d'années de co-évolution, ce nouveau *Schistosoma* adapté au *Biomphalaria* Africain va alors être introduit dans les Caraïbes et l'Amérique Latine lors de la conquête du nouveau mode et particulièrement lors de la « Traite Négrière ». Ces schistosomes en Amérique du Sud rencontrent alors un potentiel nouvel hôte, le *Protobiomphalaria* américain qui a donné l'ensemble des espèces Sud-Américaine de *Biomphalaria*. Ce *Schistosoma* africain voit alors devant lui se dresser un nouveau filtre de sélection qu'il réussit tout de même à supplanter et il s'installe alors dans les Caraïbes et l'Amérique Latine.

Cette histoire évolutive atypique va se traduire par des phénotypes d'interactions très particuliers lorsque nous étudions l'interaction entre différentes souches d'hôtes et de parasites et les bases moléculaires sous-jacentes.

« ... dorénavant un modèle d'étude... »

Que signifie modèle d'étude, organisme modèle, modèle biologique ? C'est lorsqu'une espèce, parce qu'étudiée intensément, devient une référence mondiale en termes de base de données et d'outils moléculaires disponibles pour étudier toutes ou parties de son fonctionnement tant au niveau moléculaire qu'au niveau populationnel. Aujourd'hui, la multiplication des thématiques de recherches impose qu'une certaine harmonie, voire une synergie, existe entre différentes communautés, pays et chercheurs pour aller le plus loin possible dans une thématique. La multiplication des thématiques a entraîné avec elle une explosion du nombre de modèles biologiques. L'avènement et la popularisation de nombreuses technologiques d'analyses massives ont également facilité l'apparition d'autant de thématiques que de modèles d'études.

À travers le temps, on pourrait ainsi citer les pois de Gregor Mendel qui à la fin des années 1860 inventait ainsi la génétique (qui portera son nom, Mendélienne) en comparant différents croisements de pois. Ensuite, Ernest Duchesne essayait de comprendre les interactions entre les micro-organismes, en particulier *Escherichia coli* et un *Penicillium* lorsqu'il proposa en 1897 une thèse de médecine sur la « concurrence vitale » qui inspirera Fleming dans la découverte de la pénicilline. Von Behring popularisera lui au début du 20^e siècle l'utilisation des cobayes lorsqu'il découvrit la toxine antidiptérique, avant que Thomas Hunt Morgan fasse de même avec les mouches *Drosophila* lors de la démonstration du support physique de l'héritabilité portée par les chromosomes, quelques années plus tard en 1933. De même du côté de la biologie végétale, l'apparition dans les laboratoires de

l'arabette commune (*Arabidopsis thaliana*) au début des années 1980 signa la centralisation des efforts de séquençage vers le plus petit génome végétal connu à ce jour et la révolution des organismes modèles.

Les avancées majeures réalisées grâce à la centralisation et la concentration des finances et des recherches sur quelques organismes modèles ont plus que révolutionné la recherche. Aujourd'hui, certains groupes de recherches qui veulent transposer des connaissances acquises sur ces modèles se trouvent parfois en difficulté devant l'importance des différences entre leurs organismes et le modèle référant le plus proche. Au point qu'un certain désaveu des organismes modèles peut même se ressentir actuellement, surtout avec l'avènement des technologies de séquençage massif qui permettent en quelques jours et milliers d'euros de produire un effort similaire à celui réalisé par des consortia internationaux durant des années et des millions d'euros pour séquencer par exemple le génome humain.

Il faut tout de même savoir raison garder, et rappeler que la concertation et la concentration de multiple projets vers un organisme modèle permettent de briser l'échelle du temps et de faire progresser la thématique de façon spectaculaire.

Si *Biomphalaria* est dorénavant un modèle biologique, c'est parce qu'il remplit un certain nombre des critères adéquats dont le séquençage de son génome (Adema, Hillier et al. 2017) ou de transcriptomes (Dheilly, Duval et al. 2015, Pinaud, Portela et al. 2016), mais aussi peut-être parce qu'il mérite largement plus, que l'attention qu'on lui porte.

« ...de l'évolution... »

L'évolution est bien plus qu'un concept qui oppose aujourd'hui théistes et athéistes. C'est la force naturelle qu'exerce sur toutes les générations d'organismes vivants, le temps qui passe. Ce temps lorsqu'il est pris à l'échelle géologique, c'est-à-dire de la formation de la planète, de continent ou d'espèces, forge les organismes au grè de leurs interactions et des contraintes qu'ils expérimentent. La question n'est plus de savoir si l'évolution, de Darwin ou de Jay Gould, existe ou n'existe pas, mais bien comment les multiples mécanismes (physiques, chimiques, biologiques, génétiques, etc.) présents aujourd'hui (mais peut-être pas hier ou demain) ont pesé sur les organismes pour en faire ce qu'ils étaient, sont et seront. Même à partir d'un organisme isolé dans un tube, une évolution est observable en laboratoire, lui conférant des capacités parfois bien différentes de celles qu'il avait au départ de l'expérience. Reste que l'étude de l'évolution, et les multiples théories qui ont émergé pour l'étudier, demeure probablement la discipline la plus rassembleuse de la biologie. Elle devrait toujours nous servir pour juger la pertinence biologique de tel ou tel phénomène. En quelques années de travail, il reste souvent très compliqué de pouvoir étudier l'évolution d'un organisme. Mais pour y remédier, il est possible d'utiliser des organismes qui n'ont pas la même histoire évolutive et ainsi confronter leurs compétences, leurs parcours et leurs

armes pour continuer ainsi à projeter nos recherches dans une vision évolutive de la biologie.

Dans notre contexte de recherche, les liens immunologiques qui existent entre *Biomphalaria* et *Schistosoma* nous renseignent sur l'évolution des systèmes immunitaires de *Biomphalaria*, voire même de tous les invertébrés. Ce contexte évolutif nous permet bien souvent de rattacher les résultats et découvertes sur une échelle temporelle pour ainsi comprendre comment les outils sélectionnés par l'évolution chez l'hôte ou chez le parasite ont conduit à façonner les phénotypes d'interactions que nous observons aujourd'hui.

« ... de l'immunité mémoire chez les invertébrés. »

Immunis, pour « exempté » en latin. Utilisé dans l'Empire romain pour les militaires évitant leur service ou ensuite au Moyen-âge pour les affranchis de l'impôt, ce n'est qu'au XIII^e siècle que l'immunité a pris une signification biologique, lorsqu'elle décrira le compartiment de défense d'un organisme qui lui permet de résister à des organismes extérieurs (des greffes aux agents pathogènes) et même des centaines d'années après, expliquer par exemple les rejets de greffes, les cancers, etc.

Cette immunité est en réalité plus complexe à définir, car elle ne devrait pas être décrite comme une arme offensive, mais plutôt comme un mécanisme régulateur comme le définit Thomas Pradeu (Philosophe des Sciences du groupe concept et analyse théorique de l'activation de l'immunité et frontière biologique, Immuno ConcEpT dans l'UMR51164 de l'université de Bordeaux & CNRS). L'immunité ne tue pas les organismes extérieurs pathogènes, elle permet au corps de conserver ses fonctions vitales malgré la présence d'un agent infectieux. Elle se positionne donc à l'interface des relations très étroites qu'entretiennent différents organismes entre eux. L'organisme n'est plus « séparé de son environnement, fermé à ses influences... », « sur le plan immunitaire, organisme et environnement se constituent réciproquement ». Cette définition plus intégrative, a bâti ma réflexion qui a mené le travail de toute cette thèse

Lorsqu'une perturbation d'ordre infectieux n'arrive qu'à troubler de manière transitoire un organisme, celui-ci va survivre. La capacité de conserver alors en mémoire le passage de ce perturbateur pourra à l'avenir lors d'une prochaine rencontre, lui économiser une réponse à large spectre en se focalisant sur ce qui aurait fonctionné lors de la première rencontre. La mémoire immunitaire devient alors une force de sélection pour tous les organismes qui auraient fait de leur survie un élément caractéristique de leur trait de vie.

Ainsi, chez *Biomphalaria*, les premiers phénotypes de résistance acquise par une première infestation vont être observés dès la fin des années 1990. La dénomination du phénomène va rester confuse jusqu'à aujourd'hui : priming, immunisation, résistance acquise, protection induite, mémoire immunitaire, mémoire immunitaire

innée, immunité entraînée. La bataille sémantique est lancée, en parallèle de l'exploration des bases moléculaires de cette résistance.

Aspects fonctionnel et évolutif de l'immunité mémoire chez les invertébrés : l'escargot vecteur de la bilharziose intestinale *Biomphalaria glabrata* comme nouvel organisme modèle ?

Introduction

Compartiments de l'immunité

Un organisme, comme le décrit Thomas Pradeu est un système biologique complexe et cohérent. Il est « ouvert à son environnement et est le produit de ses échanges avec lui (nutrition, respiration, micro-organismes), les barrières de l'organisme (peau, muqueuses, etc...) étant en réalité davantage des lieux d'échanges que d'isolement ». Cette définition permet d'unifier un grand nombre de compartiments et concepts qui sont souvent séparés pour définir un système immunitaire.

L'homéostasie d'un organisme est sa capacité à conserver un niveau physiologique optimal malgré la traversé de nombreux épisodes de stress, qu'ils soient abiotiques ou biotiques, chimiques ou infectieux.

Plusieurs stratégies et organes peuvent offrir à un organisme la possibilité de réguler cette homéostasie dans un environnement complexe et hostile.

Une première stratégie (Figure 2) serait de produire, de la fécondation à la mort, une batterie d'armement lui permettant de résister à des attaques extérieures. Ces caractéristiques pourraient être définies comme constitutives. Ces dernières ne changeront que lentement au long de la vie d'un individu pour faire face à un changement d'environnement. Ainsi l'organisation structurelle de la peau, le potentiel oxydant du milieu intérieur d'un individu (Espèce Réactive de l'Oxygène, ROS) ou son répertoire de peptides antimicrobien n'est pas attendu pour être modifiable.

Ce compartiment possède pourtant un taux de plasticité lui permettant de réguler et d'ajuster au mieux les dépenses énergétiques en cas de stress. Le taux d'expression d'un peptide antimicrobien (Landon, Sodano et al. 1997, Simon, Kullberg et al. 2008) ou la vitesse du catabolisme (Matthews and Battezzati 1993, Gomes-Marcondes and Tisdale 2002) pourraient être les outils d'une réponse adaptable. Cette capacité à moduler une réponse est tout de même limitée en termes de spectre d'action lié à la conservation structurale de la protéine produite. Ces molécules sont, en effet, codées dans les cellules germinales et aucune source de diversification de ces peptides antimicrobiens n'est aujourd'hui connue pour modifier leur séquence primaire et donc leur spectre d'action. Il est tout de même important de noter qu'une mutation des marques associées à l'ARN (épi-mutations), pourrait modifier la lecture de la séquence nucléotidique et donc potentiellement des acides aminés en résultant (Gordon, Satory et al. 2015), affectant potentiellement la structure fonctionnelle de telles molécules dans l'espace.

Figure 2. Exemples de réponses immunitaires

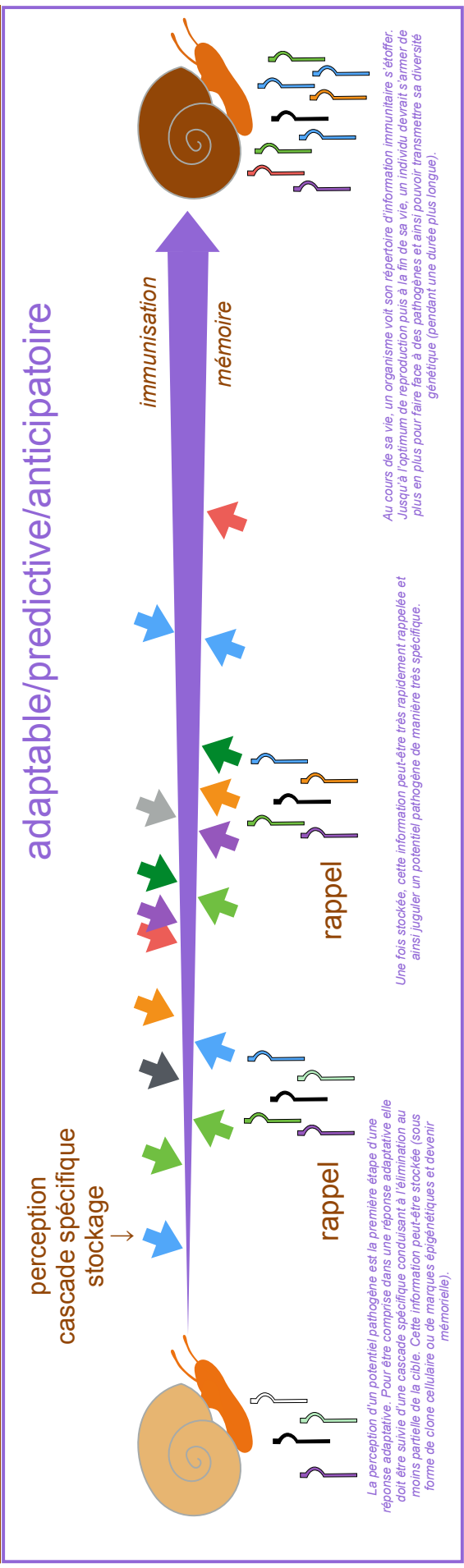
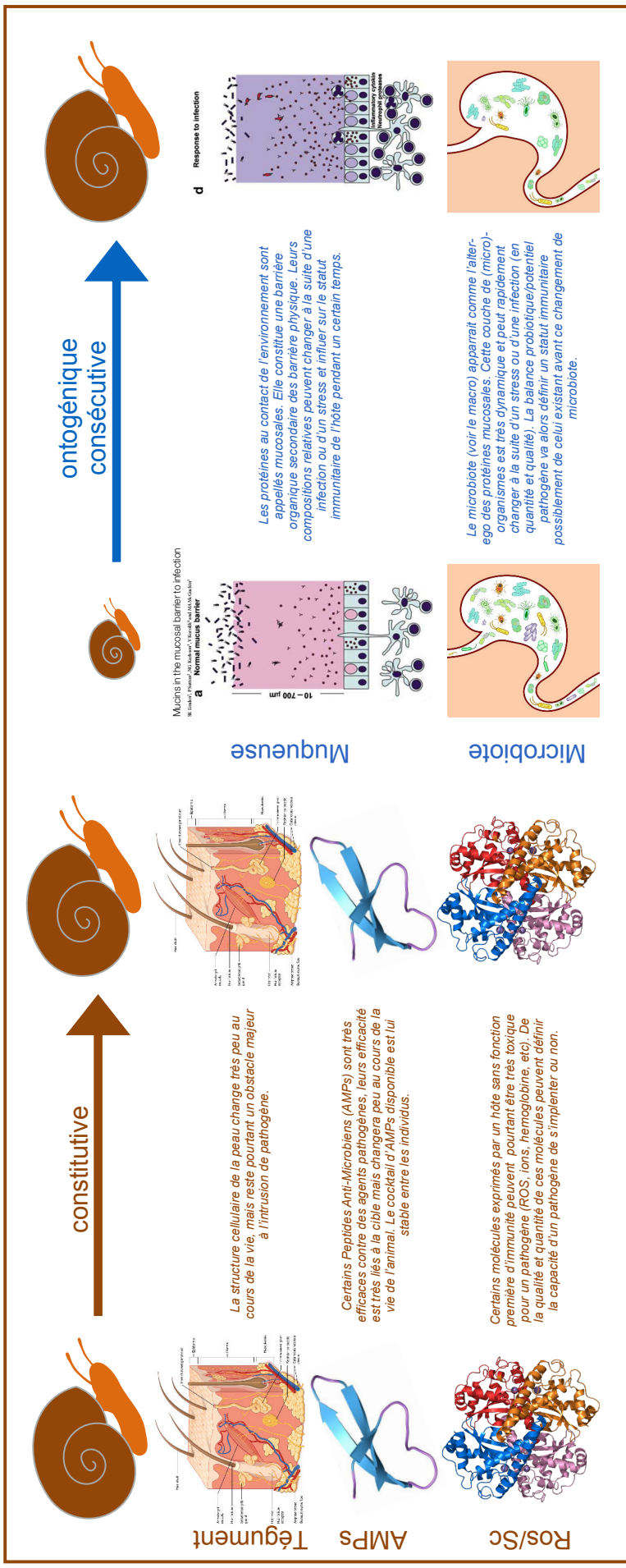


Figure 2. Le système immunitaire apparaît comme un système complexe permettant de structurer un ensemble cohérent de cellules et ainsi le faire se perpétuer malgré une confrontation à des épisodes de stress notamment infectieux. Des stratégies constitutives existent pour essayer de limiter la présence de ces stress infectieux. Par exemple le tégument de la peau constitue une barrière physique utile à la délimitation physique d'un organisme mais également une protection contre des agents infectieux opportunistes qui envahiraient ces denses surfaces d'échanges. La production de certains peptides antimicrobiens (AMP) ou d'espèces réactives de l'oxygène ou de l'azote (ROS et NOS) pourra alors définir de manière constitutive un statut immunitaire. Dans l'interaction Bg/Sm particulièrement, une histoire évolutive entre l'expression de ROS et de molécules de détoxification (ROS-scavenger) existe chez différents couples du laboratoire. Des réponses consécutives peuvent être également observées uniquement en réponse à un stress et sous la pression du système immunitaire. Ça peut être le cas d'une modification de protéines mucosales du tube digestif et ainsi modifier le microbiote associé et le statut immunitaire de l'hôte. Parmi les réponses consécutives les plus connues on peut citer la production de nombreuses molécules toxiques capables de limiter la propagation d'un agent infectieux. Une fois éliminée, la molécule en question va être traitée par le catabolisme et le système immunitaire ne conservera pas la trace de l'activité de cette molécule contre cet agent infectieux. C'est ce qui sépare une réponse consécutive d'une réponse anticipatoire telle qu'on l'imagine dans le système adaptatif des vertébrés. À la suite d'une rencontre avec un pathogène, l'hôte met en place une réponse dont il est capable de se souvenir lorsque celle-ci est performante. Lors d'une nouvelle rencontre avec ce même pathogène, l'hôte est alors capable de produire plus rapidement et en plus grande quantité cette molécule précise et ainsi limiter les dégâts que pourrait causer cet agent pathogène précédemment rencontré.

Les surfaces d'échanges telles que les muqueuses épithéliales, intestinales et respiratoires, peuvent être également considérées comme des organes importants dans l'immunité. Une des manifestations observées consécutivement à un stress infectieux est le changement de composition des protéines de la muqueuse intestinale. Cette région de l'organisme, très riche en surface d'échanges maintient une communauté d'organismes vivants très différente pour lesquels un bénéfice réciproque (entre l'hôte et la communauté) existe. Cette communauté est souvent appelée microbiote (dans les cas où ces organismes sont microscopiques) ou macrobiote (macroscopique) et forme de manière étendue un organisme issu de l'assemblage de plusieurs espèces le constituant, un holobionte. Les muqueuses, centres de régulation du microbiote, peuvent par exemple produire *de novo* des nouvelles protéines structurales Muc5ac pour remplacer des Muc2 lors d'une réponse à un nématode (Hasnain, Gallagher et al. 2013). Celles-ci ont des propriétés physiques, chimiques et biologiques très différentes et peuvent changer pendant une longue période le statut immunitaire de cet organisme (possibilité de résister à un stress infectieux).

Ces stratégies de régulation d'un stress infectieux, pourraient être qualifiées d'ontogéniques, car elles sont le résultat du développement depuis la naissance de l'organisme auquel s'ajoute chaque modification consécutive à un stress infectieux. Ces compartiments permettent donc des réponses consécutives, à la suite d'un stress pour réguler les communautés bactériennes via la modification de protéines mucosales par exemple.

Si l'on regarde l'homéothermie, le contrôle de la température du corps, lorsque la température interne du corps augmente, celui-ci produit de la transpiration qui permet de réguler l'apport calorifique à la surface de la peau et donc de le refroidir. Cette réponse est consécutive à l'augmentation de la température corporelle, il n'y a pas d'anticipation saisonnière à la transpiration. Même chose pour le bronzage, la pigmentation de la peau est le résultat de l'exposition au soleil. Le corps, pour protéger les organes profonds, produit de la mélanine dans l'épiderme pour limiter l'impact nocif des rayons ultra-violet. Cette réponse est consécutive à l'exposition au soleil. La modification de mucines respiratoires ou gastriques pourrait être comparée à ces phénomènes, mais cette fois suite à un stress infectieux.

Ces différents compartiments et stratégies de réponses à un stress infectieux, montrent la complexité de régulation d'un système immunitaire. Si historiquement, on pensait que l'immunité n'était supportée que par des cellules et des organes spécialisés, de plus en plus de travaux montrent que l'homéostasie d'un organisme nécessite une communication entre de nombreux organes et compartiments de l'organisme où l'immunité pourrait avoir un rôle déterminant.

Pourtant, ces changements consécutifs, s'ils peuvent améliorer la fitness

relative d'un organisme en lui permettant de gérer au mieux un stress infectieux, de par leur nature non-anticipatrice, ne devraient pas être transmis à la descendance. La transmission à la descendance via n'importe quel support pourrait pourtant améliorer de manière anticipée une réponse immunitaire, que ce soit par la culture (éviter un contact avec un pathogène), socialement (épouillage chez les singes), physiquement (bactérie utérine maternelle), épigénétique (petits ARN ou long ARNnc), ou protéique (antigène ou LPS). De nombreuses transmissions de la sorte ont été montrées et révèlent la pression évolutive qui pèse sur ces organismes et l'extraordinaire complexité des mécanismes qui se sont développés pour potentiellement transmettre des informations sur des pathogènes à travers des générations.

Adaptable vs adaptatif

Thomas Pradeu dans « l'immunité et l'interactionnisme biologique » définit la notion même d'un organisme, et considère que l'immunité ne serait qu'un mécanisme conciliateur : un ensemble cohérent de processus qui dirige un ensemble de cellules très différentes pour maintenir l'intégrité de l'individu, survivre et avoir potentiellement une descendance. Alors s'il est faux de considérer que toutes les réponses immunitaires mises en place par des animaux ayant eu un succès évolutif sont forcément adaptatives (apparition il y a 500 Millions d'années pour les mollusques, 320 pour les mammifères), on peut observer aujourd'hui que de plus en plus de résultats montrent que l'ensemble des organismes vivants possèdent un système immunitaire sûrement plus complexe que décrit actuellement (Discussions et Réflexions).

Mais la dénomination « adaptative » possède bien plus de sens que ne le suggère sa propre racine latine (*adaptatio*, résultat de l'action). En effet, la réponse adaptative est clairement maintenant passée sous le giron des immunologistes et leur terminologie vertébré-centrée qui implique : des lymphocytes B producteurs d'anticorps, des cellules dendritiques présentatrices d'antigènes (CPA), une sélection clonale des cellules lymphocytaires compétentes (CD4+) qui entraîne la prolifération de cellules immunocompétentes capables de porter le déterminant de pathogènes spécifiques ou de produire un récepteur approprié (CD8 ou Tc) et la nécessité de conserver ces cellules pour conserver la marque et la mémoire de cette confrontation. La sélection clonale des immunoglobulines V(D)J permet de générer une quantité de variants d'anticorps supérieure à la diversité des facteurs de pathogènes attendus, ce qui en fait le mécanisme majeur pour définir cette immunité comme anticipatoire.

Cette définition prive *de facto*, les invertébrés, les plantes et les procaryotes d'une réponse immunitaire « adaptative ». La présence d'orthologues de RAG1/2 chez les invertébrés ne suffit néanmoins pas à la production de recombinaisons similaires au système V(D)J chez ces organismes ; l'activité propre au système

immunitaire adaptatif ayant probablement été acquise à posteriori chez les vertébrés (Fugmann, Messier et al. 2006). Néanmoins, un certain nombre de réponses immunitaires chez les invertébrés et les vertébrés, sans faire appel à cette machinerie, peuvent être qualifiées d'adaptables, de prédictives, d'anticipatoires, de prospectives.

L'ensemble des êtres vivants sur la planète sont exposés à des pressions de sélection liées à leur capacité à se défendre, façonnant ainsi leurs systèmes immunitaires. Leurs systèmes immunitaires, quoique très dissimilaires doivent sélectionner des processus aux résultats très semblables. Ce ne serait donc pas le fait de posséder une armée de cellules spécialisées telle que définies par l'immunité adaptative des vertébrés ; mais d'être capable de reconnaître spécifiquement un pathogène, de l'éliminer, de garder la trace de son passage pour mieux y répondre à l'avenir, l'éliminer plus rapidement lors d'une seconde/troisième/nième rencontre, survivre et transmettre ses caractères de résistance à sa descendance. Imaginons une convergence évolutive pour un phénotype donné, où quel que soit le support de cette information (génétique, épigénétique, protéique), une réponse immunitaire adaptative et mémorielle puisse exister *sensu-stricto* : garder en mémoire la trace d'un pathogène.

Si l'immunité des mollusques, insectes ou bactéries n'est pas adaptative, mais qu'elle peut par certains traits être tout de même anticipatoire et/ou prédictive, alors on pourrait alors imaginer que différentes formes d'immunité adaptative puissent exister.

C'est-à-dire que l'organisme anticipe, prédit, prévoit qu'il pourrait être nécessaire de se défendre contre tel ou tel pathogène avec lequel il possède une histoire évolutive (parce qu'il partage la même niche écologique par exemple), cela sous-entend qu'il le recroisera sûrement au cours de sa vie, et qu'une réponse mémorielle sera plus efficace, car il a déjà produit par le passé une réponse efficace.

Anticipatoire *alias* adaptatif

Chez les invertébrés en particulier, le lien entre cellules spécialisées et réponse immunitaire s'articule surtout autour de capacités intrinsèques de ces dernières, comme une augmentation de leur activité de phagocytose (Ramirez, Garver et al. 2014, Yang, Shi et al. 2016), de leur différenciation (Rodrigues, Brayner et al. 2010), ou encore une augmentation de production de molécules toxiques (Lamiable, Arnold et al. 2016, Smith, King et al. 2016). Seules les catégories cellulaires de la réponse innée des vertébrés ont été caractérisées chez certains invertébrés. Une prolifération clonale de cellules portant un déterminant de reconnaissance spécifique (« anticorps-like ») chez ces derniers n'ayant jamais été caractérisée, ceci suggère de la part des immunologistes l'absence d'un système immunitaire adaptatif mémoriel.

Comment des organismes dépourvus de système immunitaire adaptatif (mais avec un succès évolutif semblable) sont capables de percevoir efficacement, c'est-à-dire rapidement et spécifiquement, la présence d'un pathogène (d'un acide nucléique viral à un antigène de parasite métazoaire) ? Il se pourrait que les mêmes pressions de sélection concourent à générer les mêmes adaptations chez des organismes phylogénétiquement très distants. Ainsi cela aurait pu pousser l'émergence de mécanismes mémoriels très différentes de ceux connus chez les vertébrés chez des organismes très différents.

CRISPR-Cas9

Ce système de surveillance et de mémoire immunitaire des procaryotes n'est pas retrouvé chez les eucaryotes et constitue une exception dans le règne du vivant. Il suffit à une seule bactérie d'être parvenue à achever l'intégralité de la voie CRISPR-CAS9 contre un pathogène pour conférer à l'ensemble de la colonie en devenir (sa descendance), une résistance contre celui-ci.

Les courtes répétitions palindromiques groupées et régulièrement espacées (Clustered Regularly Interspaced Short Palindromic Repeats), sont des morceaux génomiques qui sont retrouvés dans plus de 80 % de génome d'archées et 40 % de bactéries (Grissa, Vergnaud et al. 2007). Plus que de simples séquences d'ADN répétées, ces séquences correspondent à des portions de génomes viraux qui ont été intégrées aux génomes grâce à une endonucléase complémentaire Cas9, et ainsi générer le processus mémoriel. Ce système CRISPR-Cas9 s'impose véritablement aujourd'hui comme une forme d'immunité adaptative chez les procaryotes (Koonin and Makarova 2009, van der Oost, Jore et al. 2009, Marraffini and Sontheimer 2010, Terns and Terns 2011). Lorsqu'un phage viral infecte une bactérie (ou de manière synthétique sans phage), le double brin d'ADN ou d'ARN viral est reconnu par une endonucléase de type Cas1 ou Cas2 (Gasiunas, Sinkunas et al. 2014) pour démarrer le processus de mémorisation. Ces endonucléases vont reconnaître spécifiquement l'ADN/ARN étranger *via* des motifs PAM (protospacer-adjacent-motif) qui ne sont présents que dans le génome de la bactérie hôte (Gasiunas, Sinkunas et al. 2014), cliver des courtes séquences virales et les incorporer directement dans le génome bactérien. Cet ADN exogène incorporé est appelé « spacer ». La transcription de ces spacers donnera lieu à la production du CRISPR-RNA (crRNA). Ce guide crRNA lorsqu'il est pris en charge par une enzyme de type CAS9 est capable, de scanner des doubles brins d'acide nucléique, différencier l'endogène de l'exogène et détruire l'intrus lorsque la complémentarité guide/cible est établie.

Ici encore la notion de système immunitaire lorsque nous y incorporons le mécanisme de CRISPR-Cas9, ne reste pas cantonné à un rôle d'attaque contre les virus lytiques, mais un mécanisme de cohérence et de structure permettant à une colonie bactérienne d'évoluer et de perdurer malgré la présence continue d'un

agent infectieux dans son environnement. Cette forme d'immunité adaptative et mémorielle (à l'échelle de la colonie bactérienne) est produite par une seule cellule, sans l'obligation d'un organe dédié, un compartiment ou une population cellulaire spécialisée. Ces deux notions complexifient ainsi les contours de l'immunité adaptative à travers le vivant.

Immunité mémoire innée vs immunité mémoire

L'immunité mémoire, telle qu'elle est définie dans le cadre de la réponse adaptative des vertébrés repose sur la production somatique d'une diversité anticipée d'anticorps plus grande que la diversité de pathogène attendue, sa conservation puis sa réutilisation lors d'une rencontre future. Ainsi, chaque organisme est attendu pour avoir ses propres clés pour être individuellement résistant. Lorsque des organismes (bactéries, plantes, invertébrés) comme décrit précédemment sont capables de se souvenir d'une voie de réponse spécifique à un pathogène et d'y répondre plus vite et plus efficacement dans le futur (Henry, Yadeta et al. 2013, Gasiunas, Sinkunas et al. 2014, Pinaud, Portela et al. 2016), s'il n'y a pas l'utilisation des dits anticorps, on qualifiera cette réponse de mémoire immunitaire innée.

Au final, le système adaptatif des vertébrés est un système à la fois adaptatif et anticipatoire. Il est capable à la fois d'engendrer une réponse spécifique très précise contre un pathogène, mais aussi d'anticiper une diversité antigénique de pathogène grâce à son système V(D)J puis de sélectionner le variant compétent pour le garder en mémoire et s'en resservir si besoin est. Certaines espèces présentent des caractères immunitaires adaptatifs lorsque d'autres en présentent des anticipatoires. Aucun invertébré pour autant ne montre la combinaison des deux stratégies qui confirmerait un système adaptatif chez les invertébrés tel qu'il est aujourd'hui connu chez les vertébrés. Bien que mémorielle, l'immunité des invertébrés ne semble dépendre que du système immunitaire inné.

Quoique cette dénomination sémantique innée/adaptative soit aujourd'hui peut-être insuffisamment précise, elle est toujours la nomenclature la plus répandue et encore retenue.

Explorons alors ce système immunitaire constitutif, ontogénique, consécutif, adaptable, prédictif, anticipatoire, entraînable et mémoriel. Mais inné.

Support moléculaire de l'interaction *Biomphalaria glabrata* – *Schistosoma mansoni*

Reconnaissance immunitaire chez les invertébrés

« Regarde attentivement, car ce que tu vas voir n'est plus ce que tu viens de voir »
Leonardo da Vinci

La perception du monde qui l'entoure est le défi constant que n'importe quel organisme doit affronter tout au long de sa vie. La détection d'un changement de cet environnement lui permet de répondre aux différents stress aussi rapidement que possible et ainsi continuer à vivre dans la meilleure synergie possible avec son environnement.

La première étape de la vie impose un développement ontogénique, qui permet de créer tous les organes, les structurer et les faire communiquer ensemble. Cela intègre par exemple la formation d'un tégument très résistant pour la plupart des animaux (composé d'une couche complexe de cellules et de protéines variées) et capable de séparer physiquement la majorité de l'organisme de son environnement direct, la création de surfaces d'échanges dédiées (tube digestif, muqueuse en général, peau chez les animaux). Mais, si l'arme la plus efficace est une barrière physique, dans le cas où un agent pathogène (bactérien, virus dans un phage, parasite) arriverait à rompre ces barrières, il faut alors être en mesure de proposer un système de défense interne efficace capable de reconnaître très spécifiquement les différents ennemis en présence et d'y répondre. Une meilleure efficacité possible suggère que la perturbation associée doit être la plus faible possible envers l'homéostasie générale.

La perception d'un agent pathogène constitue donc un élément central du contrôle de l'homéostasie immunitaire.

Au début des années 1980, l'exploration du système immunitaire des invertébrés se concentre sur la caractérisation d'abord de peptides antimicrobiens tels que les cécropines (Qu, Steiner et al. 1982, Steiner 1982, Andreu, Merrifield et al. 1983, Dalhammar and Steiner 1984, Boman, Faye et al. 1985) ou les attacines (Hultmark, Engstrom et al. 1983, Lee, Edlund et al. 1983, Kockum, Faye et al. 1984, Boman, Faye et al. 1985).

Mais un virage scientifique se produisit à la fin des années 1980 quand les groupes strasbourgeois du Pr Jules Hoffman et ses collaborateurs (C. Hertru, JM. Reichhart, P. Bulet, J. Royet, B. Lemaitre, EA Levashina, E. Nicolas, JL. Imler, D. Ferrandon, ...) commencèrent à décortiquer les toutes premières bases moléculaires des voies de réponses immunitaires chez la mouche du vinaigre *Drosophila melanogaster*.

Ils découvrirent successivement la diptericin (Dimarcq, Keppi et al. 1988, Reichhart,

Essrich et al. 1989, Wicker, Reichhart et al. 1990, Reichhart, Meister et al. 1992, Kappler, Meister et al. 1993, Meister, Braun et al. 1994, Georgel, Kappler et al. 1995, Gross, Georgel et al. 1996), la defensin (Dimarcq, Zachary et al. 1990, Bulet, Cociancich et al. 1991, Lepage, Bitsch et al. 1991, Reichhart, Georgel et al. 1993), l'équivalent non-mammifère de NF- κ , le kappa B-related (Reichhart, Georgel et al. 1993), la metchnikowin (Levashina, Ohresser et al. 1995), la thanatin (Fehlbaum, Bulet et al. 1996) ou encore les premières cystéines-rich (Charlet, Chernysh et al. 1996, Ehret-Sabatier, Loew et al. 1996). Ces premiers résultats ont permis d'établir les premières voies immunitaires chez cette mouche et de servir de socle pour les analyses comparatives avec les autres modèles (Hoffman-Falk, Einat et al. 1983, Muskavitch and Hoffmann 1990). L'utilisation de mutants obtenus par transgénèse a également permis de préciser le rôle de chacun comme l'immune defency (*imd*) (Lemaitre, Kromer-Metzger et al. 1995, Georgel, Naitza et al. 2001) et le gène cassette *spätzle/Toll/cactus* (Lemaitre, Nicolas et al. 1996, Levashina, Langley et al. 1999, Imler and Hoffmann 2000).

Ces premières données ont alors permis d'explorer plus en détail la régulation de ces différentes armes de la réponse immunitaire et la maîtrise de la biologie cellulaire moderne y jouera un grand rôle (Ferrandon, Jung et al. 1998, Jung, Criqui et al. 2001). Rapidement, la spécificité contre différents agents pathogènes a été décrite et leurs régulations finement analysée (Basset, Khush et al. 2000, Imler and Hoffmann 2003, Kurz, Chauvet et al. 2003), tout comme le rôle potentiel des différents organes immunitaires (Tzou, Ohresser et al. 2000, Hoffmann and Jagla 2002, Kambris, Hoffmann et al. 2002).

Les connaissances sur la régulation de cette réponse immunitaire s'étoffent rapidement et la caractérisation des interactions entre les différentes voies précédemment décrites commence à se préciser (Tauszig-Delamasure, Bilak et al. 2002, Gobert, Gottar et al. 2003, Weber, Tauszig-Delamasure et al. 2003, Bischoff, Vignal et al. 2004, Ferrandon, Imler et al. 2004, Royet, Reichhart et al. 2005, Gottar, Gobert et al. 2006).

Au cours de cette décennie, ces découvertes ont permis de redéfinir les bases de l'immunité et plus particulièrement celle qui ne fait pas intervenir la genèse des anticorps somatique, la réponse immunitaire innée.

Si la plupart des réponses étudiées concernaient majoritairement les mécanismes contre des procaryotes, la réponse antivirale n'a pas été éludée (Dostert, Jouanguy et al. 2005, Deddouche, Matt et al. 2008, Eleftherianos, Won et al. 2011, Kemp, Mueller et al. 2013, Lamiable, Arnold et al. 2016). Ces progrès réalisés sur le modèle *Drosophila* ont permis d'améliorer en parallèle l'ensemble des travaux et des connaissances des scientifiques travaillant sur les modèles insectes puis aussi sur les autres modèles d'invertébrés (Barillas-Mury, Charlesworth et al. 1996, Charlet, Chernysh et al. 1996, Fehlbaum, Bulet et al. 1996, Lowenberger, Ferdig et al. 1996, Lowenberger, Smartt et al. 1999, Christophides, Zdobnov et al. 2002).

Presque 20 ans après les premiers résultats, ces travaux ont alimenté bien

plus que la recherche sur l'immunité des invertébrés (Hoffmann, Kafatos et al. 1999, Imler and Hoffmann 2000, Michel, Reichhart et al. 2001, Gottar, Gobert et al. 2002, Gobert, Gottar et al. 2003, Ferrandon, Imler et al. 2004, Gottar, Gobert et al. 2006).

En 2011, l'Académie Nobel récompensa Bruce A. Beutler (UTSoutherwestern, Texas, USA) et Jules A. Hoffman (Institut de Biologie Moléculaire et Cellulaire, Strasbourg, France) comme co-récipiendaire (1/4+1/4) de leur prix en Physiologie ou Médecine pour leurs travaux sur l'immunité innée (Beutler découvrant le Toll like receptor 4 (TLR4) majoritairement sur des cellules humaines) qu'ils partagent avec Ralph M. Steinman (1/2) (Harvard Medical School, Boston, USA) qui découvrit les cellules dendritiques et leurs rôles dans l'activation de la réponse immunitaire adaptative.

Colossaux, ces travaux ont permis d'établir les fondements de la réponse immunitaire innée chez les invertébrés puis chez les vertébrés. La première étape s'articule toujours autour de la reconnaissance immunitaire de facteurs de pathogènes. Ces molécules de reconnaissance, souvent appelées PRRs, pour Pattern Recognition Receptors, (récepteurs de reconnaissance de motifs moléculaires), englobent une grande famille de molécules très différentes partageant une fonction commune, la liaison à des molécules de pathogènes souvent nommées PAMPs (Pathogen-Associated Molecular Patterns). Les PRRs se différencient tout d'abord par leurs localisations. En effet, certains sont portés par des cellules et incorporés à leurs membranes, lorsque d'autres sont retrouvés dans le système circulant. Parmi les PRR membranaires, différentes classes sont retrouvées solubles des plantes aux animaux. Les récepteurs semblables aux Tolls (Toll-like receptors TLR) et ceux possédant un domaine C-type lectin (C-type lectin receptors CLR).

Les TLR ont porté la recherche sur l'immunité innée pendant de longues années et leurs découvertes ont révolutionné la façon de conceptualiser la réponse immunitaire. Aujourd'hui, de nombreux TLR (TLR1-TLR23) ont été caractérisés par la présence d'un domaine extracellulaire contenant le récepteur composé de motifs riches en répétition de leucines (LRR), un domaine transmembranaire pour se lier à la cellule spécialisée de l'immunité et un domaine intracellulaire activateur de la transduction du signal. La nature même des motifs LRR entre autres, est à l'origine d'une spécificité de reconnaissance. Ainsi, les TLR9 sont capables de se lier à des molécules exogènes comme de l'ADN viral, alors que les TLR 3 et 5 s'associent à de l'ADN double brin et à une protéine bactérienne de type flagellin (Akira and Takeda 2004, Ferrandon, Imler et al. 2004, Imler and Zheng 2004, Pasare and Medzhitov 2004).

Aujourd'hui, des arguments génomiques suggèrent que ces gènes Toll sont en réalité partagés en trois classes avec des trajectoires évolutives très différentes dictées par un rôle physiologique primordial (définir la polarité dorso-ventrale des mouches par exemple, (Anderson, Jurgens et al. 1985)) prenant le pas sur leur rôle dans la réponse immunitaire. Les orthologues de Toll-3/4 montrent chez différentes

espèces qu'ils ont subi des pressions de sélections diversifiantes pour leur rôle dans l'immunité lorsque les Toll qui ne possèdent pas de rôle immunitaire restent sous sélection purifiante (Levin and Malik 2017).

Bien plus que la faible diversité de reconnaissance portée par un seul PRR et son arrangement de LRR, c'est la combinaison possible de différentes quantités et variétés de TLR qui semble permettre d'augmenter les capacités de reconnaissance chez les organismes pourvus de TLR (Gangloff 2012). De nombreux chercheurs dont Ruslan Medzhitov proposent d'ailleurs que les interactions physico-chimiques de ces molécules rendent possible des associations plus combinatoires qu'aléatoires, permettant d'offrir à ces organismes des possibilités de reconnaissance bien supérieures à celles qui pouvaient être attendues auparavant (Medzhitov and Janeway 2000, Medzhitov and Janeway 2000, Medzhitov and Janeway 2000, Mushegian and Medzhitov 2001, Barton and Medzhitov 2002, Pasare and Medzhitov 2004, Hargreaves and Medzhitov 2005). Néanmoins, l'implication de ces molécules dans la reconnaissance immunitaire (c'est-à-dire l'action de lier un facteur d'un pathogène de manière spécifique) se résume très souvent pour un grand nombre de modèles à la simple observation de l'expression au niveau transcriptomique de ces facteurs de reconnaissance présumés. Cette absence de validation au niveau des interactions protéines/protéines laisse présager une complexité encore supérieure dans la manière qu'est réalisée la reconnaissance de pathogène chez les invertébrés. C'est dans ce sens que notre groupe a proposé une méthode simple pour explorer les molécules de reconnaissance chez *Biomphalaria glabrata* (Chapitre 1).

Les CLR, pour « C-Type lectin receptors », sont des lectines possédant une forte affinité pour les carbohydrates (CBP, carbohydrate-binding protein) dépendante du calcium. Ces CLR possèdent des fonctions très variées comme l'adhésion entre cellules, la reconnaissance immunitaire ou l'apoptose. Ce rôle étant intimement lié avec leur localisation cellulaire : membranaire ou soluble (Drickamer 1999, Geijtenbeek and Gringhuis 2009, Dambuza and Brown 2015, Pees, Yang et al. 2016). Deux sous-classes ont été caractérisées en fonction du type de récepteurs de ces CLR. On distingue ainsi les porteurs de domaine ayant une affinité pour le mannose nommé récepteur du mannose alors que les autres liants plutôt le galactose sont nommés AR : « asialoglycoprotein receptor family ».

Concernant les CLR caractérisés dans la reconnaissance immunitaire, leurs fonctions dépendent fortement de la nature des différents CLR activés, mais également de leurs interactions réciproques donnant lieu à des dimérisation/mulimérisation permettant d'augmenter les capacités de reconnaissance et de signalisation pour la cellule (Testi, Pulcinelli et al. 1990, Lopez-Botet, Perez-Villar et al. 1997, Mizuno, Fujimoto et al. 1997, Kastrup, Nielsen et al. 1998, Boyington, Riaz et al. 1999, Wang, Kong et al. 2001, Watson, Christou et al. 2009, Su, Singh et al. 2010, Keebaugh and Schlenke 2012).

Concernant les PRRs, ces derniers ne sont pas exclusivement associés aux

membranes cellulaires, deux familles importantes existent : les NOD-like récepteurs (NLR) et les RIG-I-like récepteur (RLR). Les premiers ont été décrits pour leur implication dans la réponse apoptotique et inflammatoire (Inohara and Nunez 2003, Inohara, Chamaillard et al. 2005, Strober, Murray et al. 2006). Ces protéines sont, en effet, capables de générer des oligomères pour activer des voies de caspases impliquées elles-mêmes dans des voies d'activation de cytokines (IL-A, par exemple ou NF-kB). Les RLRs sont quant à eux impliqués dans les réponses antivirales en se liant aux ARN viraux et plus particulièrement à ceux portant des marques 5'-triphosphate.

Des PRR et d'autres acteurs de l'immunité innée ont également été décrits au coup par coup chez d'autres espèces ou d'autres groupes comme la voie de réponse Jak-STAT (Darnell, Kerr et al. 1994, Leung, Qureshi et al. 1995), la voie de la mélanisation (Ashida, Kinoshita et al. 1990), les Toll d'*Anopheles* (Luo and Zheng 2000, Christophides, Zdobnov et al. 2002, Luna, Wang et al. 2002, Imler and Zheng 2004), d'*Aedes* (Luna, Hoa et al. 2003) ou la voie IMD de *Tribolium* (Zou, Evans et al. 2007). D'autres molécules de reconnaissance ont été décrites notamment chez les plantes où des orthologues de PRR et plus précisément de Toll ont été caractérisés (Ausubel 2005, Kagan 2014).

En la matière, l'escargot *Biomphalaria glabrata* a vu rapidement augmenter la caractérisation d'un grand nombre de molécules de reconnaissance immunitaires, orthologues d'autres invertébrés. Ainsi, les Toll (Zhang, Zeng et al. 2007, Zhang and Coultas 2011, Sullivan and Belloir 2014, Pila, Tarrabain et al. 2016) et les premières lectines ont été caractérisées (Zhang, Adema et al. 2004, Hanington, Forys et al. 2010).

Si la recherche de molécules homologues entre différentes espèces a permis d'avancer rapidement sur les grandes fonctions immunitaires pour la plupart des organismes non-modèles, la découverte de molécules spécifiques à chaque genre a été moins explorée. Or, ces molécules de reconnaissance sont nombreuses chez les invertébrés et sont presque toujours caractéristiques de chaque genre (Dscam d'Arthropode, les Biomphalysines de *Biomphalaria*, etc). Certaines de ces molécules sont hautement polymorphes et diversifiées ce qui était inattendu jusqu'à lors pour une réponse immunitaire innée.

Les clés de l'immunité anticipatoire chez *Biomphalaria*

« Anticiper le malheur sans faire partager les affres de l'anticipation, là est le véritable héroïsme » Daniel Pennac

Certains invertébrés, au même titre que les vertébrés, sont capables de générer des molécules immunitaires si diversifiées qu'ils seraient ainsi capables de répondre à la grande diversité des déterminants moléculaires de pathogènes qui leur font face. Ceci étant le propre de ce que l'on peut attendre d'un système adaptatif. La qualité extraordinaire du système immunitaire adaptatif des vertébrés est de produire un répertoire de molécules de reconnaissance, évolutivement éprouvées, comme étant plus diversifiées que la diversité de pathogènes qu'ils vont rencontrer. La sélection et l'adaptation d'un système moléculaire complexe permettant de créer à partir de quelques gènes de la lignée somatique, une grande diversité de molécules potentielles de l'immunité devient alors un argument important de la présence d'un véritable système adaptatif, si cette diversité peut être sélectionnée fonctionnellement pour être incorporée dans une réponse à long terme. Ainsi chez de nombreux organismes, des molécules hautement variables et/ou polymorphes ont été caractérisées : les VLR (Variable Lymphocytes Receptors, (Pancer, Amemiya et al. 2004) d'Agnathes, les VBCP (Variable Chitin-binding protein, (Cannon, Haire et al. 2002, Cannon, Haire et al. 2005, Dishaw, Mueller et al. 2008)) d'Amphioxus, les SRCR (Scavenger receptor Cystein-Rich, (Pancer 2000)) et SP185/333 d'oursins ou les Dscam (Down Syndrom Cell Adhesion Molecules, (Yamakawa, Huot et al. 1998, Agarwala, Nakamura et al. 2000, Schmucker, Clemens et al. 2000, Agarwala, Ganesh et al. 2001) d'arthropodes jusqu'aux Fibrinogen-related proteins (FREPs) de mollusque et plus précisément de *Biomphalaria* (Monroy, Hertel et al. 1992, Hertel, Stricker et al. 1994, Leonard, Adema et al. 2001, Zhang, Leonard et al. 2001, Zhang and Loker 2003, Zhang, Adema et al. 2004). Pour FREP3 par exemple, lorsqu'on s'intéresse à son domaine Immunoglobuline (IgSF1), deux individus ne partagent qu'une version commune d'ADNc parmi leurs 45 et 37 versions respectives d'ADNc. Puisque chaque membre de la famille des FREP n'étant constitué que d'1 à 5 loci et qu'il n'y a qu'un seul exon qui constitue ce domaine, cette diversité pourrait reposer sur des mécanismes de recombinaison ou de fusion au niveau transcriptionnel (trans-splicing) (Leonard, Adema et al. 2001, Zhang and Loker 2003, Zhang, Adema et al. 2004). Un grand nombre de points chauds de mutation indique également un niveau supplémentaire de diversification de ces molécules (Zhang, Adema et al. 2004).

Chez de nombreux organismes, des mécanismes très différents ont été sélectionnés pour générer de la diversité sur ces récepteurs potentiels de l'immunité, présageant de pressions de sélection fortes et ciblées sur ces familles de molécules (Armitage, Kurtz et al. 2017).

La tentation de comparer ces familles à des nouvelles familles de récepteurs

immunitaires semblables aux anticorps de vertébrés est grande, malgré le manque de validation fonctionnelle de ces familles. Aujourd'hui, malgré le fait qu'un grand nombre de travaux montre une implication de ces familles dans le cadre d'une réponse immunitaire, leur rôle fonctionnel de reconnaissance est toujours fortement controversé. En effet, il est envisageable que des molécules, parce que nécessaires aux pathogènes pour leur pénétration/évasion dans l'hôte ou bien dans l'organisation et la mise en place du système nerveux et des neurones de l'hôte, comme les DScam par exemple, aient subi des pressions de sélection importantes conduisant à de la diversification et/ou du polymorphisme. Ceci ne signifie pas pour autant qu'elles soient impliquées dans une interaction spécifique avec un ligand de pathogène, et dans la reconnaissance immunitaire. Ainsi, la FREP1 d'*Anopheles* (qui n'a du nom que le domaine fibrinogène, mais pas les caractéristiques propres aux FREP de mollusques) a été décrite comme une molécule nécessaire à différentes espèces de *Plasmodium* pour s'ancrer à la matrice périvitroïque de l'hôte définitif et ainsi traverser l'épithélium (Zhang, Niu et al. 2015). Par contre, le FREP9 a été montré comme se liant en dimère aux sporozoïtes de *Plasmodium* dans le tube digestif du moustique, et que sa sous-expression était liée à une augmentation du nombre de parasites circulants (Dong and Dimopoulos 2009). Si un phénomène de pression de sélection entraînant de la diversification sur ces deux FREP est justifié pour limiter l'infection avec *Plasmodium*, cela ne sous-entend pas que ces deux FREP aient un rôle de reconnaissance immunitaire à l'image d'un anticorps. De même pour les DScam, leur rôle dans la reconnaissance immunitaire est très souvent contesté (Armitage, Kurtz et al. 2017).

Pour citer textuellement Louis du Pasquier (Armitage, Kurtz et al. 2017), « certains ont eu tendance à comparer des pommes et des oranges ». Les premiers résultats concernant les DScam ont été pondérés par leurs auteurs pour éviter de surestimer leurs rôles encore non validés (Du Pasquier 2005, Armitage, Kurtz et al. 2017). De plus, l'élément central qui différencie le système anticipatoire des vertébrés de celui des invertébrés est moins la capacité de produire un bon variant correspondant à un épitope donné que la capacité de le sélectionner, de l'amplifier, de le conserver et de le réactiver plus tard si besoin est. Si certains considèrent qu'il n'y a pas de raison pratique, fonctionnelle, pour que les DScam soit des récepteurs spécifiques d'antigènes (Brites and Du Pasquier 2015, Armitage, Kurtz et al. 2017), ce qui est vrai ou faux chez DScam ne l'est peut-être pas pour les FREP, TLR, ou d'autres récepteurs potentiels de reconnaissance.

Sans sélection puis conservation et rappel d'un variant polymorphe utile, peut-on considérer cette stratégie comme adaptative tel qu'on le définit dans le système immunitaire des vertébrés ? Peut-on imaginer que ces organismes aient sélectionné des mécanismes de diversification, mais pas, ou pas encore, l'intégralité du processus de sélection clonale ?

Une diversification massive (« shot-gun ») pourrait être sélectionnée pour créer de la diversité au sein de ces molécules de reconnaissance. On peut imaginer

qu'à la suite d'une infection par un agent pathogène, l'ensemble de ces familles de reconnaissance soit fortement induit dans l'idée qu'au moins un variant soit compétent : c'est-à-dire parfaitement adapté à la reconnaissance d'un facteur donné d'un pathogène. Lors d'une prochaine rencontre avec le même pathogène deux stratégies s'offrent alors à cet hôte : produire de la même manière une diversité shot-gun qui devrait permettre, comme la première fois, d'exprimer le variant compétent pour reconnaître la cible ; et /ou sélectionner le variant compétent de la première rencontre. Sélectionner, ou de produire à nouveau un variant précédemment produit contre un pathogène serait une caractéristique d'un système immunitaire adaptatif. Augmenter les capacités de diversification pour générer un polymorphisme supérieur et avoir plus de chances de détecter la présence d'un pathogène lors d'une prochaine rencontre serait défini comme un système consécutif, bien loin de caractéristiques anticipatoires.

Une autre possibilité existe pour augmenter un répertoire potentiel de reconnaissance sans utiliser de mécanismes de diversification, cette augmentation repose sur la capacité pour ces molécules de s'associer pour former des hétéromères de 2, 3, 4 ou plus de variants, et ainsi augmenter *de-facto* le répertoire de récepteurs disponibles. On peut alors spéculer que dans le cadre d'une résistance acquise, l'individu pourrait conserver en mémoire la bonne combinaison (quantité et qualité) de variants nécessaires pour former les bons hétéromères compétents lors d'une prochaine rencontre.

En plus des mécanismes de diversification, puis d'interactions directes avec un facteur de pathogène, les molécules diversifiées d'invertébrés devront montrer la capacité d'être sélectionnées pour générer une réponse compétente et être réutilisées plus tard dans le cadre d'une réponse adaptative et mémorielle.

La diversification reste un élément central d'un système immunitaire anticipatoire et la découverte de ces familles chez des invertébrés a totalement changé la vision que nous avons de l'immunité chez ces organismes.

Immunité mémoire innée chez les Invertébrés

Les premiers phénotypes de résistance acquise, c'est-à-dire qu'un individu puisse devenir résistant à la suite d'une première stimulation immunitaire ont été montrés dans un grand nombre de taxons parmi les invertébrés. Ainsi, *Anopheles*, *Aedes*, *Drosophila*, *Tenebrio*, *Bombyx*, *Tribolium*, *Daphnia*, *Crassostrea* ou *Biomphalaria* sont capables de mettre en place une réponse immunitaire permettant de mieux répondre à une seconde infection (Kurtz and Franz 2003, Moret and Siva-Jothy 2003, Sadd and Schmid-Hempel 2006, Pham, Dionne et al. 2007, Roth, Sadd et al. 2009, Rodrigues, Brayner et al. 2010, Pope, Powell et al. 2011, McTaggart, Wilson et al. 2012, Portela, Duval et al. 2013, Zhang, Qiu et al. 2014, Dubief, Nunes et al. 2017). Alors que pour d'autres interactions, *Daphnia* (Duneau, Ebert et al. 2016), *Hetaerina* (González-Tokman, González-Santoyo et al. 2010) ou *Formica*

(Reber and Chapuisat 2012) les expériences n'ont montré aucune protection induite par une première rencontre avec le pathogène. Cette stimulation peut être très différente en fonction des modèles, mais repose sur une première rencontre avec un agent pathogène vivant ou atténué, voire un extrait protéique de ce dernier. L'absence de réponse observée pour certaines espèces d'invertébrés pourrait jeter le doute sur l'aspect non-généraliste de ces phénomènes. Aussi, des études comparatives devront être réalisées pour mieux comprendre la conservation de tels mécanismes chez l'ensemble des invertébrés.

« Spécificité de l'Immunité mémoire innée chez les Invertébrés »

Comment doit-on alors définir la spécificité de la réponse immunitaire, particulièrement lorsque l'on s'intéresse à la réponse immunitaire mémoire des invertébrés ? La majorité des travaux réalisés sur l'immunité mémoire des invertébrés a amené autant de résultats que de méthodes pour les découvrir. Cela suggère que la force évolutive pesant sur ces organismes nous permet d'observer aujourd'hui, presque qu'autant de mécanismes moléculaires que d'interactions (Coustau, Kurtz et al. 2016).

La spécificité d'une réponse immunitaire, impliquée ou non dans une réponse mémorielle, pourrait pourtant être définie en fonction de la distance phylogénétique séparant les pathogènes utilisés entre la première et la deuxième stimulation pour ainsi différencier un eucaryote d'un procaryote (Deleury, Dubreuil et al. 2012), deux groupes bactériens gram + et - (Dhinaut, Chogne et al. 2017), deux espèces d'un même pathogène (Portela, Duval et al. 2013), deux stades de développement d'un même parasite (Pinaud, Portet et al, soumis) ou différents constituants membranaires (Imler, Tauszig et al. 2000, Ferrandon, Imler et al. 2004, Miyashita, Kizaki et al. 2014, Sullivan and Belloir 2014).

La spécificité de l'immunité mémoire innée des invertébrés doit donc être observée pour chaque espèce au regard des communautés de pathogènes qui lui sont associés et non en fonction de la disponibilité de telle ou telle souche commerciale de micro-organismes. Cette question évolutive ou écologique des interactions qui doit induire une réflexion concernant la spécificité de la réponse, comme celle *in-fine*, de la spécificité de reconnaissance.

Support d'une mémoire immunitaire

« Le souvenir est un compromis... » Tommaso Landolfi

Au demeurant, le souvenir est un compromis entre ce qu'on va véritablement garder en mémoire et ce qu'on va devoir oublier. Il faut donc savoir synthétiser, garder le principal.

Toute la problématique de la mémoire et en particulier de la mémoire

immunitaire, repose moins sur la problématique du temps que doit durer le souvenir que sur son support physique. Comment conserver la trace d'un pathogène le plus virulent que vous ayez croisé dans votre vie et sur lequel l'hôte a brillamment pris le dessus. Une trace d'ADN, une protéine de surface, une enzyme de virulence, les supports potentiels de cette mémoire immunitaire peuvent être nombreux. À ce jeu, le système adaptatif des bactéries CRISPR-Cas9 est particulièrement efficace puisqu'il suffit d'un morceau de brin d'ADN exogène qui sera intégré à son propre génome pour s'en souvenir, le mobiliser et ainsi résister à une nouvelle agression (Koonin and Makarova 2009).

Une autre possibilité de garder une trace d'un pathogène pourrait reposer sur une réponse maintenue, comme la réponse systémique acquise (SAR) des plantes (Ryals, Neuenschwander et al. 1996). Après avoir rencontré un pathogène virulent, ces plantes vont activer une réponse immunitaire à large spectre qui sera gardée à un haut niveau d'activité pendant un temps suffisamment long pour éliminer ce pathogène du milieu de vie, s'il se représentait (Conrath, Beckers et al. 2015). Cette réponse pourrait être maintenue durant toute la vie de l'individu, le coût du maintien étant compensé par l'avantage de la résistance à des pathogène toujours présents dans le milieu (van Hulten, Pelsler et al. 2006, Luna, Bruce et al. 2012).

Cette réponse même temporaire, reste tout de même coûteuse en énergie. Une réponse inductible serait énergétiquement plus efficace. Certaines cellules du système immunitaire inné des vertébrés, comme les macrophages (Mc) ou les Natural Killers (NK), voient ainsi après une stimulation immunitaire leur paysage chromatinien profondément modifiés pour passer d'un statut immunitaire sensible à un statut tolérant voire résistant. Cela signifie qu'à la suite d'une stimulation, une cellule est capable de modifier l'accessibilité de certaines portions génomiques pour se préparer à faire face à un pathogène. Lors d'un potentiel challenge immunitaire, les portions génomiques seront plus facilement mobilisables pour permettre la production des facteurs de l'immunité. La sélection de différents paysages épigénétiques est issue de centaines de générations d'interactions et pourrait au regard de l'évolution être considérée comme une réponse anticipatoire.

Concernant le temps de mémoire de cette résistance, peu de données existent actuellement même si tout semble indiquer que l'information du changement chromatinien est conservée puis transmise aux cellules concernées, avec une implication potentielle d'interleukine et de miRNA (communication personnelle Mihai Netea). Chez les vertébrés, ce remodelage épigénétique des cellules de l'immunité innée a été décrit sous le nom d'immunité entraînée (Netea, Quintin et al. 2011, Netea and van der Meer 2017). Des similarités concernant le support moléculaire de cette mémoire immunitaire innée et l'immunité innée identifiée chez *Biomphalaria* pourraient être envisagées (Chapitre 3).

Au sein des invertébrés, la durée de cette résistance acquise est d'ailleurs un grand débat. En effet, il est quasiment impossible de comparer les systèmes entre eux. En effet, comment comparer les quelques heures de résistance chez un

organisme vivant quelques heures/jours doivent être comparé à la résistance pérenne de *Biomphalaria* qui conserve la résistance tout sa vie qui dure plusieurs mois voire années ?

Si la réponse immunitaire adaptative des vertébrés est décrite comme retardée d'une dizaine de jours après la stimulation, peu d'information sont connues chez les invertébrés. Une équipe mexicaine en 2016 a essayé de compiler des résultats de résistance induite par une primo-stimulation (Jorge Contreras-Garduno, Humberto Lanz-Mendoza et al. 2016), chez un grand nombre d'organismes et a déterminé que cette résistance pouvait exister dès les premières heures (Wu, Zhao et al. 2014, Wu, Li et al. 2015), un à deux jours (Zhao, Wu et al. 2013, Garbutt, O'Donoghue et al. 2014, Shi, Lin et al. 2014) et jusqu'à plusieurs semaines après la première stimulation (Lin, Chen et al. 2013, Portela, Duval et al. 2013, Contreras-Garduno, Rodriguez et al. 2014). Aucune cohérence entre les traits de vies, ni entre les stratégies d'établissements écologiques traditionnellement évoquées (stratégies r ou K) n'a pu être corrélée à cette diversité de réponse. Aussi, il existe une incohérence sur la durée du priming chez les invertébrés (Milutinovic and Kurtz 2016), ce qui pourrait suggérer la possibilité d'une convergence évolutive de mécanismes très différents concourant tous à une résistance acquise au cours de la vie.

Enfin chez les vertébrés, la résistance acquise par une primo-stimulation, (immunisation ou vaccination) permet à un organisme de monter une réponse adaptative et devenir résistant pour plusieurs années. Le système articulé autour d'une sélection clonale de cellules portant des anticorps spécifiques de l'agent pathogène concerné, permet au cours du temps, de remplacer les cellules en fin de vie par de nouvelles possédant l'exacte copie de l'anticorps et ainsi conserver cette marque relativement longtemps.

Quid chez les invertébrés ?

Contrainte de l'immunité mémoire

**« Se rappeler quelque chose est encore le meilleur moyen de ne pas l'oublier »
Pierre Dac**

La durée fonctionnelle d'une résistance acquise pose un problème fondamental de la sélection évolutive pesant sur ce système. Sur un continuum évolutif, on peut croiser des organismes incapables de produire une réponse immunitaire mémorielle ou de garder une trace d'un pathogène ; et d'autres capables d'induire une réponse maintenue pendant un court ou un long moment. Ces différences doivent être comparées par rapport à l'unité de sélection de l'organisme en question. En effet, un vertébré comme l'homme peut vivre plusieurs dizaines d'années, mais l'immunisation prophylactique (telle qu'elle est pratiquée en

société sous la forme de la vaccination) et la sélection clonale qui en découle nécessitent un rappel de cet agent vaccinant tous les 10 à 15 ans pour produire une réponse immunitaire suffisamment efficace contre des pathogènes ciblés à l'échelle populationnelle.

Il faut également rappeler que de nombreux scientifiques se refusent à considérer le système immunitaire comme une arme agressive, utile à l'élimination des agents étrangers. Il préfère considérer le système immunitaire comme le chef d'orchestre minutieux de l'organisation et de la communication entre les très nombreuses cellules diversifiées produites par l'homme (l'individu) et celles qui lui sont associées (microbiote, macrobiote, holobionte). Les maladies auto-immunes seraient donc la représentation du dérèglement de cette organisation cellulaire. En l'absence d'actions réciproques envers les organismes associés, un système immunitaire adaptatif se retournerait contre son possesseur conduisant aux conséquences néfastes énoncées précédemment. Il faut tout de même citer l'exemple des cancers chez l'homme, qui à la suite de mutation nucléaire (avec une conséquence génétique, épigénétique, etc) provoque un changement de communication entre ces cellules et le système immunitaire. Il est alors difficile de limiter la croissance de ces cellules qui deviennent incontrôlables tout en conservant leur capacité à prospérer (Cairns, Harris et al. 2011) mais refusant de se laisser dicter la loi de l'homéostasie immunitaire

Le cancer impose une réflexion directe sur la mémoire immunitaire, comme les cellules tumorales ne sont que l'expression de composés cellulaires endogènes, à l'inverse d'agents pathogènes exogènes qui sont très différents structurellement des cellules saines, il est quasiment impossible de conserver une trace d'un cancer passé pour mieux s'y défendre dans le futur. Si des solutions thérapeutiques contre le cancer sont parfois nommées vaccins, elles ne ciblent pas des antigènes polymorphes de cellules cancéreuses, mais des composés essentiels à la croissance cellulaire comme l'EGF (epidermal growth factor).

Mais alors si l'immunité mémoire a besoin de se rappeler au bon souvenir des pathogènes anciennement éliminés, où commence et s'arrête la frontière entre l'immunité mémoire et la perpétuelle stimulation d'un hôte par des pathogènes ?

Il y a en effet des pathogènes qui ne sont rencontrés que très rarement au cours de la vie, le virus de la varicelle pour les enfants en bas-âge, la mononucléose infectieuse pour les adolescents, certains pathogènes multi-résistants lors d'infection nosocomiale de personnes âgées ou immunodéprimées. Ces pathogènes représentent une cohorte d'agents infectieux qui existe dans la communauté humaine en Europe par exemple, et passer outre ces infections permet d'être protégé pour l'ensemble de la période de sensibilité pour l'infection de ce pathogène. Par exemple, la varicelle, cause à l'âge adulte des dégâts très importants sur la fécondité, mais son infection est bénigne chez les jeunes (Buvelot, Lebowitz et al. 2016). Dans ces cas-là, il est donc attendu que pour franchir la classe

d'âge supérieure, la résistance mémorielle à ces pathogènes est une étape primordiale pour réduire les conséquences potentielles de la réduction de fitness de l'individu. Conserver une trace immunitaire de ce pathogène est donc essentielle pendant le plus de temps possible après la maturité sexuelle.

Concernant les pathogènes qui ont sélectionné un grand taux de polymorphisme, comme la grippe (influenza), l'immunisation, qu'elle soit prophylactique ou consécutive à une infection, ne devrait générer d'action protectrice que pour la saison ou quelques autres, et ne justifie pas à l'échelle évolutive une pression de sélection pour acquérir une mémoire immunitaire efficace pendant des décennies. En effet, ce type de pathogènes ayant sélectionné de la diversification active dans la population au niveau de leurs protéines de surfaces, il est très difficile pour le système immunitaire adaptatif de lutter avec ce type d'interactions (Alizon 2013, Fraser, Lythgoe et al. 2014). Enfin, reste les agents pathogènes intimement liés aux traits de vie humains comme *Helicobacter pylori* ou *Escherichia coli*, inféodés aux surfaces d'échanges de l'organisme, ces interactions plutôt réciproques peuvent basculer en interactions pathogènes dès que l'hôte est immunodéprimé ; se transformant alors en ulcère gastrique, gastro-entérite ou septicémie. Il est donc plutôt envisageable pour ce type d'interaction durable, qu'un phénomène de tolérance voire d'incorporation comme celles décrites dans la théorie de la discontinuité antigénique énoncée par Thomas Pradeu (Pradeu, Jaeger et al. 2013, Pradeu and Vivier 2016), se réalise au fil de l'évolution. Cette fin de réponse peut être vécue comme l'acceptation de cet organisme dans le giron de l'individu, de l'holobionte, du méta-organisme. Ces interactions durables qui peuvent conduire soit à une élimination soit à une incorporation de l'organisme extérieur, pourraient être perçues comme une exposition quasi-perpétuelle de l'hôte à une grande diversité d'agents potentiellement pathogènes, et ne justifie pas d'avoir sélectionné évolutivement une immunité mémoire.

Dans le modèle *Biomphalaria/Schistosoma*, le phénomène de résistance acquise est observé à la suite d'une infestation naturelle où le premier parasite va se développer normalement, résultant en une exposition continue à des antigènes du parasite qui continue de se développer chez le mollusque. Pourtant, la résistance induite par cette réponse mémorielle est activable également par vaccination (Chapitre 2).

La confrontation entre un pathogène et un système immunitaire ultra-spécifique capable de se souvenir de variants spécifiques signifie que la seule manière d'infecter à nouveau ce même hôte est de diversifier les molécules support de cette reconnaissance chez le parasite, ceci forçant *in-fine* la diversification des protéines impliquées dans la reconnaissance des pathogènes par les hôtes (Chapitre 2).

Support cellulaire de l'immunité

Chez les vertébrés, trois grands types de cellules ayant des fonctions différentes ont été caractérisés pour leur rôle direct d'interaction avec des agents pathogènes. On retrouve ainsi les granulocytes (neutrophiles + éosinophiles + basophiles), les phagocytes (macrophages + neutrophiles + cellules dendritiques) et enfin les NK (pour Natural Killer). Si elles sont toutes circulantes, elles peuvent être associées à des compartiments plus spécifiques comme les cellules dendritiques très présentes dans les muqueuses. Les cellules dendritiques ont la capacité de présenter les antigènes de pathogènes éliminés pour activer la réponse immunitaire adaptative, comme un trait d'union entre la réponse innée et adaptative. Les cellules NK quant à elles, participent au renouvellement cellulaire de l'organisme en traquant les cellules immunitaires infectées par un virus ou un parasite intracellulaire (Wolf, Sherratt et al. 2017). Des résultats récents suggèrent que les NK peuvent coopérer avec des macrophages dans le cadre de surveillance de cancer par exemple (Walajtys-Rode and Dzik 2017).

L'activité des cellules immunitaires est relativement conservée à travers les phyla et elles sont souvent retrouvées chez les invertébrés sous le nom générique d'hémocytes (Blandin, Shiao et al. 2004, Williams 2007, Cavalcanti, Filho et al. 2012). Plusieurs infra-populations ont ainsi pu être caractérisées en fonction de leurs caractéristiques morphologiques ou fonctionnelles. Ainsi, chez bon nombre d'invertébrés, de nombreux types cellulaires ont été caractérisés comme des lamellocytes (hyalinocytes), des plasmatocyte (blast-like cells), des cellules cristales (granulocytes) (Rizki and Rizki 1959). Le rôle exact des différentes populations d'hémocytes dans les voies de réponses immunitaires est encore mal connu chez la plupart des invertébrés. En réponse à un pathogène donné, le compartiment hémocytaire est sollicité soit par une augmentation de la phagocytose, encapsulation et/ou l'expression de gènes d'intérêts comme chez *Biomphalaria* (Cheng and Auld 1977, Stumpf and Gilbertson 1978, Bayne, Buckley et al. 1980, Yoshino 1983, Boswell and Bayne 1985, Yoshino and Granath 1985, Noda and Loker 1989, Adema, Arguello et al. 1994, Coustau and Yoshino 1994, Agner and Granath 1995, Fryer and Bayne 1996, Humphries and Yoshino 2003) comme chez d'autres invertébrés dont *Tenebrio* (Jones 1957), *Drosophila* (Rosetto, Engstrom et al. 1995, Franc, Dimarcq et al. 1996, Schulz and Fossett 2005), *Anopheles* (Hernandez, Lanz et al. 1999, Danielli, Loukeris et al. 2000, Rodrigues, Brayner et al. 2010, Smith, King et al. 2016) ou *Aedes* (Hillyer and Christensen 2002).

Encore aujourd'hui il n'y a pas de consensus concernant l'immunité cellulaire chez les invertébrés. Il reste toujours difficile de suivre par exemple la dynamique des sous-populations, car à l'inverse des vertébrés, aucun marqueur de surface n'existe chez *Biomphalaria*. L'acquisition de marqueurs moléculaires pour étudier ces cellules demeure jours après jours une nécessité.

L'immunité mémoire, une mémoire pour plusieurs générations ?

« Ne pas transmettre ses expériences, c'est castrer l'avenir » Marin Karmitz

Il est maintenant établi que certains invertébrés comme *Tenebrio*, *Biomphalaria* ou *Anopheles* aient pu développer une réponse immunitaire qui puisse être mémorielle et spécifique, et ce, malgré l'absence de mécanismes orthologues de l'immunité adaptative telle que nous les connaissons chez les vertébrés.

En considérant qu'un individu exposé à une communauté de pathogènes ait réussi à y faire face, celui-ci a tout intérêt à transmettre à ses congénères et/ou à sa descendance cette résistance induite et/ou acquise (Moret and Schmid-Hempel 2001, Singh, Criscoe et al. 2015).

Il a alors été montré plusieurs types d'immunité sociale pour répandre cette information de résistance au sein de sa communauté, sa colonie ou son groupe social. Les primates par exemple, réalisent une toilette respectueuse de la fourrure qui permet à la fois d'établir des liens sociaux dans la communauté, mais aussi d'éliminer les ectoparasites (Henazi and Barrett 1999). Les termites *Zootermopsis* (Traniello, Rosengaus et al. 2002) ou les fourmis *Camponotus* (Hamilton, Lejeune et al. 2011) et *Lasius* (Konrad, Vyleta et al. 2012) voient leur sensibilité à un pathogène diminuer lorsqu'un individu contaminé est introduit dans la colonie. Il n'est pas encore établi si cette protection induite repose sur une immunisation sociale par transfert direct de l'agent pathogène ou si cette transmission pourrait être portée par de la trophallaxie, c'est-à-dire la régurgitation d'éléments nutritifs pour la communauté, qui pourrait contenir des éléments immunisants (Thorne and Traniello 2003). Chez les abeilles du genre *Apis*, des soins sont pratiqués via des mouvements abdomen contre abdomen très intenses qui permettent de faire tomber certains ectoparasites grâce à l'action combinée de la chaleur et des frottements (Traniello, Rosengaus et al. 2002). De même, les drosophiles sont capables d'augmenter le phénomène de recombinaison pour accélérer la diversification de leurs descendances à la suite d'un stress infectieux (Kacsoh, Lynch et al. 2013) afin d'activer des voies immunitaires cohérentes. Si cette méthode semble aléatoire, elle est similaire au phénomène SOS des bactéries qui favorise la mutation aléatoire pour acquérir de nouvelles caractéristiques génétiques afin de mieux répondre à un environnement stressant (Schaaper and Loeb 1982, Matic, Rayssiguier et al. 1995).

La transmission d'informations permettant d'améliorer une réponse immunitaire peut donc s'opérer de manière latérale au sein d'une même génération comme l'immunité sociale, mais peut également être réalisée vers les générations suivantes. Dans ce cas, on parle de transfert trans-générationnel d'immunité de manière générale ou de priming trans-générationnel (TGIP) chez les invertébrés. Cette transmission a été montrée chez les vertébrés où des anticorps sont retrouvés dans le lait maternel à destination du nourrisson (Hanson and Soderstrom 1981). Ces transferts existent également chez de nombreuses espèces d'invertébrés (Little, O'Connor et al. 2003, Sadd, Kleinlogel et al. 2005, Moret 2006, Sadd and Schmid-

Hempel 2007, Roth, Joop et al. 2010, Zanchi, Troussard et al. 2011, Hernandez Lopez, Schuehly et al. 2014, Fisher and Hajek 2015, Pigeault, Vezilier et al. 2015, Tate and Graham 2015, Trauer-Kizilelma and Hilker 2015) pour lesquels les bases moléculaires commencent à être caractérisées. La nature des molécules transférées est multiple et peut provenir soit d'un transfert venant de la mère, du père, de molécules associées aux bactéries (LPS), de bactéries directement, voire de molécules immunitaires (Sadd and Schmid-Hempel 2007, Haine, Moret et al. 2008, Zanchi, Troussard et al. 2011, McNamara, van Lieshout et al. 2014, Vantaux, Dabire et al. 2014, Dubuffet, Zanchi et al. 2015, Salmela, Amdam et al. 2015, Tate and Graham 2015, Pigeault, Garnier et al. 2016, Dhinaut, Chogne et al. 2017). Dans le cadre de l'interaction *Biomphalaria/Schistosoma* des résultats préliminaires n'ont pas montré de priming trans-générationnel (communication personnelle) mais des expériences complémentaires mériteraient d'être effectuées afin de confirmer/infirmes ces résultats.

Si la terminologie rapproche *de-facto* le priming avec son alter-ego « priming trans-générationnel », il est envisageable que les mécanismes sous-jacents pour ces deux mécanismes soient fortement différents entre priming et priming trans-générationnel.

Travail de recherche

Édito

C'est pour son rôle de *Biomphalaria glabrata* comme vecteur de *Schistosoma mansoni* et donc de la bilharziose qui a le plus intéressé la communauté scientifique ces dernières dizaines d'années bien plus que l'étude de son immunité.

Pourtant, l'utilisation de *Biomphalaria* se justifie entièrement dans l'étude de l'évolution des systèmes immunitaires mémoires chez les invertébrés.

Cette interaction et surtout l'immunité de l'hôte repose sur bien plus que les facteurs décrits jusqu'à présent. Ainsi, dans le **Chapitre 1**, je vais tâcher d'expliquer en quoi l'interaction *Biomphalaria/Schistosoma* repose aujourd'hui à la fois sur des facteurs protéiques, enzymatiques et même nucléotidiques pour exprimer les relations immunologiques entre cet hôte et son pathogène.

Cet escargot est capable de générer une réponse immune mémorielle qui lui confère une résistance acquise contre le même pathogène, pour toute la durée de sa vie. Une grande partie du travail de la thèse, a consisté à étudier finement ce phénotype de résistance acquise et à décrire ses premières bases moléculaires. **Le Chapitre 2** va donc s'atteler à décrire le changement phénotypique et transcriptomique qui pourrait expliquer l'acquisition de la résistance, en résumé, les bases moléculaires de l'immunité innée mémoire chez *Biomphalaria glabrata*. De même, les premiers indices concernant les bases mécanistiques de la spécificité envers différents parasites seront exposés tout comme le déterminant protéique privilégié pour expliquer le phénotype immunitaire humoral observé lors du challenge immunitaire, les biomphalysines, et l'histoire évolutive qui les concerne.

Cette immunité montre finalement des proximités fonctionnelles avec une autre immunité décrite chez les vertébrés, l'immunité entraînée. **Le Chapitre 3** aura pour objectif de présenter les premiers résultats laissant présager que *Biomphalaria glabrata* et particulièrement ses hémocytes, subissent des modifications de structures chromatinienne pour se préparer à répondre à une seconde infestation.



Enfin, dans la **Discussion et les Réflexions**, nous poserons les bases de différentes questions qui interrogent la communauté scientifique actuellement. L'immunité mémoire est-elle l'apanage unique des vertébrés ? Quelle est la cause évolutive qui aurait pu forcer les organismes à acquérir une immunité mémoire ? L'immunité entraînée peut-elle être une synapomorphie chez les eucaryotes et être donc bien plus qu'un phénomène mémoriel spécifique des cellules de l'immunité innée des vertébrés ?

Chapitre 1

Acte1, Scène 1 : présentation des personnages

*Cette thèse s'est particulièrement intéressée aux supports moléculaires de la réponse immunitaire produite par un escargot vecteur *Biomphalaria* : Quelles sont les armes sélectionnées chez cet hôte pour contrer les potentielles armes des pathogènes qui lui font face ?*

➤ Integrated Multi-Omic Analyses In *Biomphalaria-Schistosoma* Dialogue Reveal The Immunobiological Significance Of FREP-SmPoMuc Interaction.

Portet A , Pinaud S , Tetreau G, Galinier R, Cosseau C, Duval D, Grunau C, Mitta G, Gourbal B.

Developmental and Comparative Immunology, 2017 doi: 10.1016/j.dci.2017.02.025

↪ Specific Pathogen Recognition by Multiple Innate Immune Sensors in an Invertebrate

Tetreau G , Pinaud S , Portet A , Galinier R, Gourbal B, Duval D.

Frontiers Immunology, 2017 doi: 10.3389/fimmu.2017.01249

➡➡ Arm Race Between Endogenous miRNAs From Host And Circulating Xeno-Mirna Of Parasites.

Pinaud S, Lassalle D, Chaparro C, Portet A, Allienne JF, Gourbal B, Duval D.

En preparation

 premiers auteurs ➤ article publié ↪ article soumis ➡➡ article en préparation

Le parasite *Schistosoma mansoni* dépend obligatoirement du mollusque *Biomphalaria* pour se développer et se transmettre à l'hôte définitif. Cette interaction obligatoire signifie qu'il existe des pressions de sélection qui vont peser sur ces deux protagonistes, favorisant positivement la virulence du pathogène, ou positivement les défenses immunitaires de l'hôte. De plus, l'histoire évolutive complexe entre *Biomphalaria* et *Schistosoma* a forcé des interactions atypiques avec la confrontation d'un filtre de sélection potentiellement très différent entre ces deux protagonistes.

Ainsi, chaque souche d'hôte disponible au laboratoire révèle une compatibilité spécifique de chaque parasite différent qui lui est présenté. On s'attend néanmoins à ce que les interactions sympatriques connaissent de meilleurs succès d'infection que leurs homologues allopatriques, dues à une histoire co-évolutive entre les deux protagonistes. La disponibilité de ces couples hôtes/parasites nous permet d'étudier l'évolution des mécanismes moléculaires à la base de l'interaction. Nous pouvons étudier la course aux armements ou l'adaptation qui existe entre les molécules de l'hôte et celles du parasite. Ce polymorphisme de compatibilité suggère également qu'il existe chez l'hôte une diversité du répertoire de molécules de défense qui se confronte à la diversité du répertoire de facteurs de virulence chez le parasite

Ces différents contextes évolutifs ont d'ailleurs été utilisés pour mieux appréhender les mécanismes moléculaires de l'adaptation locale par des approches intégratives (Voir Projets Parallèles : Portet, Pinaud et al, soumis). Les pathogènes avec une histoire évolutive commune avec leur hôte sont dits en sympatrie (récupérer l'un et l'autre dans le même site géographique sur le terrain), et vont alors posséder un grand nombre de mécanismes adaptés pour expliquer le succès de l'infestation. En effet, dans le cas de ces interactions, une immuno-modulation est observée pendant leur installation chez l'hôte. À l'inverse, les interactions allopatriques montrent que cette discussion semble moins tenue à l'échelle moléculaire et se traduit par l'impossibilité pour le parasite de se soustraire de la réponse immunitaire du mollusque. Pourtant, ces différentes interactions sont compatibles et le parasite arrive à se développer en utilisant des stratégies très différentes. Une attention particulière a été portée sur les ARN non-codants de longueur proche de 21nt et qui peuvent réguler de nombreuses voies comme celle du métabolisme, la réponse aux stress ou la réponse immunitaire. De manière intéressante, nous constatons que le parasite sympatrique produit plus de miARNs que son homologue allopatrique et que ces miARNs sont potentiellement capables de cibler l'expression de nombreux gènes immunitaires et ainsi expliquer l'immunosuppression (Voir Projets Parallèles : Portet, Pinaud et al., soumis). Des études d'interactome ont permis également de comparer l'affinité des molécules d'un même hôte (BgBRE) pour des extraits parasitaires issus de parasite compatible (*SmBRE*) ou incompatible (*SmGUA*) (Roger, Mitta et al. 2008, Mone, Gourbal et al. 2010). Cette approche expérimentale a permis de mettre en évidence d'une part,

une molécule hautement diversifiée et polymorphe venant de l'hôte et contenant à la fois un domaine fibrinogène et un domaine immunoglobuline, les Fibrinogen Related Proteins (FREPs) (Roger, Mitta et al. 2008, Mone, Gourbal et al. 2010). D'autre part, une molécule hautement diversifiée et polymorphes du parasite semblant leur faire face, des mucines dénommées SmPoMuc (Roger, Mitta et al. 2008).

Ces protéines hautement diversifiées et polymorphes ont laissé présager une pression de sélection importante suggérant qu'elles pourraient être au cœur du système de reconnaissance immunitaire entre hôte et parasite. Cette expérience a également révélé la présence d'un autre partenaire dans cette interaction immunologique, une BgTEP (thioester-containing-protein), une protéine de la superfamille des TEP qui contient entre autres les protéines du complément, les alpha-2-macroglobulines ($\alpha 2M$) et les autres TEP d'invertébrés (Portet, Galinier et al, soumis dans *Frontiers in Immunology*, Voir projets parallèles).

➤ Une revue du travail effectué depuis la découverte de ces deux acteurs a été publiée dans *Developmental and Comparative Immunology* (Portet, Pinaud et al. 2017). Cette synthèse présente les différentes approches « omiques » utilisées pour explorer cette interaction : génomique, transcriptomique, épigénomique, métabolomique et protéomique et révèle entre autres que : (i) des facteurs environnementaux exprimés à travers des déterminants épigénétiques pouvaient réguler ces deux facteurs, (ii) un haut niveau de polymorphisme associé à une faible expression des variants de SmPoMuc est caractéristique des souches de parasites les plus infectieuses, (iii) un haut niveau d'expression de FREP quel que soit le niveau ou la quantité de variants est caractéristique des souches de mollusques les plus réfractaires à l'infection (voir Projet Parallèle, (Galinier, Roger et al. 2017)).

La conclusion de cette synthèse souligne que la seule interaction moléculaire entre FREP et SmPoMuc ne peut pas expliquer le polymorphisme de compatibilité. L'invalidation des FREP par injection de siRNA chez une souche résistante de *Biomphalaria* (BS90) permet de rendre sur la moyenne des études 21 % des escargots sensibles (Hanington, Forsys et al. 2010, Hanington, Forsys et al. 2012). Ces résultats sont les premiers qui démontrent l'implication des FREP dans la réponse immunitaire (formation de complexe immun), et la résistance contre des trématodes. Ce phénotype partiel suggère également que cette résistance est portée par d'autres facteurs de reconnaissance que seulement les FREP.

➤ Afin d'explorer ces autres molécules candidates impliquées dans la reconnaissance et la potentielle activation de la réponse immunitaire en fonction de différents types de pathogènes, une méthode simple a été développée au laboratoire. Cette méthode d'interactome entre de l'hémolymphe sans cellule et des pathogènes vivants entiers (gram+, gram-, levure, métazoaires) a été couplée à une approche de protéomique comparative qui a permis de révéler des répertoires

différents mais non-exclusifs de molécules immunitaires pouvant enrichir le nombre de molécules impliquées dans l'expression du polymorphisme de compatibilité (Tetreau, Pinaud et al. 2017).

➔L'analyse de cette interaction s'est ensuite focalisée sur le dialogue moléculaire entre des acides nucléiques. Particulièrement, les petits ARN (sRNA) sont aujourd'hui bien connus pour être des séquences d'acides nucléiques simple brin, capable de modifier l'expression générale d'un gène, mais non-codantes pour des protéines (Vasudevan, Tong et al. 2007, Vasudevan, Tong et al. 2008). Ces petits ARN et en particuliers les microARN sont produits sous la forme d'un pri-miRNA qui est la forme transcrite du génome via la polymérase Pol II (Figure 3, (Kim 2005)). Cette forme va alors être maturée en un pré-miRNA produit puis exporté dans le cytoplasme via un complexe de « cropping » incluant l'enzyme Drosha et l'exportine 5 (EXP5). Dans le cytoplasme, sa structure secondaire en tige boucle va être reconnue par un complexe contenant entre autres une endonuclease (Dicer) responsable du clivage et de la production d'une forme courte d'une vingtaine de nucléotide en moyenne. Ce brin court appelé aussi microARN (miARN) sera chargé dans un dernier complexe protéo-enzymatique nommé RISC. La proximité nucléique entre ce brin d'ARN et des portions génomiques ou transcrites va permettre via la mise en contact par le complexe RISC de reconnaître spécifiquement une zone par complémentarité de séquence nucléique (ARN ou ADN). Cette séquence reconnue est nommée cible du miARN. En fonction des protéines associées à ce complexe, cette hybridation (totale ou partielle) pourra modifier la durée de vie de l'ARN, l'activité de transcription ou de traduction associé à ce transcrit, l'ouverte/fermeture chromatinienne de la zone, le changement de marque d'histone spécifique, etc (Pasquinelli 2012, Chhabra 2015, Taguchi 2015, Spolverini, Fuchs et al. 2017).

Aujourd'hui, l'exploration des sRNA montre qu'ils ont un rôle de plus en plus important au cœur du dialogue moléculaire hôte/parasite (LaMonte, Philip et al. 2012, Xu, Ai et al. 2012, Dennison, BenMarzouk-Hidalgo et al. 2015, Fromm, Trelis et al. 2015, Liu, Hao et al. 2017). Chez *Schistosoma*, il a été décrit que des acides nucléiques, et plus précisément des longs ARN non codants, des ARN de transferts (tRNA) et des miRNA étaient produits et inclus dans des vésicules libérées par les parasites dans le sang des hôtes définitifs infectés (Xue, Sun et al. 2008, Copeland, Marz et al. 2009, Gomes, Cabral et al. 2009, Huang, Hao et al. 2009, Hao, Cai et al. 2010, Simoes, Lee et al. 2011, Nowacki, Swain et al. 2015). Si ces acides nucléiques sont capables de cibler la défense immunitaire de l'hôte définitif, il est tout à fait envisageable qu'ils aient également un rôle similaire chez le mollusque en participant par exemple à l'expression du polymorphisme de compatibilité. Nous avons donc entrepris d'étudier les profils de régulation des miARN, des résultats préliminaires sont donc présentés provenant à la fois de l'hôte et du pathogène pour comprendre le dialogue moléculaire au niveau des acides nucléiques dans ce couple *Biomphalaria/Schistosoma* (Pinaud et al. en préparation).

À partir de données de séquençage de petite taille sRNAseq (small-RNA sequencing) nous avons pu entre autres prédire de nouveaux miRNA et observer leur régulation dans différentes conditions d'infection. Nous avons par exemple montré qu'un miRNA de *Biomphalaria glabrata* qui participe à la réponse anti-parasitaire est en réalité contenu dans un ARN ribosomique r-miARN comme sporadiquement montré dans d'autres modèles (Son, Kumar et al. 2013, Chak, Mohammed et al. 2015, Yoshikawa and Fujii 2016). De manière intéressante, nous avons observé la présence d'ARN endogène de parasites dans le compartiment hémolympatique de son hôte. Ces derniers sont qualifiés de xéno-miRNA et ont probablement été sélectionnés afin de perturber les réponses immunitaires de l'hôte. Néanmoins, face à une infestation parasitaire, l'hôte est capable de réguler sa réponse immunitaire via ses miARNs et moduler un certain nombre de facteurs clés tel que les Biomphalysines (Galinié, Portela et al. 2013)(Pinaud, Tetreau et al. en préparation, voir Chapitre 2).

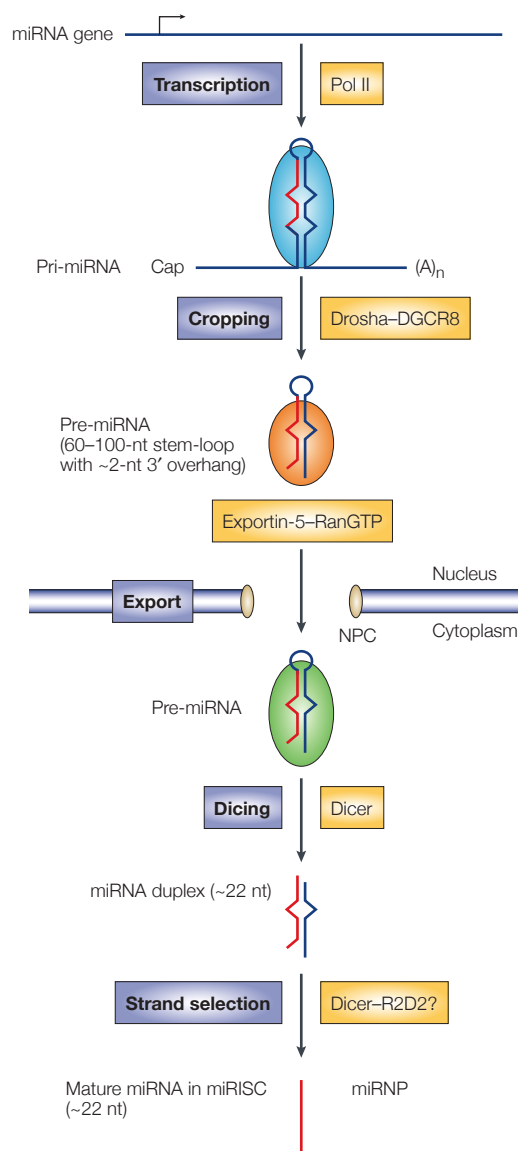




Figure 3. **Biogenèse des microARN**
d'après *MicroRNA Biogenesis: coordinated
cropping and dicing*
dans *Nature Reviews Molecular Cell Biology*
par Narry Kim en 2005
10.1038/nrm1644

Chapitre 1

Acte1, Scène 1

« L'art de chercher l'inattendu, c'est de cela dont est faite la Science ».
Fotis Kafatos, 2008

➤ **Integrated Multi-Omic Analyses In *Biomphalaria-Schistosoma* Dialogue Reveal The Immunobiological Significance Of FREP-SmPoMuc Interaction.**

Portet A , Pinaud S , Tetreau G, Galinier R, Cosseau C, Duval D, Grunau C, Mitta G, Gourbal B.

Developmental and Comparative Immunology, 2017 doi: 10.1016/j.dci.2017.02.025

↪ **Specific Pathogen Recognition by Multiple Innate Immune Sensors in an Invertebrate**

Tetreau G , Pinaud S , Portet A , Galinier R, Gourbal B, Duval D.

Frontiers Immunology, 2017 doi: 10.3389/fimmu.2017.01249

➤➤ **Arm Race Between Endogenous miRNAs From Host And Circulating Xeno-Mirna Of Parasites.**

Pinaud S, Lassalle D, Chaparro C, Portet A, Allienne JF, Gourbal B, Duval D.

En preparation

 premiers auteurs ➤ article publié ↪ article soumis ➤➤ article en préparation



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

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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.

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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 (Tennessen 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. unoinfective) 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 (Tennessen 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

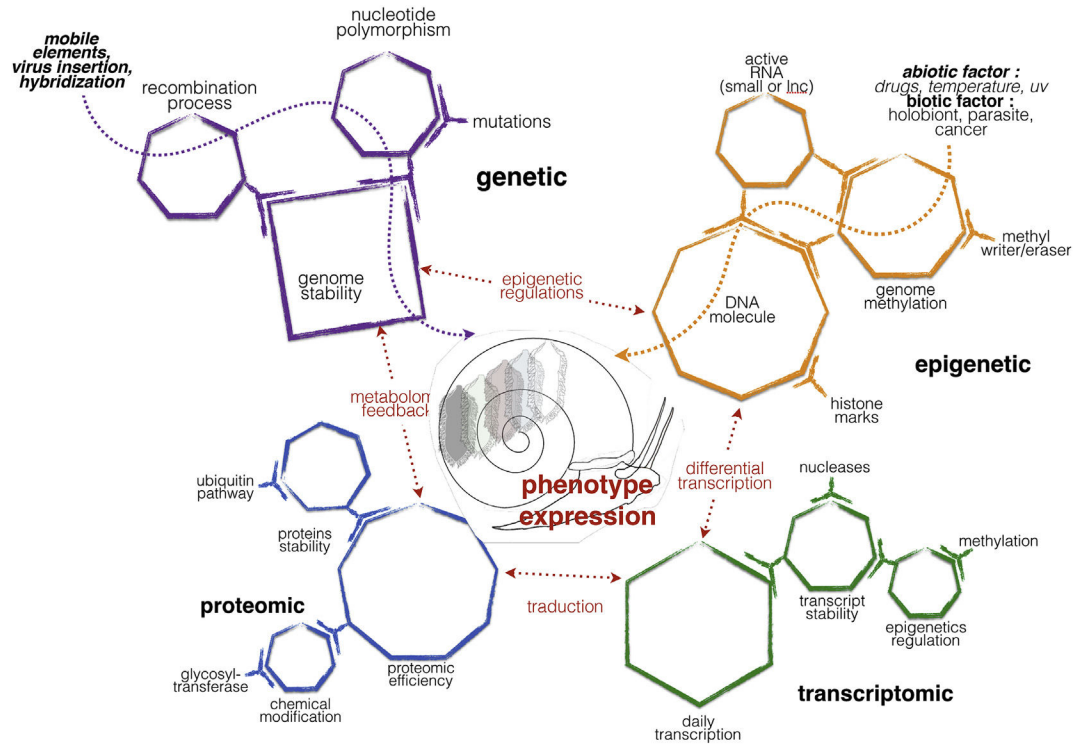


Fig. 1. Integrative multi-Omics approaches of host-parasite interaction.

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 (*SmPoMucs*)

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 (*SmPoMucs*) (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 *SmPoMucs* derived from a limited set of genes. *SmPoMucs* 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 *SmPoMucs* 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 *SmPoMucs* 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* 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* 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 *SmPoMucs*?

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 intra-molluscal 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*) (Galinié 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

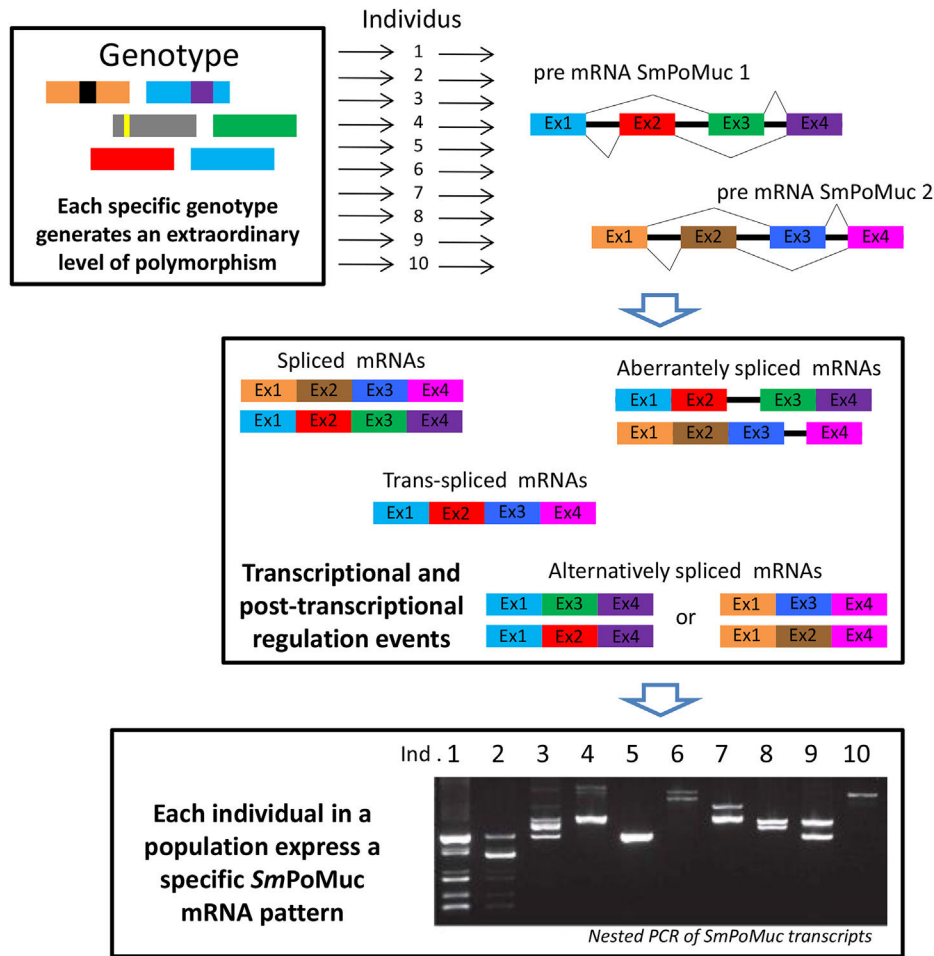


Fig. 2. Transcriptional *SmPoMuc* polymorphism defines a specific *SmPoMuc* pattern for each individual in a population of *S. mansoni* parasites. Hypothetical exon/intron structures of *SmPoMuc*s were used here to describe the transcriptional processes involved in the genesis of *SmPoMuc* diversity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

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 *SmPoMuc*s 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 *SmPoMuc*s, 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 *SmPoMuc* key candidates of the compatibility polymorphism and matching phenotype process, being potential pathogen antigens recognized by the host recognition receptors.

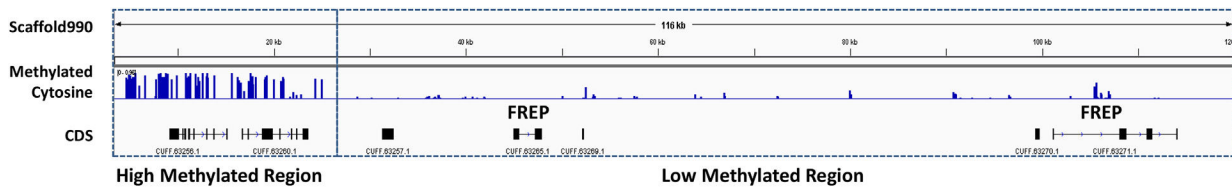


Fig. 3. Genome extract of the *B. glabrata* genome assembly: LGUN_random_Scaffold990:3335-119,948. Methylated cytosines are represented by blue bars. The height of the bar represents the percentage of methylation obtained for the methylated cytosines. The highest percentage of methylation observed in 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 *SmPoMuc*. CO-IP assays were thus conducted using proteins from *Biomphalaria* hemolymph, parasite extract containing *SmPoMuc* and antibody raised against C-terminal part of *SmPoMuc*. 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).

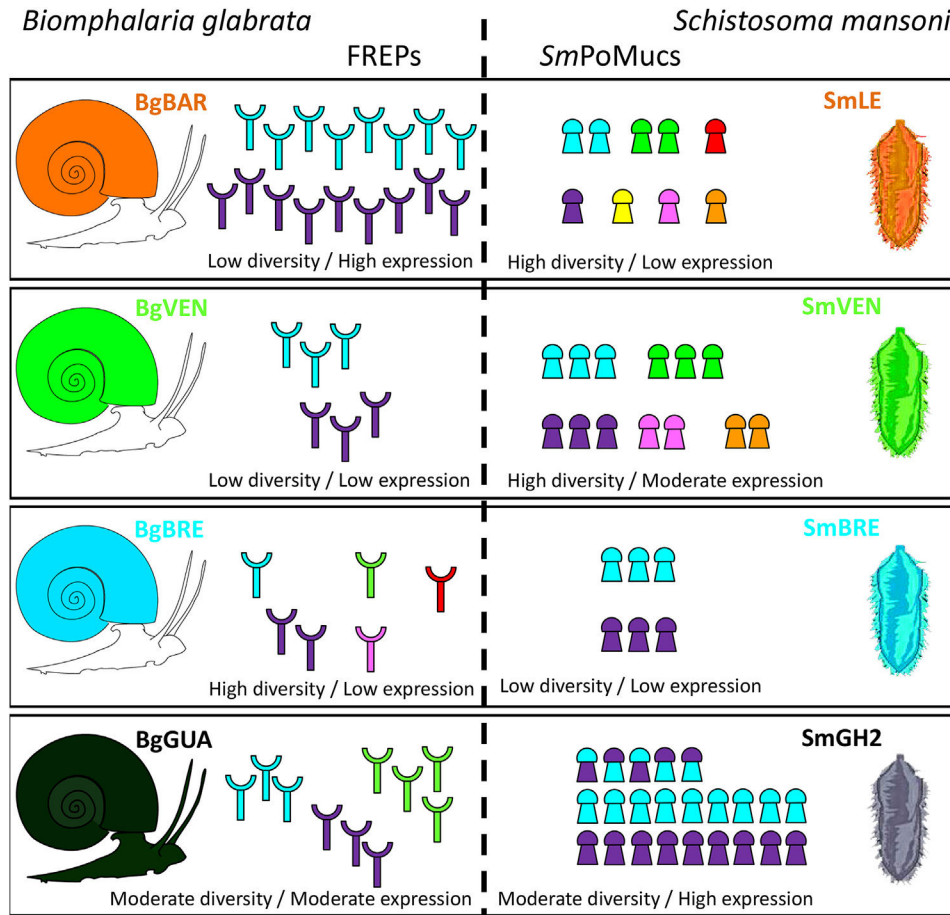
Nevertheless, such multimerization could be an additional process for increasing the mollusc pathogen recognition repertoire, by generating a wide range of multimers each with a different level of parasite antigen specificity. As such, it could increase the potential for specific recognition of parasite 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/*SmPoMuc* 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 (*SmPoMuc*s) (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 *Schistosoma* 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 *SmPoMuc*s 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 *SmPoMuc*s (Fig. 4). In this context, the increased diversification combined with the decrease expression levels of the *SmPoMuc*s 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 *SmPoMuc*s 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 *SmPoMuc*s. We could hypothesis 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 infect 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.

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Chapitre 1

Acte1, Scène 1

« L'art de chercher l'inattendu, c'est de cela dont est faite la Science ».
Fotis Kafatos, 2008

➤ Integrated Multi-Omic Analyses In *Biomphalaria-Schistosoma* Dialogue Reveal The Immunobiological Significance Of FREP-SmPoMuc Interaction.

Portet A 🍏, Pinaud S 🍏, Tetreau G, Galinier R, Cosseau C, Duval D, Grunau C, Mitta G, Gourbal B.

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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

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*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.

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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*, pattern-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

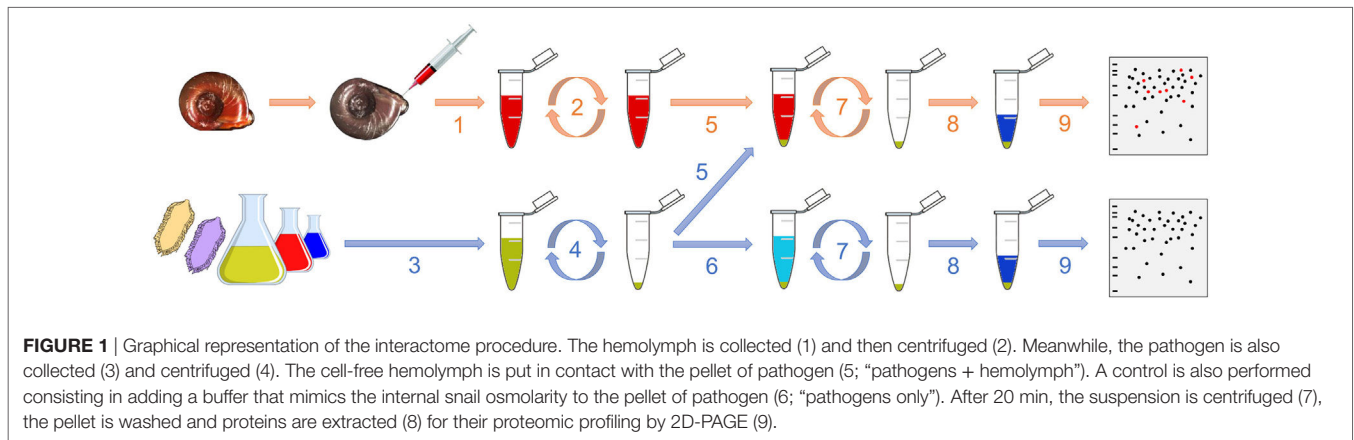
Snail Rearing

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. The laboratory and experimenters possessed an official certificate from 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).

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 $2,000 \times g$ 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 $2,000 \times g$ for 10 min and used as a control for intact cells; 2. hemocytes vortexed and centrifuged at $2,000 \times g$ for 10 min corresponded to the hemolymph preparation procedure of the interactome; 3. hemolymph sonicated (70% for 5 s) and then centrifuged ($2,000 \times g$ 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 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 miracidia 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 μ 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 μ L of ammonium bicarbonate (25 mM) and it was incubated at room temperature for 30 min on a rocking agitator (500 rpm). Finally, 150 μ L of a solution of ammonium bicarbonate (12.5 mM) and acetonitrile (50%) was added to the spot. After 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 μ L of sequencing grade modified trypsin (Promega) and incubated on ice for 30 min. The excess of trypsin was discarded and 50 μ L of ammonium bicarbonate (25 mM) was added. Digestion was performed overnight at 30°C. The 50 μ 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 μ 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 μ 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 μ 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 (3–75% 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 mm long \times 75 μ 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 MS_n scans with the same precursor at \pm 30 s retention time window and \pm 50 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 (FDR \leq 1) for peptide and protein cutoff (-logP \geq 20 and number of unique peptides \geq 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 μ 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 μ 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 TBS (500 mM Tris-HCl, 1.5 M NaCl, 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 (mAbGEa, 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 5 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).

Yeast Clotting by Incubation with Cell-Free Hemolymph

Yeast cells were cultured in Sabouraud medium as described above. They were washed twice with CBSS. Yeasts were then resuspended either in CBSS or in cell-free hemolymph for 20 min or 3 h. Preparations were deposited on microscope slides for plating and were then labeled with DAPI and phalloidin as described above. They were observed using a Zeiss LSM 700 microscope.

RESULTS AND DISCUSSION

An Original and Simple Method

Generally, the identification of host molecules that can bind or recognize a set of pathogen determinants is performed by global pull-down assays. Such global interactome approach consists in the incubation of native or denatured protein extracts from both the host and the pathogen. The resulting interacting protein complexes are then separated through differential centrifugation steps, revealed by SDS-PAGE and identified by MS (27–29). Although powerful, this strategy suffers from several flaws, mainly associated with the extraction procedure itself which might (i) affect the nature of protein interactions by changing their conformation and (ii) promote forced interaction between proteins that would not encounter each other *in vivo*. Therefore, a part of the interactions observed can be essentially artificial and experimentally biased. To bypass these problems, we propose a new and simple interactome procedure in a cell-free hemolymph context that tends to mimic biological interactions between pathogens and soluble host proteins (Figure 1). Indeed, entire living pathogens were exposed to circulating humoral factors already present in cell-free hemolymph freshly extracted from naïve snails and they were incubated at 26°C, which corresponds to the environmental and internal temperature of this ectothermic organism. Therefore, only proteins present at the surface of the pathogen are recognized in a biologically realistic context. Moreover, the short time (20 min) chosen allows focusing exclusively on the very first step of innate immune response and avoiding the pathogen to respond to the attack from the immune factors, which could affect pathogens' proteomic profiles and bias the analysis. As a control, only spots that were present in the three “pathogen + hemolymph” replicates and absent in the three “pathogen only” replicates were considered for the analysis of each pathogen studied.

Each MS/MS profile was confronted to both the databases of the host and of the pathogen. This ensured that the approach reliably enabled the identification of host's interacting molecules while limiting the risk of false positives. No significant matches were observed against any of 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.

Identification of a Large Variety of Interacting Proteins

This approach allowed the identification of a total of 109 spots exclusively identified in “pathogen + hemolymph” samples for the five pathogens tested (Figure 2; Figure S1 in Supplementary Material). These spots provided a significant match to 34 unique accession numbers, referring to 23 different proteins (Table S1 in Supplementary Material). Each pathogen was recognized by a specific, but overlapping, set of circulating proteins in mollusk's hemolymph (Figure 3). Specific recognition proteins to a given pathogen must be expected since each class of pathogen express at their surface specific and different structural motif also called pathogen-associated molecular patterns (PAMPs). The best known PAMPs are lipopolysaccharide from Gram-negative bacteria, lipoteichoic acid or peptidoglycan from Gram-positive bacteria, mannan-derived molecules or glycan from fungi, and fucosylated or glycoprotein receptors from *Schistosoma* sp. (30). Surprisingly, we identified numerous proteins not known to be involved in pathogen recognition and/or killing (extracellular matrix proteins, protease, and carbohydrase enzyme). Considering that some of these proteins are generally considered as intracellular molecules, a possible explanation for their presence could be that host's cells were damaged during the hemolymph collection (although non-invasive) and/or during the centrifugation step. A dual-staining with DAPI and phalloidin of hemocytes revealed no difference between fresh hemocytes and vortexed ones that were intact, as compared to sonicated hemocytes that were totally disrupted (Figure 4). This confirmed that the procedure of preparation of cell-free hemolymph did not damage the cells and that all interacting proteins from the snails were naturally present in the extracellular compartment of the hemolymph.

We, thus, propose to classify the snail interacting proteins identified into three different categories based on their nomenclature and known primary function: (i) molecules previously described as primary pathogen recognition molecules able to trigger an immunological response, with potential additional lytic activity [immune recognition factors (IRF)], (ii) proteins whose primary role is not pathogen sensing but are involved in other physiological functions [non-canonical proteins interacting with pathogens (NCIP)], and (iii) enzymes implicated in the metabolism of a wide range of molecules enzymes interacting with pathogens (EIP).

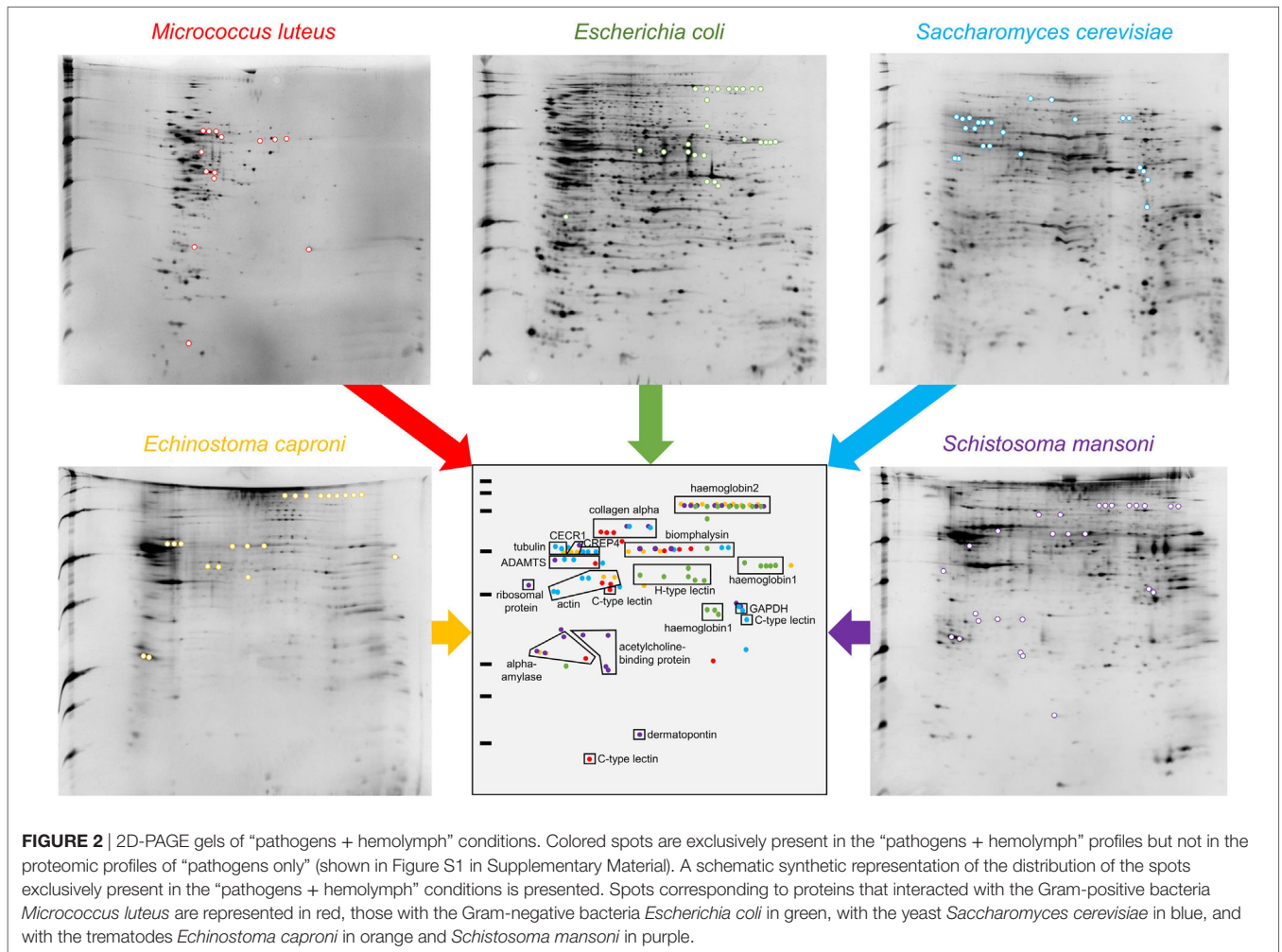


FIGURE 2 | 2D-PAGE gels of “pathogens + hemolymph” conditions. Colored spots are exclusively present in the “pathogens + hemolymph” profiles but not in the proteomic profiles of “pathogens only” (shown in Figure S1 in Supplementary Material). A schematic synthetic representation of the distribution of the spots exclusively present in the “pathogens + hemolymph” conditions is presented. Spots corresponding to proteins that interacted with the Gram-positive bacteria *Micrococcus luteus* are represented in red, those with the Gram-negative bacteria *Escherichia coli* in green, with the yeast *Saccharomyces cerevisiae* in blue, and with the trematodes *Echinostoma caproni* in orange and *Schistosoma mansoni* in purple.

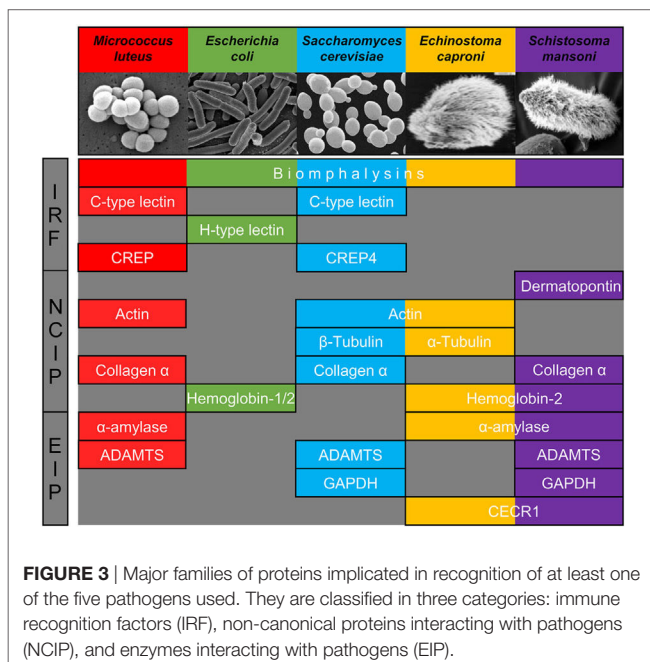


FIGURE 3 | Major families of proteins implicated in recognition of at least one of the five pathogens used. They are classified in three categories: immune recognition factors (IRF), non-canonical proteins interacting with pathogens (NCIP), and enzymes interacting with pathogens (EIP).

Pathogen Sensing by Soluble Immune Receptors and Atypical Toxins (IRF)

Among the IRE, two different families of proteins are identified: lectins and biomphalysin (Figure 2). Lectins represent a large family with a wide variety of evolutionarily conserved structures and some of them have been described as involved in immune 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

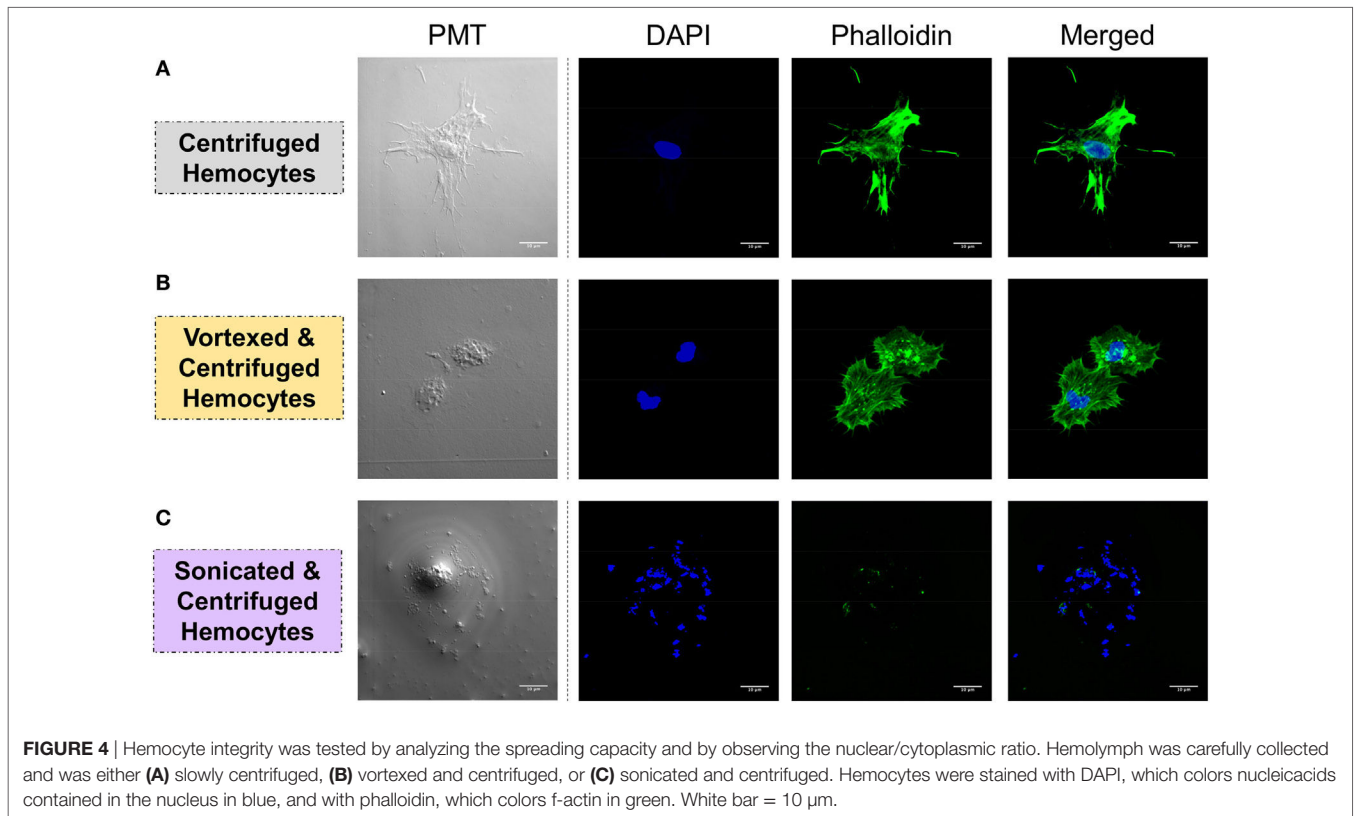


FIGURE 4 | Hemocyte integrity was tested by analyzing the spreading capacity and by observing the nuclear/cytoplasmic ratio. Hemolymph was carefully collected and was either **(A)** slowly centrifuged, **(B)** vortexed and centrifuged, or **(C)** sonicated and centrifuged. Hemocytes were stained with DAPI, which colors nucleicacids contained in the nucleus in blue, and with phalloidin, which colors f-actin in green. White bar = 10 μ m.

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 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 intramolluscan 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 β -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 destructure 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

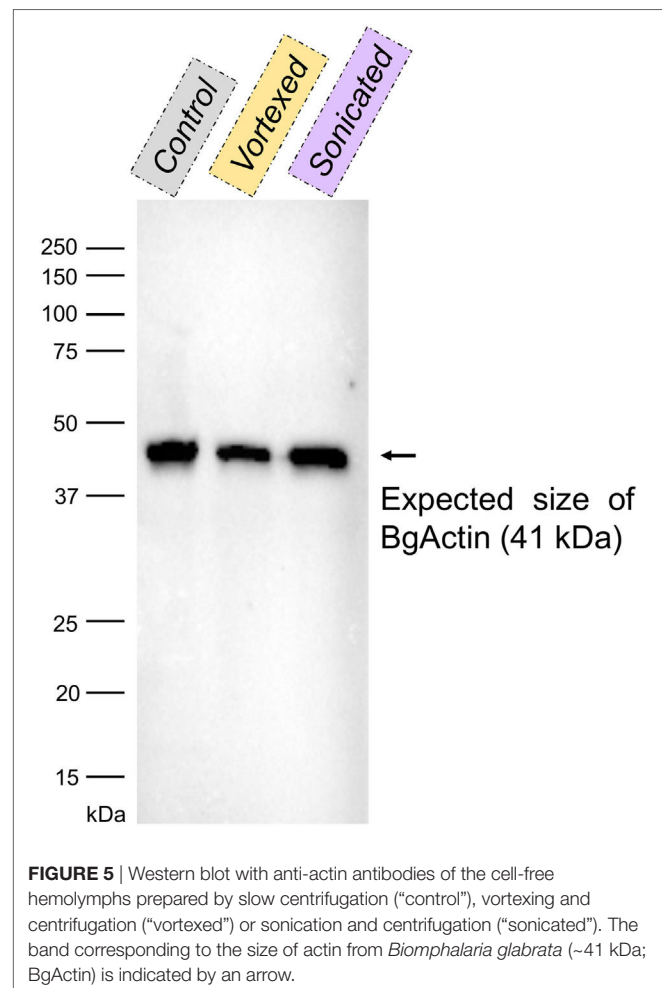


FIGURE 5 | Western blot with anti-actin antibodies of the cell-free hemolymphs prepared by slow centrifugation (“control”), vortexing and centrifugation (“vortexed”) or sonication and centrifugation (“sonicated”). The band corresponding to the size of actin from *Biomphalaria glabrata* (~41 kDa; BgActin) is indicated by an arrow.

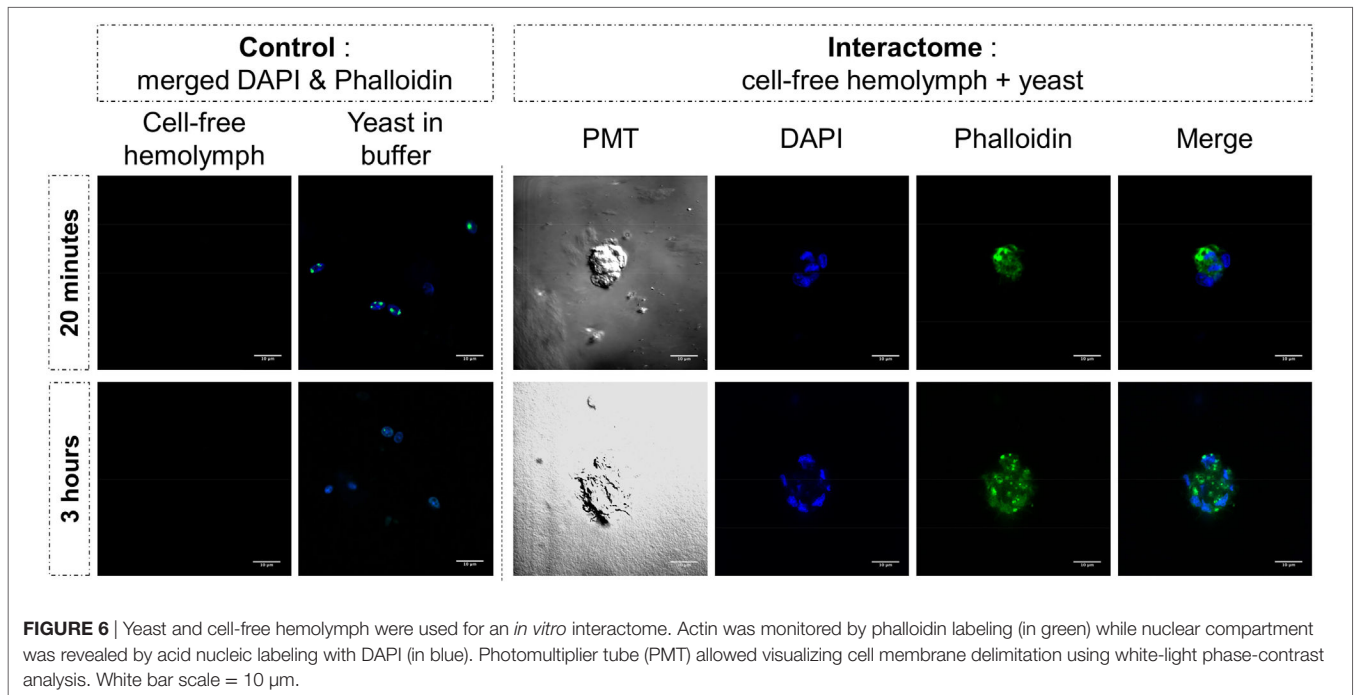


FIGURE 6 | Yeast and cell-free hemolymph were used for an *in vitro* interactome. Actin was monitored by phalloidin labeling (in green) while nuclear compartment was revealed by acid nucleic labeling with DAPI (in blue). Photomultiplier tube (PMT) allowed visualizing cell membrane delimitation using white-light phase-contrast analysis. White bar scale = 10 μ m.

functionality that can be liberated from larger proteins are named cryptides. Those derived from 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 role of molecules that interacted with pathogens since they were constitutively present in hemolymph of uninfected (naïve) snails. Indeed, most of the studies are based on the differential analysis (i.e., uninfected vs infected, or infected by different pathogens) of the host immune response (efficient or not) leading to a list of genes whose immunological function is rarely demonstrated. Moreover, if functional invalidation (gene knock-out, siRNA-mediated gene silencing, and mutants) already demonstrated the requirement of such molecules during the immune response, the first step of pathogens binding is still rarely studied (11, 12, 33).

Each pathogen was recognized by a specific, although partially overlapping set of interacting proteins from the mollusk (Figure 3; Table S1 in Supplementary Material). Most of pathogens' perception involved at least three different families of proteins from two of the three protein categories described (Figure 3). Such contrasting sets of binding proteins, in terms of diversity and quantity, suggest that specificity of immune detection quickly occurs at a fine scale. The recognition of the same pathogen by several different sensors with a high degree of specificity suggests that these molecules are part of different host defense pathways that can interact with each other (1). Such interactions can take three different forms: by cooperation, leading to the more efficient engagement of the same effector mechanism, by complementation, allowing to trigger different complementing effector mechanisms or by compensation, where one pathway compensates the deficiency of another one (59). The real involvement of these proteins in pathogen recognition, as expected in parasite antigen/host receptor interaction, is still not demonstrated and will require specific investigation of downstream process for each candidate identified. Thus, these pathways might contribute to assess the danger for which they have been exposed and leading *in fine* to discriminate symbiotic organisms from pathogens (60). Simultaneous activation of distinct recognition pathways would enable a concerted and appropriate response to tolerate or eliminate such or such intruder. Another aspect of the molecular interaction not yet described and evaluated in invertebrates is the temporal dynamic of pathogen perception by soluble immune factors. Can this recognition be immediate and frozen once and for all, or require gradual biochemical and structural maturation to recruit other more specialized immune factors? The dynamic of structuration of soluble immune complexes by analyzing interactome at different times must be explored to answer this question. In this study, we show that different biochemical interactions between the external surface of pathogens and host molecules occur within just 20 min of interaction. This supports the idea of a first wave of pathogen detection that we called "sensing," a prerequisite for the subsequent activation of immune system. This sensing step appears additive but also epistatic by the number of various biological functions involved and suggests a cooperative crosstalk for a specific immune response (1). The relative function of the IRE, NCIP, and EIP, whether they are implicated in pathogen recognition, immune complex maturation, and/or triggering of immune response, will require further investigation. The method developed herein allowed reaching the early step of pathogen sensing, validating the binding ability of

several IRE, and opening opportunity in model systems to deeper study their activity in the immune response pathways.

In summary, the present data constituted an important step toward a better understanding of the pathogen sensing and immune specificity in invertebrates. It clearly demonstrates that innate immune response in invertebrate is not supported by a unique class of immune factors but rather by a panel of molecules involved in diverse biological functions and able to bind specifically to a range of distinct pathogens. Notably, it involves some dual immune proteins able to play a role in both pathogen binding and clearance. This work does not intend to provide an extensive description of all sensing molecules but it definitely opens the way to a better integrative biological overview of molecules necessary to initiate an orchestrated immune response against pathogens in both model and non-model organisms.

AUTHOR CONTRIBUTIONS

GT, SP, and DD designed the research; AP, RG, and BG substantially participated in conception and improvement of research; SP, AP, and DD performed interaction experiments; GT performed the 2D-SDS-PAGE experiments and qualitative analysis; SP and AP performed the Western blots, fluorescent labeling, and microscope observations; all authors contributed to the analysis and interpretation of the results; GT, SP, and DD led the manuscript writing; all authors participated to manuscript writing, editing, and critical reviewing; and they all approved the final draft.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://www.frontiersin.org/article/10.3389/fimmu.2017.01249/full#supplementary-material>.

FIGURE S1 | 2D-PAGE gels of "pathogens + hemolymph" and "pathogens only" for each of the five pathogens used. Arrows are indicating spots exclusively present in the "pathogens + hemolymph" profiles but not in the proteomic profiles of "pathogens only," which represents proteins from *Biomphalaria glabrata* hemolymph that participated in the recognition of pathogen's proteins.

TABLE S1 | Protein identification of the 109 spots revealed only in "pathogens + hemolymph" gels as compared to "pathogens only" gels. For each spot, the $-10\log P$ values of proteins and peptides are indicated, together with the top BLAST hit in NCBI nr database, the conserved domains of the sequence retrieved (performed with NCBI CD-search available at <https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) and the pI and molecular mass (calculated with the ExPASy "Compute pI/Mw tool" available at http://web.expasy.org/compute_pi).

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Chapitre 1

Acte1, Scène 1

« L'art de chercher l'inattendu, c'est de cela dont est faite la Science ».
Fotis Kafatos, 2008

➤ **Integrated Multi-Omic Analyses In *Biomphalaria-Schistosoma* Dialogue Reveal The Immunobiological Significance Of FREP-SmPoMuc Interaction.**

Portet A 🍏, Pinaud S 🍏, Tetreau G, Galinier R, Cosseau C, Duval D, Grunau C, Mitta G, Gourbal B.

Developmental and Comparative Immunology, 2017 doi: 10.1016/j.dci.2017.02.025

↪ **Specific Pathogen Recognition by Multiple Innate Immune Sensors in an Invertebrate**

Tetreau G 🍏, Pinaud S 🍏, Portet A 🍏, Galinier R, Gourbal B, Duval D.

Frontiers Immunology, 2017 doi: 10.3389/fimmu.2017.01249

➡➡ **Arm Race Between Endogenous miRNAs From Host And Circulating Xeno-Mirna Of Parasites.**

Pinaud S, Lassalle D, Chaparro C, Portet A, Allienne JF, Gourbal B, Duval D.

En préparation

Les résultats préliminaires qui devraient être intégrés à l'article ci-dessus sont placés dans la discussion de ce chapitre sous la forme de résultats/discussions.

🍏 premiers auteurs ➤ article publié ↪ article soumis ➡➡ article en préparation

Acte1, Scène 1 : Discussions

Le succès ou l'échec de l'interaction entre *Biomphalaria* et *Schistosoma*, une fois le filtre de rencontre passé, est dépendant d'un très grand nombre de facteurs dont certains sont encore inconnus aujourd'hui. Afin de faciliter l'étude de cette interaction, des expériences de protéomique massive ont été réalisées pour identifier les protéines à la fois de l'hôte et du parasite qui pouvaient interagir (Roger, Mitta et al. 2008). Dans cette approche, il était attendu de pouvoir identifier des équivalents fonctionnels d'antigènes et d'anticorps dans ce modèle invertébré (Mone, Gourbal et al. 2010). En comparant en particulier des profils protéomiques d'hôtes et de parasites avec des phénotypes très distincts « compatible, le parasite se développe » et « incompatible, le parasite ne se développe pas », il a été identifié que la seule différence observable était une protéine du parasite appelée SmPoMuc. Ces mucines ont été décrites pour être sécrétées par la glande apicale des sporocystes primaires et jouer un rôle dans la pénétration et la migration du parasite dans les tissus de l'hôte (Roger, Grunau et al. 2008, Roger, Mitta et al. 2008). Néanmoins, le rôle des SmPoMucs n'a jamais été clairement démontré fonctionnellement par une invalidation chez *Schistosoma* alors que les outils d'invalidation se perfectionnent chez cette espèce (He, Cai et al. 2012, Collins, Wang et al. 2013, Wang, Collins et al. 2013).

Suite à des expériences de co-IP dirigées contre ces mucines, plusieurs acteurs de l'interaction ont pu être caractérisés dont la TEP et les FREP. Si le premier pourrait avoir un rôle d'opsonine et être impliqué dans la genèse de la capsule hémocytaire, la caractérisation des FREP quant à elle demeure partielle. Cette famille très polymorphe et diversifiée, pourrait porter la fonction de reconnaissance des déterminants antigéniques de pathogène. Néanmoins, leur invalidation ne conduit jamais à expliquer 100 % des phénotypes et suggère fortement l'implication d'autres molécules. Dans ce contexte de nombreuses expériences globales ont essayé d'identifier de nouveaux partenaires moléculaires pouvant être impliqués dans ce dialogue moléculaire entre *Biomphalaria* et son parasite *Schistosoma*. Malheureusement, ces approches se sont heurtées à des limites expérimentales ne permettant pas de compléter définitivement le scénario de ces interactions.

En effet, la plupart de ces expériences d'interactome consistent souvent à extraire des protéines du pathogène et de l'hôte puis à les mettre en contact avant de les séparer par centrifugation différentielle, un contexte physiologique très éloigné de celle d'une interaction naturelle (Mone, Gourbal et al. 2010, Wu, Dinguirard et al. 2017). Or, la moindre de ces étapes pourrait à la fois forcer des protéines à interagir alors qu'elles ne pourraient biologiquement jamais se rencontrer (faux positif) ou au contraire masquer des interactions entre des protéines qui interagiraient naturellement (faux négatif). Ainsi, un protocole a été réalisé en mettant simplement en contact des pathogènes vivants avec de l'hémolymphe de *Biomphalaria glabrata*. Cette mise en contact se déroule à température physiologique de l'hôte (26°C) et

pendant un temps très court (20mn) pour ne capter que les interactions fortes de cette interaction complexe. Les différences entre pathogènes seuls et pathogènes incubés avec l'hémolymphe sont alors identifiées par protéomique en deux dimensions (2D-Page). Seuls les protéines surnuméraires par rapport à l'interaction avec le pathogène seul ont été identifiées en spectrométrie de masse. Parmi les cinq agents pathogènes testés (*Escherichia coli*, *Micrococcus luteus*, *Saccaromyces cerevisiae*, *Schistosoma mansoni* et *Echinostoma caproni*), nous avons identifié de nombreuses lectines, des Biomphalysines et de nombreuses autres molécules pour lesquelles une fonction primaire dans la reconnaissance immunitaire avait rarement été décrite.

En très peu de temps (20mn), de nombreuses protéines de types lectines et des molécules à double rôle reconnaissance/effecteur viennent se fixer à des déterminants du pathogènes et ce parfois, de manière spécifique en fonction de la nature même du pathogène. Cette reconnaissance entraîne rapidement la création d'un complexe immun auquel participe aussi des protéines non décrites comme ayant des fonctions de reconnaissance immunitaire. Il s'agit de variants d'hémoglobine, d'actine ou de collagène ou encore d'enzymes, dont le rôle est difficilement justifiable dans la reconnaissance immunitaire. La maturation de ce complexe immun pourrait être nécessaire pour déclencher une cascade supportant une réponse immunitaire efficace. Par exemple, un clivage de la protéine TEP pour laquelle un rôle d'opsonine est proposé, a été observée après 3h au contact de pathogène vivant (Portet, Galinier et al. en préparation) pendant la potentielle constitution/maturation du complexe immun.

Un des résultats surprenants de cette étude est l'absence des FREP dans ces résultats d'interactomes, quelle que soit la nature du pathogènes testée. Dans le cadre de l'interaction avec *Schistosoma*, il serait cohérent de tester la même procédure avec le même stade parasitaire ayant été utilisé dans l'étude précédente réalisée en 2010, à savoir des sporocystes primaires (Monet et al., 2010) et non pas avec des miracidia. Notre hypothèse concernant l'absence des FREP dans cette interaction est que *Biomphalaria* n'aurait pas investi dans la reconnaissance d'un stade transitoire, le stade miracidium. Des hypothèses d'écran de fumée immunogène (Roger, Mitta et al. 2008) voire d'immunosuppression causée en partie par la libération dans le plasma de molécules active pendant la perte des plaques ciliées au cours de la transformation miracidium/Sporocyste (Lodes and Yoshino 1990, Yoshino, Brown et al. 2014), viennent enrichir cette hypothèse.

Il serait également intéressant d'augmenter le temps d'interaction pour appréhender la dynamique de la formation de ces complexes à la surface des pathogènes. De même, des études protéomiques montrent une transformation majeure des protéines de surfaces et notamment leurs glycanes exposés à la surface du parasite, suggérant un changement de pattern de reconnaissance nécessaire pour l'hôte pour être capable d'identifier un miracidium ou un Sp1 (Peterson, Hokke

et al. 2009, Yoshino, Wu et al. 2012).

Avec le recul, il serait maintenant intéressant d'utiliser : (i) des temps d'interactions différents pour suivre la formation du complexe immun comme par exemple 10mn, 1h et 3h, (ii) utiliser des hémolymphes issues d'animaux primés voir challengés par différents pathogènes, (iii) utiliser des anticorps dirigés contre des récepteurs potentiels pendant des infestations naturelles pour essayer de modifier les capacités de liaison et donc de reconnaissance de l'hôte.

Concernant la dynamique de la formation de la capsule, il est intéressant de faire un parallèle avec les résultats de protéomique concernant la TEP. Au contact de pathogènes plus particulièrement 3h après cette interaction, la BgTEP subit un clivage supplémentaire (Portet, Galinier et al. soumis) qui pourrait avoir un rôle dans les capacités de reconnaissance de cette molécule.

De même, si la protéomique comparative par l'utilisation de gels 2D présente l'intérêt d'observer directement les spots surnuméraires, les protéines de très faibles ou de très hautes tailles ou faiblement représentées ont tendance à mal se focaliser et ont donc du mal à migrer. De même, les protéines présentant une taille et un point isoélectrique (IEF) identiques à une protéine de pathogène ne seront pas visibles sur un gel et ne seront pas identifiées. Une alternative pourrait être l'utilisation d'une méthode de séquençage massif des protéines appelée Label-Free. Cette méthode permet d'identifier plusieurs milliers de peptides par échantillons et à l'avantage de s'affranchir des défauts précédents. Elle possède malgré ses propres défauts dont la nécessité de séquencer à la fois les protéines de l'hôte et du pathogène. La complexité de l'échantillon de l'hôte pourrait alors masquer les différences surnuméraires observées par l'utilisateur de gels 2D.

Afin d'aller plus loin dans la caractérisation de ces complexes formés à la surface des pathogènes, il serait intéressant de réaliser des co-IP à l'aide d'anticorps dirigés contre les différents candidats identifiés dans cette étude comme par exemple les C-type lectines ou les Biomphalysines. Dans le même contexte, l'utilisation des agents pathogènes *Escherichia coli*, *Micrococcus luteus* et *Saccaromyces cerevisiae* pourrait être contestable et l'utilisation de la seule souche pathogène de bactérie identifiée chez *Biomphalaria* pourrait être proposée (Duval, Galinier et al. 2015).

L'exploration du compartiment hémolymphatique (hémocyte + plasma) de l'hôte a également permis de révéler la présence d'un acteur supplémentaire dans le dialogue moléculaire Bg/Sm qui n'est pas protéique mais nucléique : les microARN (miARN). Si la présence de miARN endogènes pouvait être attendue comme régulateur de la physiologie de l'hôte (Lindsay 2008, Vasudevan, Tong et al. 2008), la présence de miARN exogènes et circulants du parasite fut plus inattendue. Il était décrit que ces petits ARN exogènes pouvaient réguler la réponse immunitaire d'un hôte (Buck, Coakley et al. 2014) et que *Schistosoma* était capable de produire des miARN via des vésicules de sécrétions identifiées chez l'hôte vertébré (Nowacki, Swain et al. 2015), mais chez son hôte intermédiaire rien de tout cela n'avait été

démonstré.

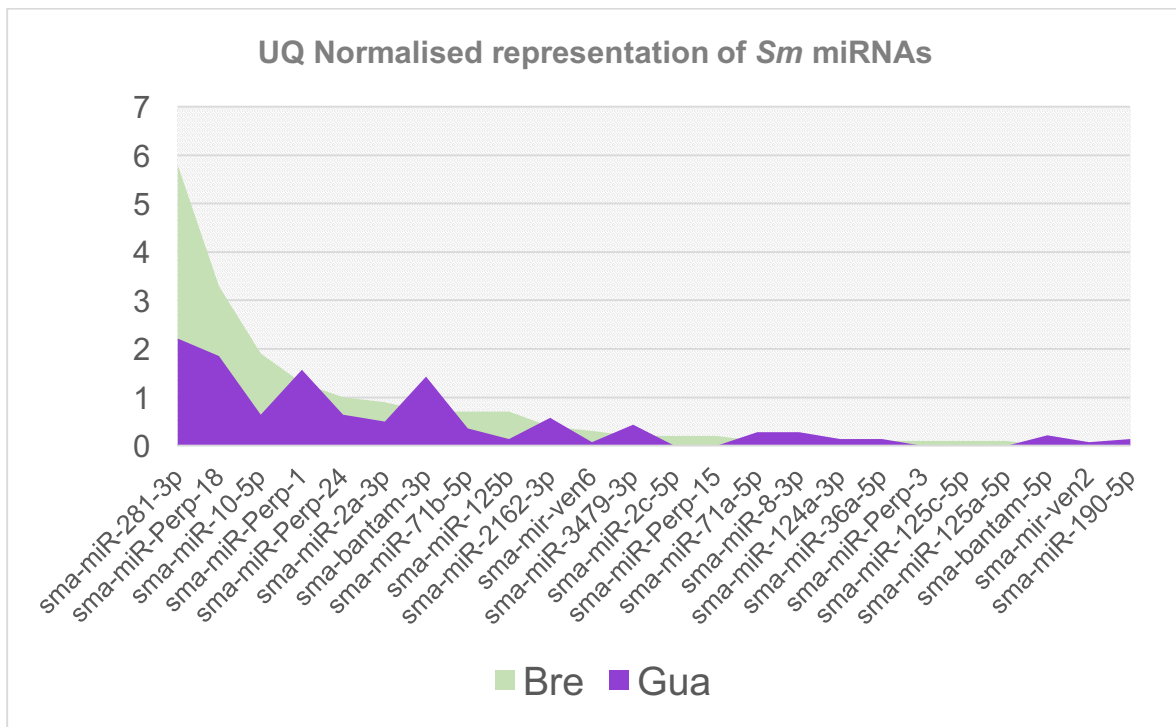


Figure 4. **Représentation des miARN de *Schistosoma* chez une souche SmBRE ou SmGUA.** Diagramme de représentation normalisées des transcrits de miRNA de *Schistosoma*. Chaque colonne correspond à l'expression d'un miRNA en réponse à une primo-infestation par un parasite SmBRE (100% compatible, vert) ou par un parasite SmGUA (5% compatible, violet).

En effet, jusqu'à aujourd'hui, aucun miARN exogènes circulants dans l'interaction *Biomphalaria/Schistosoma* n'avaient jamais été décrits. Ces miARN sont parfois appelés xéno-miARN pour leur provenance étrangère (latin xeno) à l'hôte. Dans ce travail, nous avons pu montrer en comparant des couples où le succès d'interaction est différent (compatible BgBRE/SmBRE et incompatible BgBRE/SmGUA) que la stratégie du parasite en terme d'expression de répertoires de miARN variait fortement.

En effet, les parasites sympatriques libèrent des ARN non-codants dont la séquence a été sélectionnée pour interférer avec des éléments de la réponse immunitaire de leur hôte (Figure 4). À l'inverse, les parasites allopatriques, ne produisent pas la même diversité de miARN (Pinaud, Lassalle *et al.*, en préparation). Cette diversité pourrait se retrouver également dans la nature des cibles potentielles de ces miARN lorsqu'on les prédit avec des algorithmes tel que miranda. En effet, leur analyse montre que le parasite sympatrique serait capable de cibler chez l'hôte une plus grande diversité de transcrits impliqués dans la réponse immunitaire que son homologue allopatrique.

Le même travail est a réalisé sur les miRNA de l'hôte et il sera important d'étudier l'action croisée des miRNA de l'hôte sur le parasite et inversement.

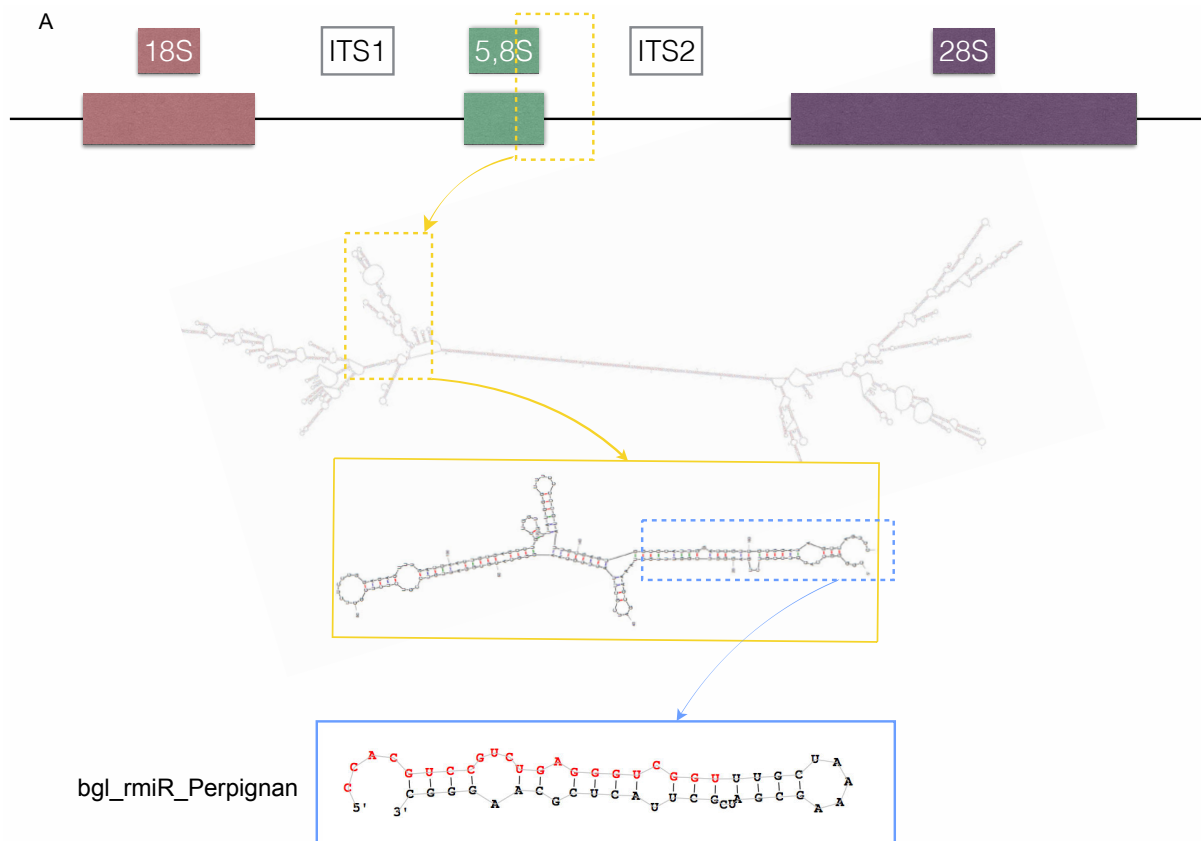


Figure 5. Cas particulier du r-miR-Perpignan (en rouge). Le bgl_rmiR_Perpignan voit la tige-boucle de son précurseur (cadre bleu) être positionnée à l'interface entre la portion 5,8S et les l'ITS2 de l'ARN ribosomique de *Biomphalaria glabrata* (cadres jaune).

Ce plan expérimental a également permis de prédire un nouveau miARN de *Biomphalaria* qui a la particularité d'être localisé dans un ARN ribosomique (Figure 5), on parle de r-miARN, tout en ayant un rôle potentiel dans l'interaction hôte/parasite.

Ce plan expérimental, bien que suffisant pour obtenir des premiers résultats très intéressants, mériterait d'être étoffé pour caractériser la dynamique d'excrétion de ces différents miARN pendant l'interaction. Ainsi, une extraction de petits ARN des différents stades parasitaires pourrait être proposée dans le but de les injecter à des hôtes naïfs et ainsi voir si leurs capacités de résistance sont altérées par des potentiels petits ARN transférés.

Un travail de confirmation des cibles prédites par des algorithmes d'interactions est maintenant nécessaire pour aller plus loin dans la caractérisation de ces éléments régulateurs. Pour cela, il pourrait être proposé d'utiliser des systèmes hétérologues pourvus de la machinerie de biogénèse des miARN (comme les cellules HeLa) où à la fois le miARN et la cible potentielle sont fusionnés avec des gènes rapporteurs différents qui pourraient être transfectés (GFP, Cherry). En cas d'interaction avérée entre le miARN et la séquence cible, le signal de fluorescence porté par le transcrit présentant la séquence cible serait moins intense

(Thomson, Bracken et al. 2011).

La validation des cibles prédites, associée à la caractérisation transcriptomique des miARN de l'hôte et du parasite permettra à l'avenir de soumettre un article sur le rôle potentiel des acides nucléiques du type miARN dans le dialogue moléculaire entre *Biomphalaria* et *Schistosoma*.

Ce chapitre révèle que l'interaction *Biomphalaria/Schistosoma* est dépendante de bien plus de facteurs que simplement des récepteurs immuns de l'hôte et des facteurs de virulence du parasite, tels que les FREP et les SmPoMuc. Cette interaction dépend également d'un grand nombre de lectines (FREP, C-type, H-lectine), de protéines capables de lier un complexe immunitaire et pourtant précédemment peu discuté dans la réponse immunitaire (hémoglobine, collagène), d'enzymes capables d'assurer la maturation de ce complexe pour amorcer la réponse immunitaire, d'acide nucléique circulant. De nombreux facteurs sont peut-être toujours à déterminer comme les modifications post-transcriptionnelles nécessaires à l'activation des protéines potentiellement responsables de la reconnaissance immunitaire comme la phosphorylation ou l'ubiquitinylation.

Chapitre 2

Résister à l'opresseur

« L'art de chercher l'inattendu, c'est de cela dont est faite la Science ».
Fotis Kafatos, 2008

Biomphalaria possède la particularité de devenir résistant à la suite d'une première infestation : quel est le support moléculaire de cette résistance et de cette spécificité ?

➤ **A shift from Cellular to Humoral Responses Contributes to Innate Immune Memory in the Vector Snail *Biomphalaria glabrata*.**

Pinaud S, Portela J, Duval D, Nowacki FC, Olive MA, Allienne JF, Galinier R, Dheilily NM, Kieffer-Jaquinod S, Mitta G, Théron A, Gourbal B.
PLoS Pathogens, 2016 doi: 10.1371/journal.ppat.1005361

↻ **Polymorphic sets of immune receptors support specificity in the innate immune memory of the Schistosomiasis vector snail, *Biomphalaria glabrata*.**

Pinaud S, Portet A, Portela J, Allienne JF, Saint-Beat C, Arancibia N, Galinier R, Duval D, Gourbal B.
En preparation pour Molecular Ecology

↻ **Diversified aerolysin toxins in the vector snail *Biomphalaria glabrata*: an innovative family of immune weapons acquired by horizontal.**

Pinaud S, Tetreau G, Portet A, Chaparro C, Galinier R, Gourbal B, Duval D.
Soumis dans Genome Biology, Automne 2017

➤ article publié ↻ article en préparation

Dès la fin des années 1990, l'équipe d'André Théron qui s'intéresse alors aux interactions écologiques entre *Biomphalaria* et *Schistosoma* sur le terrain, s'est aperçu que dans une certaine interaction entre un escargot infesté par son parasite sympatrique Brésilien *BgBRE/SmBRE*, il était impossible d'infester à nouveau un escargot par la même souche de parasite (Sire, Rognon et al. 1998). Cette observation de ce nouveau phénotype était particulièrement intéressante dans la mesure où sur le terrain, il était souvent considéré que la très faible prévalence chez les escargots infestés devait s'expliquer par un filtre de rencontre très étroit entre l'hôte intermédiaire et le parasite, et qu'à la suite d'une rencontre plutôt rare, seul le polymorphisme de compatibilité pesait sur le succès ou l'échec de l'infestation. Récemment pourtant, de nouveaux résultats montrent dans des zones d'endémies que des escargots non-émetteurs de cercaires sont pourtant positifs par diagnostic PCR. Ceci suggère que ces mollusques ont été exposés plus fréquemment que suspecté jusqu'à alors, à une grande diversité de parasites en réussissant tout de même à ne pas être infestés (Allan, Dunn et al. 2013). Cela pourrait suggérer qu'une nouvelle composante puisse jouer un rôle dans la transmission du parasite sur le terrain, la capacité pour l'hôte à devenir résistant à une nouvelle infestation.

Deux hypothèses contraires ont alors été proposées pour tenter d'expliquer ce phénomène : (i), soit il existe une résistance immunitaire acquise de l'hôte à la suite de la première stimulation, ou alors, (ii) il s'agit d'une compétition parasitaire entre le premier parasite infestant et les prochains tentant de coloniser la niche écologique du premier (Sire, Rognon et al. 1998). Afin d'infirmer l'une ou l'autre de ces hypothèses, de nombreuses expérimentations ont été menées pour stimuler l'immunité de l'hôte sans dépendre du développement du parasite de la primo-infection. L'utilisation de particules d'or pour mimer les lésions faites lors de la migration de sporocystes secondaires pouvant expliquer une réaction inflammatoire responsable de cette résistance acquise. L'utilisation de miracidia irradiés aux U.V. capables d'infester le mollusque mais incapables de se développer en sporocystes primaires n'induisent aucune résistance acquise (Portela, Duval et al. 2013). A l'inverse, l'injection d'interleukine 1 humaine (Connors, de Buron et al. 1998) ou d'extrait parasitaire induit une résistance significative chez le vecteur (Portela, Duval et al. 2013) montrant que cette résistance peut être portée par des molécules propres à l'hôte et très certainement liée à la réponse immunitaire. D'ailleurs, ce phénotype de résistance est d'autant plus important que la distance génétique séparant les parasites utilisés pour la première et la seconde stimulation est faible voire nulle. Cette résistance est qualifiée de priming génotype-dépendant (Portela, Duval et al. 2013). Ainsi, lorsqu'on utilise la même souche pour les deux stimulations, on parle de priming homologue, une résistance totale est observée au

challenge immunitaire (à la deuxième rencontre). Par contre cet effet protecteur diminue lorsqu'on augmente la distance génétique neutre entre le parasite utilisé pour la première rencontre et celui utilisé pour la deuxième rencontre ; dans le cadre d'un priming hétérologue. Le changement d'espèce de Schistosome (SmBRE - > SROD) diminue encore de moitié la protection associée à la première rencontre (Portela, Duval et al. 2013).

➤ Une étude incluant de la transcriptomique massive (RNAseq), de la protéomique comparative (2D-PAGE) et une invalidation fonctionnelle, a montré que cette résistance acquise était portée par un « shift » (une bascule) immunitaire qui permettait aux escargots de modifier profondément la nature de la réponse de défense qu'ils expriment entre la primo-infection et le challenge (Pinaud, Portela et al. 2016). En effet, lors d'une première rencontre, une réponse cellulaire d'encapsulation par les hémocytes est observée, alors que lors d'une seconde rencontre, les parasites sont éliminés par un processus humoral puisque aucune capsule n'est alors observée. L'analyse transcriptomique a d'ailleurs montré que ce shift se traduisait par une régulation atypique où peu de gènes sont induits lors de la première stimulation alors qu'un large cluster de gènes spécifiques est différentiellement régulé lors de la seconde rencontre. Dans le cas d'une compétition larvaire entre parasites, des facteurs protéiques de parasites auraient pu être attendus dans le plasma du mollusque, mais les analyses protéomiques n'en ont relevé aucun. Cette absence de facteurs protéiques de parasite dans le plasma du mollusque renforce l'idée d'une acquisition de la résistance par un processus immunitaire de l'hôte indépendant de toute action ou effet du parasite primo-entrant.

↳ Dans le cas d'une réponse génotype-dépendant, nous avons pu montrer que de nombreuses molécules « récepteurs potentiels de pathogènes » (lectines, des protéines de reconnaissances de peptidoglycanes, des récepteurs portant des répétitions riches en leucine, etc.) ou effecteurs comme la biomphalysin, étaient spécifiquement régulées en fonction du pathogène utilisé pour le challenge. La complexité et diversité de ces répertoires pourraient supporter le phénotype de résistance totale observé spécifiquement dans les interactions homologues (Pinaud, Portet et al., en préparation).

Cette résistance acquise ne semble pas non plus être médiée par une modification des populations cellulaires. En effet, l'analyse des populations cellulaires par cytométrie en flux (FACS) montre qu'il n'y a pas de différences dans les types de populations cellulaires disponibles dans le plasma à la suite d'une première ou seconde rencontre avec le parasite. Cela semble indiquer que cette résistance acquise et la spécificité qui en découle pourraient être portées non pas par une nouvelle communauté cellulaire, mais par une reprogrammation d'un sous-type cellulaire hémocytaire.

↳ Découverte lors d'expérience de protéomique, la biomphalysin une toxine

de type beta-Pore Forming Toxin (β -PFT) proche structurellement des aérolysines d'*Aeromonas hydrophyla* (Galinier, Portela et al. 2013) possède une activité antiparasitaire en créant des pores dans les membranes cellulaires. Son activité lytique est améliorée par la présence d'un facteur plasmatique encore non caractérisé (Galinier, Portela et al. 2013). Par des approches transcriptomiques et protéomiques, nous avons caractérisé plusieurs isoformes de cette biomphalysin. C'est la raison pour laquelle nous nous sommes aussi focalisés sur la caractérisation de cette nouvelle famille. Des analyses bio-informatiques de recherche de domaine (Pfam) sur le génome en cours d'annotation (Adema, Hillier et al. 2017) ont pu révéler la présence de 23 membres de la famille des Biomphalysine chez *Biomphalaria glabrata* (Pinaud, Tetreau et al, soumis).

L'ensemble de ces résultats apportent les premières bases moléculaires capables d'expliquer les mécanismes impliqués dans la résistance acquise de *Biomphalaria* à la suite d'une première infestation par *Schistosoma*. Ils décrivent particulièrement l'implication de molécules de reconnaissance et leurs potentielles combinaisons avec des effecteurs d'un nouveau genre chez les invertébrés pouvant être à l'origine d'un haut niveau de spécificité de cette immunité innée mémoire chez *Biomphalaria glabrata*.

Chapitre 2

Résister à l'opresseur

➤ **A shift from Cellular to Humoral Responses Contributes to Innate Immune Memory in the Vector Snail *Biomphalaria glabrata*.**

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↻ Polymorphic sets of immune receptors support specificity in the innate immune memory of the Schistosomiasis vector snail, *Biomphalaria glabrata*.

Pinaud S, Portet A, Portela J, Allienne JF, Saint-Beat C, Arancibia N, Galinier R, Duval D, Gourbal B.

En preparation pour Molecular Ecology, Été 2017

↻ Diversified aerolysin toxins in the vector snail *Biomphalaria glabrata*: an innovative family of immune weapons acquired by horizontal.

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RESEARCH ARTICLE

A Shift from Cellular to Humoral Responses Contributes to Innate Immune Memory in the Vector Snail *Biomphalaria glabrata*

Silvain Pinaud¹, Julien Portela¹, David Duval¹, Fanny C. Nowacki^{1,2}, Marie-Aude Olive¹, Jean-François Allienne¹, Richard Galinier¹, Nolwenn M. Dheilly^{1,3}, Sylvie Kieffer-Jaquinod⁴, Guillaume Mitta¹, André Théron¹, Benjamin Gourbal^{1*}

1 University of Perpignan Via Domitia, IHPE UMR 5244, CNRS, IFREMER, University of Montpellier, Perpignan, France, **2** Institute of Biological, Environmental & Rural Sciences, Aberystwyth University, Aberystwyth Ceredigion, United Kingdom, **3** School of Marine and Atmospheric Sciences, Stony Brook University, Stony Brook, New York, United States of America, **4** Plate-forme d'analyses protéomiques EDyP-Service, Laboratoire de Biologie à Grande Echelle UMR-S 1038 Inserm/CEA/UJF CEA, Grenoble, France

* benjamin.gourbal@univ-perp.fr



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Abstract

Discoveries made over the past ten years have provided evidence that invertebrate anti-parasitic responses may be primed in a sustainable manner, leading to the failure of a secondary encounter with the same pathogen. This phenomenon called “immune priming” or “innate immune memory” was mainly phenomenological. The demonstration of this process remains to be obtained and the underlying mechanisms remain to be discovered and exhaustively tested with rigorous functional and molecular methods, to eliminate all alternative explanations. In order to achieve this ambitious aim, the present study focuses on the Lophotrochozoan snail, *Biomphalaria glabrata*, in which innate immune memory was recently reported. We provide herein the first evidence that a shift from a cellular immune response (encapsulation) to a humoral immune response (biomphalysin) occurs during the development of innate memory. The molecular characterisation of this process in *Biomphalaria/Schistosoma* system was undertaken to reconcile mechanisms with phenomena, opening the way to a better comprehension of innate immune memory in invertebrates. This prompted us to revisit the artificial dichotomy between innate and memory immunity in invertebrate systems.

Author Summary

Schistosomiasis is the second most widespread tropical parasitic disease after malaria. It is caused by flatworms of the genus *Schistosoma*. Its life cycle is complex and requires certain freshwater snail species as the intermediate host. Given the limited options for treating *S. mansoni* infections, much research has focused on a better understanding of the immunobiological interactions between the invertebrate host *Biomphalaria glabrata* and its parasite *S. mansoni*. Recently, we demonstrated the existence of a time-dependent and

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genotype-dependent acquired innate immune memory in *B. glabrata* snails. A primo-infection of the Lophotrochozoan vector snail, *Biomphalaria glabrata*, with *Schistosoma mansoni* totally protected the snail against a secondary challenge. Learning more about the immunobiological interactions between *B. glabrata* and *S. mansoni* could have important socioeconomic and public health impacts by changing the way we attempt to eradicate parasitic diseases and prevent or control Schistosomiasis in the field.

Introduction

The environment of an invertebrate is filled with complex and changing populations of microorganisms, including potential pathogens. This engenders selective pressures comparable to those experienced by Gnathostomes [1], and means that invertebrates should possess sophisticated immune systems capable of dealing with such pathogens. Indeed, recent studies have shown that the immune defenses of invertebrates are more complex and specific than previously thought, and the existence of innate immune memory or priming has been suggested [2–6].

To date, the observations of invertebrate innate immune memory have been mainly phenomenological and based on ecological or phenotypic studies, and little work has addressed the potential molecular and cellular mechanisms underlying these processes. The innate immune system of invertebrates includes the barrier functions of the epithelium, the cellular immune response and the humoral defense response [7,8]: the former refers to mucosal immunity and hemocyte responses (phagocytosis, encapsulation, melanization), while the latter includes pathogen recognition receptors (PRRs), antimicrobial peptides, coagulation, and the production of cytolytic molecules or reactive intermediates of oxygen and nitrogen [7,8]. During the primary infection of an invertebrate, the pathogen is recognized at the first encounter and the cellular and humoral defense responses are coordinated to neutralize the intruder. To the best of our knowledge, the existing studies investigating the molecular mechanisms of innate immune memory in invertebrates have all suggested that the cellular immune response and/or hemocyte phagocytosis is/are improved upon a subsequent encounter with the same pathogen [9–11]. For example, in *Porcellio scaber*, enhanced phagocytosis was demonstrated after a first encounter with different bacteria species (*Bacillus thuringiensis*/*Escherichia coli*) [12]. For *Anopheles gambiae*, hemocytic differentiation evidenced by increased mRNA levels of hemocyte-specific genes, such as thioester-containing protein 1 (TEP1) and leucine rich repeat immune protein 1 (LRIM1) was reported following initial exposures to *Plasmodium* and bacteria [5]. In shrimp, bacterial challenge was followed by an enhanced cellular immunity characterized by a significant increase in the percentage of phagocytic cells [13]. The innate immune memory response in invertebrates has been previously described as being involved in two mechanisms, namely:

- i. a process of acquired resistance or sustained response that consists of a long-lasting protection against a later challenge that persists even if the pathogen is neutralized; or
- ii. a recalled response that consists of the ability to store information of previously met pathogens and recall it later to generate a faster and more powerful response against a subsequent exposure to the same pathogen.

However, the international community working on invertebrate innate immunity believes that the existing innate immune memory observations cannot be used in isolation. Instead, they should be used to construct hypotheses that need now to be exhaustively tested with

rigorous functional, cellular, biochemical and molecular methods, until all alternative explanations are eliminated [10,14]. To fully understand the capabilities of invertebrate immune systems, we must use global molecular approaches at the whole-organism level to investigate the mechanisms that form the basis for innate immune memory, while being guided by the existing observations and phenotypic descriptions. Without this demonstration, the existence of immune memory process in invertebrates will remain controversial and doubted by many immunologists [for the polemic, see [14,15]].

Recently, we demonstrated that a primo-infection of the Lophotrochozoan vector snail, *Biomphalaria glabrata*, with *Schistosoma mansoni* protected completely the snail against a homologous secondary challenge [4]. Total protection was reached 10 days after primo-infection, and was maintained for the rest of the snail's lifespan. Our findings provided evidence for the existence of a time-dependent acquired innate immune memory in *B. glabrata* snails [4,16]. Moreover, we used homologous and heterologous challenges to demonstrate that this innate immune memory was genotype-dependent, in that the protection decreased with increasing neutral genetic distance between the parasites used for the primo-infection and the secondary challenge [4].

Here, we sought to investigate the molecular mechanisms of innate immune memory in *B. glabrata* in response to *S. mansoni* infections. We precisely describe the snail's immune response phenotypes, then characterize the molecular determinants involved in *B. glabrata* innate immune memory via a global transcriptomic approach and a targeted proteomic analysis of plasmatic factors performed in combination with RNA interference. This work provides the first global investigation of the molecular processes supporting innate immune memory in an invertebrate model. Moreover, *Biomphalaria* snails have an important role in the transmission of Schistosomiasis the second most widespread human parasitic disease after malaria causing 200,000 deaths annually. More emphasis on snail-related research, on the role of snails and parasite intramolluscan larval stages in transmission is essential for changing the way we attempt to eradicate parasitic diseases [17,18]. Learning more about the immunobiological interactions between *B. glabrata* and *S. mansoni* could have important socioeconomic and public health impacts by contributing to the discovery of new ways to prevent and/or control Schistosomiasis diseases by limiting the parasite in the field.

Materials and Methods

Further details are provided in [S1 Appendix](#) and [Fig 1](#).

Snail and parasite strains

The present work utilized a strain of *Biomphalaria glabrata* originating from Brazil (BgBRE), along with its 100% compatible sympatric strain of *Schistosoma mansoni* (SmBRE). Both were as previously described [19].

Innate immune memory protocol

One hundred and forty BgBRE snails were primo-infected with 10 miracidia of SmBRE each; 60 were secondarily challenged 25 days later with 10 miracidia of SmBRE each. For the RNA-seq approach, pools of 20 BgBRE snails were recovered at 1, 4, 15, and 25 days post primo infection (DPPI); these were designated 1DPPI, 4DPPI, 15DPPI and 25DPPI, respectively. Twenty more snails were recovered at each of 1, 4 and 15 days after the secondary challenge; equimolar amounts of each of these experimental groups were joined together into a single sample designated as days post-secondary challenge (DPC) sample. Two pools of 20 uninfected snails (designated naive1 and naive2) were sampled and used as control replicates. For the

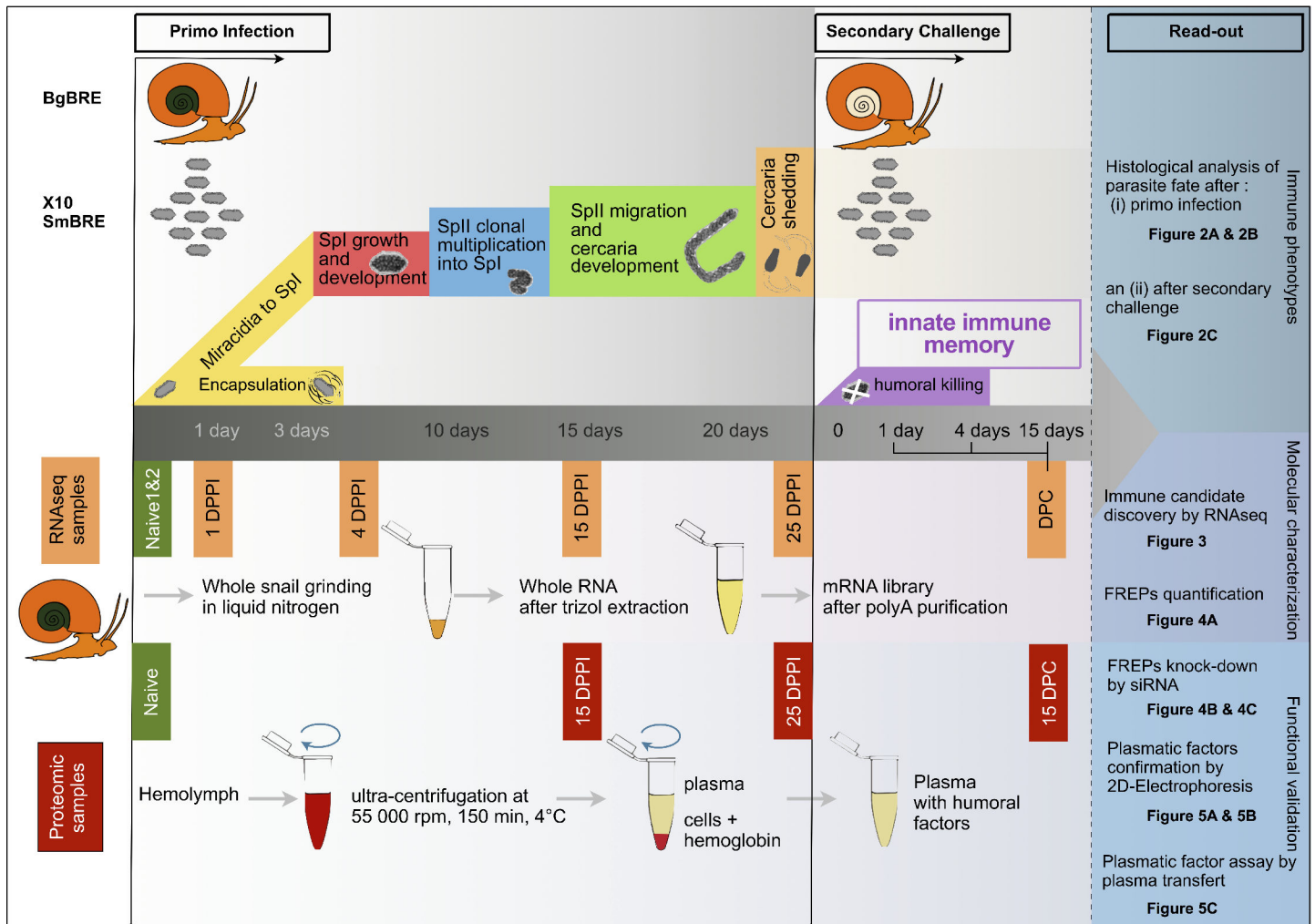


Fig 1. Overview of experimental procedures. Innate immune memory experiments were carried out. For primo-infection, Brazilian *Biomphalaria glabrata* (BgBRE) snails were individually exposed to 10 miracidia of their sympatric Brazilian *Schistosoma mansoni* trematode parasite (SmbRE). Following infection depending on the compatibility status of the snail/parasite couples, some of the miracidia were encapsulated by the hemocytes (snail immune cells) or developed into primary sporocysts (intra-molluscan stage of the parasite). Intramolluscan parasite stages include two generations of sporocysts (primary sporocyst (SPI) and secondary sporocyst (SPII)) and the production of cercariae. SPII developed inside SPI and migrated to reach the snail hepato-pancrea. Cercariae developed inside SPI and migrated back into the snail to reach the aquatic environment. Twenty-five days after primo-infection, the snails were challenged for a second time with again 10 SmbRE miracidia. In this case all miracidia degenerated in snail tissues, demonstrating the activation of a humoral immune response. Immune phenotypes observed during innate immune memory process were analyzed using a histological approach (see Fig 2A, 2B & 2C). In order to explore the molecular mechanisms of innate immune memory several experimental procedures were designed. A RNAseq experiment was realized with samples recovered from uninfected snails (Naive 1, Naive 2), samples recovered at 1, 4, 15 and 25 days post primo-infection (DPPI) and at 1, 4 and 15 days post-secondary challenge (DPC) (see Fig 3). Based on RNAseq results, functional validation of the FREP immune recognition receptor was undergone. First, individual quantification was made for all FREPs annotated on transcriptomic analysis (see Fig 4A). FREP knockdown was then carried out by siRNA injection, normalized by siGFP and monitored by Q-RT-PCR (see Fig 4B & 4C). Finally, to confirm the involvement of plasmatic factors in innate immune memory, snail hemolymph was recovered (Naive, 15, 25 DPPI and 15 DPC) and plasmatic fraction was characterized by 2D-gel electrophoresis (see Fig 5A & 5B). Plasma samples were also injected to naive snails to demonstrate that immune protection could be acquired following primed snail plasma transfer (see Fig 5C).

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proteomic approach, 200 BgBRE snails were primo-infected and secondarily challenged with 10 miracidia (per round) of SmbRE. Plasma sampling was performed on the same schedule described for the RNAseq experiments. Five biological replicates (5 independent pooled plasmas from 10 snails) were recovered from naive snails (controls), and 15DPPI, 25DPPI and 15DPC snails.

Histological procedures

Forty-eight hours after primo-infection or secondary challenge with 10 miracidia of SmBRE, snails were fixed in Halmi's fixative (mercuric chloride 4.5%, sodium chloride 0.5%, trichloroacetic acid 2%, formol 20%, acetic acid 4% and 10% of picric acid-saturated aqueous solution). Fixed mollusks were dehydrated and embedded in paraffin as previously described [20,21]. Transverse histological sections (10- μ m thick) were cut and stained using azocarmine G and Heidenhain's azan. The following serial steps were used: (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 subjected to microscopic examination.

Whole-body RNA sequencing

Total RNA was extracted from naive1, naive2, 1DPPI, 4DPPI, 15DPPI, 25DPPI and DPC samples using nitrogen and the TRIzol reagent (Sigma Life Science, St. Louis Missouri, USA). mRNAs were sequenced in paired-end, 72-bp read length, with three samples multiplexed per lane, using an Illumina Genome Analyzer 2 (Montpellier Genomix (MGX), Montpellier, France).

Transcriptome assembly

De novo transcriptomes were assembled using high-quality reads (phred > 29) from all seven sequenced samples and an in-house pipeline created using the Velvet (v1.2.01), Oases (v0.2.04) and CDhit EST (v4.5.4) programs [22]. A consensus reference transcriptome was optimized using various parameters, including k-mer length, insert length and expected coverage, as previously described [22]. To suppress non-molluscan transcripts, a BLAST-based transcriptome subtraction strategy was used. Transcripts were compared against the *B. glabrata* draft genome, and transcripts with identities and coverages of less than 80% were deleted. This led to a final subtraction of 45.1% of the transcripts, most of which corresponded to *S. mansoni*. The final size of the transcriptome was 159,711 transcripts.

Differential expression analysis of transcripts

Quality reads (Phred score >29) were aligned to the assembled transcriptome using the C++ script, Bowtie2 (v2.0.2) (mapping quality score 255), which was run locally using the Galaxy Project server [23]. The DESeq2 software [24] (v2.12; <http://www.bioconductor.org/packages/release/bioc/html/DESeq2.html>) was run under the default settings to compare duplicate samples from uninfected snails (naive1 and naive2) versus infected samples to quantify differential gene expression (P value < 0.1). A heatmap was constructed to analyze transcript expression patterns (log₂ fold change) using Hierarchical Ascending Clustering (HAC) with Pearson correlation, as applied by the Cluster 3.0 [25] and JavaTreeView software packages. We used quantitative real time PCR (Q-RT-PCR) to ascertain RNAseq results. For that purpose the correlation between the rpkm (reads per kilobase per million mapped reads) of RNAseq and the ct (cycle threshold) of Q-RT-PCR was tested and confirmed (see S1 Fig). Primer sequences used in Q-RT-PCR were available in S3 Table.

FREP RNA interference

Small interfering RNAs (siRNAs) were used to knock down FREP2 (GenBank accession number gi|16303186), FREP3 (gi|18389116) and FREP4 (gi|16303188). Three siRNA duplexes (Eurogentec) were used in conjunction with InvivoFectamine transfecting agent (Invitrogen, CA). Two hundred ng of pooled FREP duplex siRNA or GFP siRNA (used as control) were

injected into the cardiac sinus of *B. glabrata* snails as previously described [26]. One, Two and four days following siRNA injection, the knock-down efficiency was confirmed by Q-RT-PCR analysis of FREP2, -3 and -4 mRNA expression. For phenotypic analysis, 90 BgBRE snails were infected with 10 miracidia of SmbRE. Twenty-one days after primo-infection, these snails were individually injected with or without siFREPs or siGFP. Four days later, each snail was subjected to a secondary challenge with 10 miracidia of SmbRE. Fifteen days post-challenge, snails were fixed and the parasite prevalence was determined by identifying schistosome primary sporocysts (SpI), in snails tissue as previously described [27,28].

Proteomic screening of primed snail plasma

Hemolymph was collected from the head-foot region of 50 BgBRE snails (5 pools of 10 snails, 5 biological replicates) at each time point of the infection process (Naïve, 15DPPI, 25DPPI, and 15DPC). Hemocytes were pelleted by centrifugation at 2500 rpm for 15 min at 4°C, and discarded. Hemoglobin was separated from the plasma by ultracentrifugation at 55,000 rpm for 2.5 h at 4°C and the supernatant plasma samples were recovered. Protein concentration of the samples were estimated using a 2D Quant kit (GE Healthcare life sciences). The plasma (5 biological replicates for each of the 4 time points) was subjected to 2D gel electrophoresis on 12% SDS-PAGE gels using 100 µg of plasma, denaturing buffer, and 17 cm IPG Strips, pH 3–10 non-linear gradient (BioRad). Gels were stained with mass spectrometry (MS)-compatible silver staining, and comparative analysis of digitized proteome maps was performed using the PDQuest 7.4.0 image analysis software (Bio-Rad). Spots showing obvious qualitative and quantitative (at least 2-fold) differences were excised from the gel and characterized by nanoscale capillary liquid chromatography on an Ultimate 3000 coupled to a LTQ-Orbitrap tandem mass spectrometer (nanoLC-MS/MS) mapped to Swiss prot-trembl and the *Biomphalaria glabrata* Brazil transcriptome (<http://ihpe.univ-perp.fr/enseignement/>). A protein was considered to be correctly identified if at least two peptides were confidently matched to database sequences with an overall Mascot score greater than 50 [29].

Plasma transfer

Plasma samples were recovered from 15DPPI or naïve snails as described above. PBS-snail solution (8.41 mN Na₂HPO₄, 1.65 mN NaH₂PO₄, 45.34 mM NaCl) was used as the control injection. Twenty µl of each sample (naïve plasma, 15DPPI plasma, or TBS Tween solution) were injected into 25 BgBRE snails per group. One group of 48 BgBRE snails was left untreated and used as a control for infection. Fifteen days after injections, the snails in the four experimental groups were infected with 10 miracidia/snail of SmbRE. This period of 15 days was used to ensure that an observed phenotype was not a direct effect of the injected molecules, but rather reflected their ability to activate the snail's immune response pathways. Fifteen days after infections, snails were dissected and the parasite prevalence was quantified as described above.

Statistical analysis

To test for significant differences in prevalence, Fisher's exact test was used, with $P \leq 0.05$. For the proteomic approach, statistically significant quantitative differences between spots were tested using a Mann-Whitney U test, as applied with the PDQuest 7.4.0 software (BioRad).

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

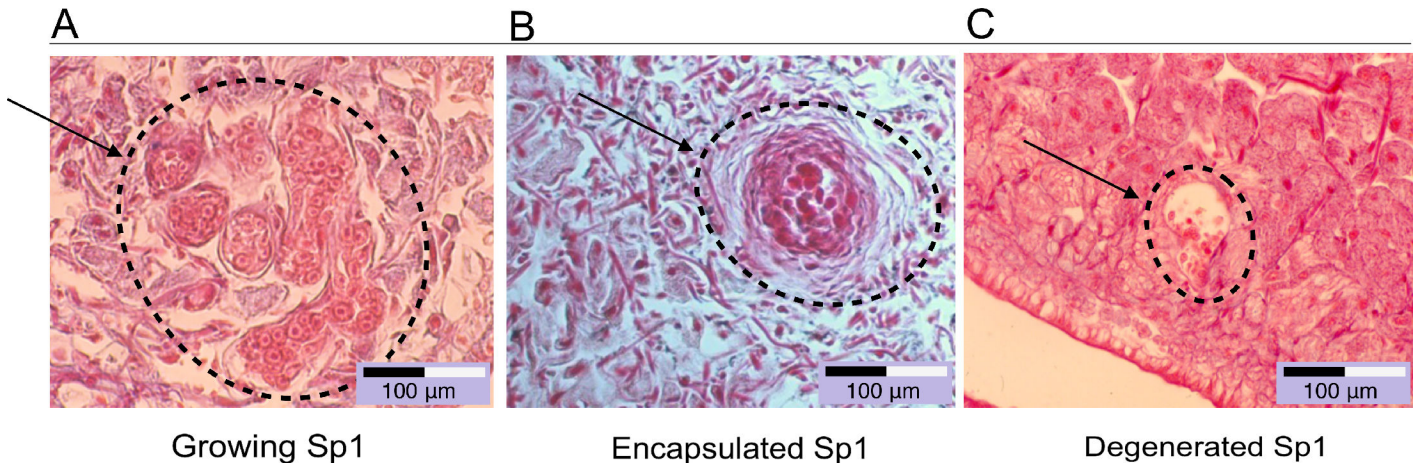


Fig 2. The immune response of *B. glabrata* to *S. mansoni* infection. The Brazilian strain of albino *B. glabrata* (BgBRE) is 100% susceptible (for 10 miracidia and upwards) to its corresponding strain of *S. mansoni* (SmBRE). When a snail is infected with 10 miracidia of *S. mansoni* within the same individual compatible and incompatible interactions occur, 3 to 4 miracidia develop normally in the snail's tissues while the others are recognized and encapsulated by the snail's cellular immune response. A. Six-day-old sporocyst in a compatible interaction. B. Encapsulated sporocyst 48 h after primary infection in an incompatible interaction. C. Six-day-old sporocyst in a primed snail. Primed BgBRE are 100% protected against a secondary challenge with SmBRE. Sporocysts from secondary challenge were neutralized by immune humoral factors.

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experiments on animals, animal housing, and animal breeding (# A66040; decree # 87–848, October 19, 1987; and authorization # 007083).

Results

Histological inspection of *B. glabrata* immune response phenotypes

Three types of immuno-biological interactions were observed following infection of *B. glabrata* by *S. mansoni*. After primo-infection, a compatible interaction was characterized by the ability of miracidia to penetrate, transform into sporocysts and develop normally in snail tissues (30 to 40% of the entering miracidia produce SPI) (Fig 2A). In an incompatible interaction, miracidia were immediately recognized, encapsulated and killed by hemocytes; in this case, a multi-cellular capsule could be observed surrounding the parasite (60 to 70% of the entering miracidia were killed) (Fig 2B). Finally, in primed snails subjected to a secondary challenge, the sporocysts degenerated in the snail tissues. Encapsulation was never observed nor was the accumulation of hemocytes observed near the parasite (100% of the entering miracidia were killed by humoral factors) (Fig 2C). These findings indicate that a cellular immune response was the main outcome following a primo-infection, whereas an exclusively humoral immune response was found in primed snails subjected to a secondary challenge (Fig 2).

A whole-snail transcriptomic approach for investigating the molecular basis of innate immune memory

The RNAseq values were validated by a correlation analysis performed with our quantitative Q-RT-PCR data (S1 Fig, S3 Table). We obtained a good correlation ($R^2 = 0.768$), indicating that the representations of transcripts in our RNAseq results appeared to be proportional to those obtained through Q-RT-PCR amplifications.

This RNAseq experiment allowed us to identify 1887 differentially expressed transcripts, most of which were found in the 1DPPI, 25DPPI and DPC samples (Fig 3, S1 Table). At 1DPPI, most of the differentially expressed transcripts were under-represented (Fig 3). This

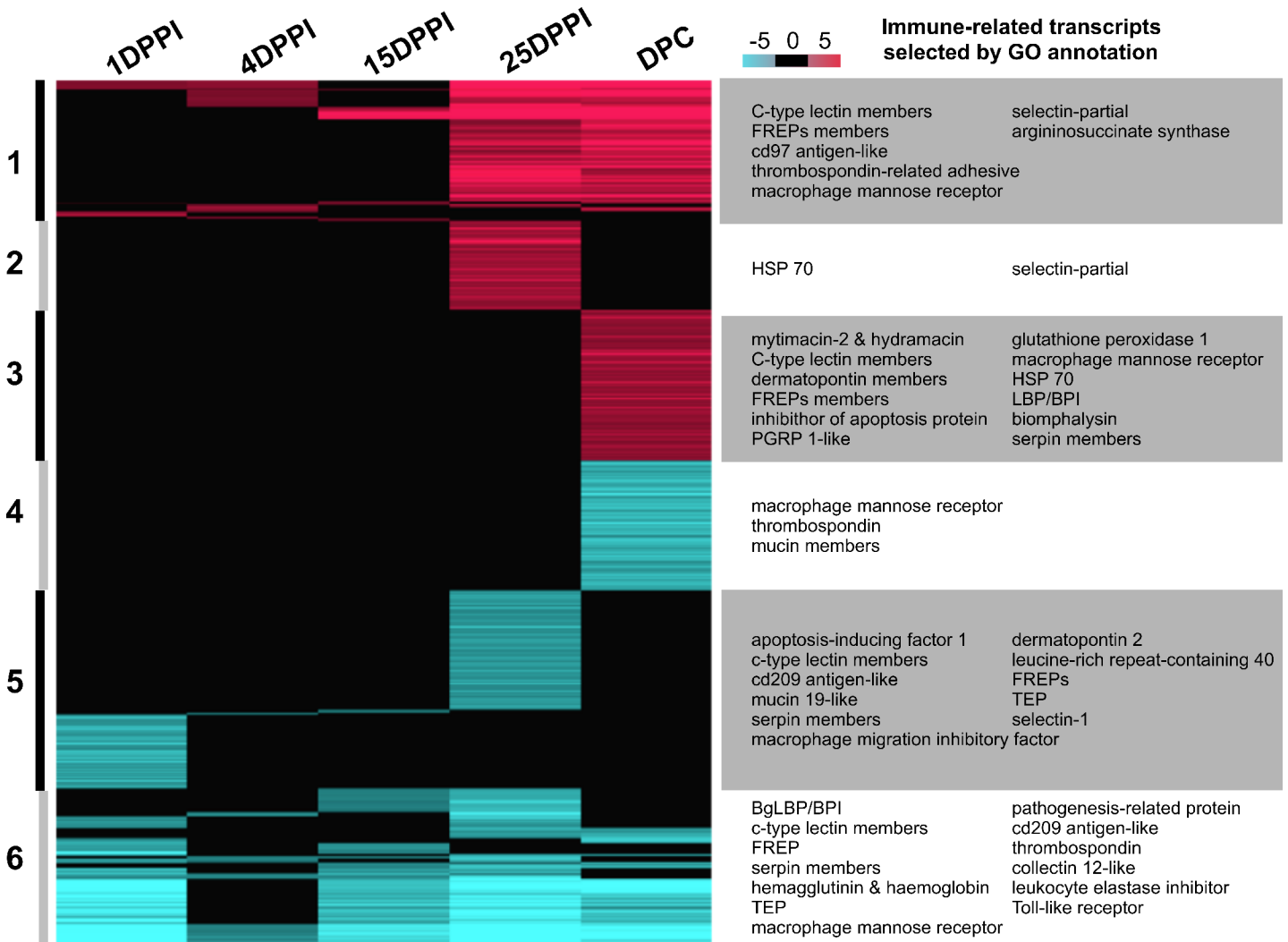


Fig 3. RNAseq analysis of the innate immune memory response of *B. glabrata* to *S. mansoni*. Heatmap showing differentially represented transcripts compared to naïve snails, as identified by DESeq2 analysis ($p < 0.1$). Color scale indicates the Log₂FC ratio from under-represented (blue) to over-represented (red) transcripts. Transcripts were grouped into six clusters based on their expression patterns during the process of innate immune memory. Samples were recovered at 1DPPI, 4DPPI, 15DPPI and 25DPPI following primo-infection. Following secondary challenge samples were recovered at 1 day, 4 days and 15 days and pooled into DPC sample. Six clusters are identified: Cluster 1: transcripts over represented more than once all along infection and challenge. Cluster 2: transcripts exclusively over represented in single one condition. Cluster 3: transcripts exclusively over represented after immune challenge (DPC). Cluster 4: transcripts exclusively under represented after immune challenge (DPC). Cluster 5: transcripts exclusively under-represented in single one condition. Cluster 6: transcripts under represented more than once all along infection and challenge. FREP: Fibrinogen-related protein, HSP: Heat-shock protein, PGRP 1-like: Pathogenesis-related protein 1-like, LBP/BPI: lipopolysaccharide-binding protein/bactericidal/permeability-increasing protein, BgLBP/BPI: *Biomphalaria glabrata* LBP/BPI, TEP: thioester-containing protein.

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could reflect the immuno-suppression/modulation induced by *S. mansoni* parasites within the first hours of an infection, when the parasites develop their molecular mimicry strategy [30,31]. At 25DPPI and DPC, the proportions of over- and under-represented transcripts were more comparable (Fig 3). Hierarchical clustering was used to sort the differentially represented transcripts into six clusters, three each corresponding to over-represented transcripts (clusters 1–3) and under-represented transcripts (clusters 4–6) (Fig 3, S1 Table). Cluster 2 included transcripts that were specific to the primo-infection and not activated following the secondary challenge. Clusters 1 and 3 appeared to be the most promising possible sources of innate

immune memory candidates. Cluster 1 comprised transcripts that were over-represented following primo-infection and remained highly expressed throughout infection (sustained response); it included immune molecules known to be involved in the cellular immune response (macrophage mannose receptors, thrombospondin), as well as certain pathogen recognition receptors (PRRs) (selectins and C-type lectins). In contrast, cluster 3 comprised transcripts that were over-represented exclusively following the secondary challenge (Fig 3), including certain PRRs (FREPs, C-type lectins) and circulating immune effectors (anti-microbial peptides such as mytimacin and LBP/BPI, and biomphalysin). Interestingly, clusters 4, 5, and 6, which contained the under-represented transcripts, included immune molecules belonging to the same immune functional groups represented in clusters 1 to 3 (Fig 3). This may indicate that there is a trade-off among the immune variants involved in the specific response to *S. mansoni* parasites, with some members of the same family (i.e., variants or isoforms) being down-regulated to permit the over-expression of other variants. In cluster 4, notably, most of the under-represented transcripts were involved in the cellular and epithelial immune response (e.g., macrophage mannose receptors, extracellular matrix compounds, mucins). This indicates a down regulation of epithelial (mucosal immunity) and cellular immunity two major components of innate immune response following secondary challenge. The complete list of differentially represented transcripts is presented in S1 Table.

Loss-of-function, FREP-mediated RNA interference

To estimate total FREP expression (Fig 4A) over the entire innate immune memory process based on our RNAseq data, we summed the fold changes for all the differentially represented FREP transcripts (identified in Fig 3). A huge induction (5.096-fold) of FREPs was observed following the secondary challenge (DPC, Fig 4A). SiRNA-mediated knockdown of FREPs were used in combination to ascertain the role played by these FREP candidates in innate immune memory. Knockdown of FREPs was confirmed by Q-RT-PCR; the results were normalized with respect to the mRNA expression levels in naïve snails, and compared to the corresponding levels in siGFP-control-injected snails (Fig 4B). The best knockdown level was reached at 96 h post-siRNA injection, when we observed a decrease of 7.98-, 1.56- and 3.60-fold change for FREP 2, 3 and 4, respectively (Fig 4B). The effect of FREP knockdown on the innate immune memory phenotype was assessed in a typical homologous innate immune memory assay. In untreated snails, the parasite prevalence after secondary challenge was 0% (Fig 4C), indicating that innate immune memory had effectively protected them against a subsequent exposure to *S. mansoni*. Injection of siGFP did not significantly change the parasite prevalence after secondary challenge (4%; Fig 4C). Following injection of the FREP siRNA, however, 15% of the snails became infected with *S. mansoni* following secondary challenge (Fisher's exact test $P < 0.05$) (Fig 4C). Thus, FREP knockdown partially altered the innate immune memory phenotype, rendering primed snails more susceptible to *S. mansoni* infection.

Global proteomic comparison of primed snail plasma

To validate our RNAseq results, we focused our attention on the plasmatic compartment of *B. glabrata*, which should contain the actors of the humoral innate immune memory response. We performed global comparative 2D gel electrophoresis on plasma samples obtained from naïve, 15DPPI, 25DPPI, and 15DPC snails. Our quantitative and qualitative bioinformatic analyses identified 29 differentially expressed spots corresponding to 62 different proteins (Fig 5A, S2 Table).

A hit map of the quantitated expression ratios was generated for the entire infection process (Fig 5B, S2 Table). Four clusters were identified (Fig 5B): the proteins in cluster 1 were up-

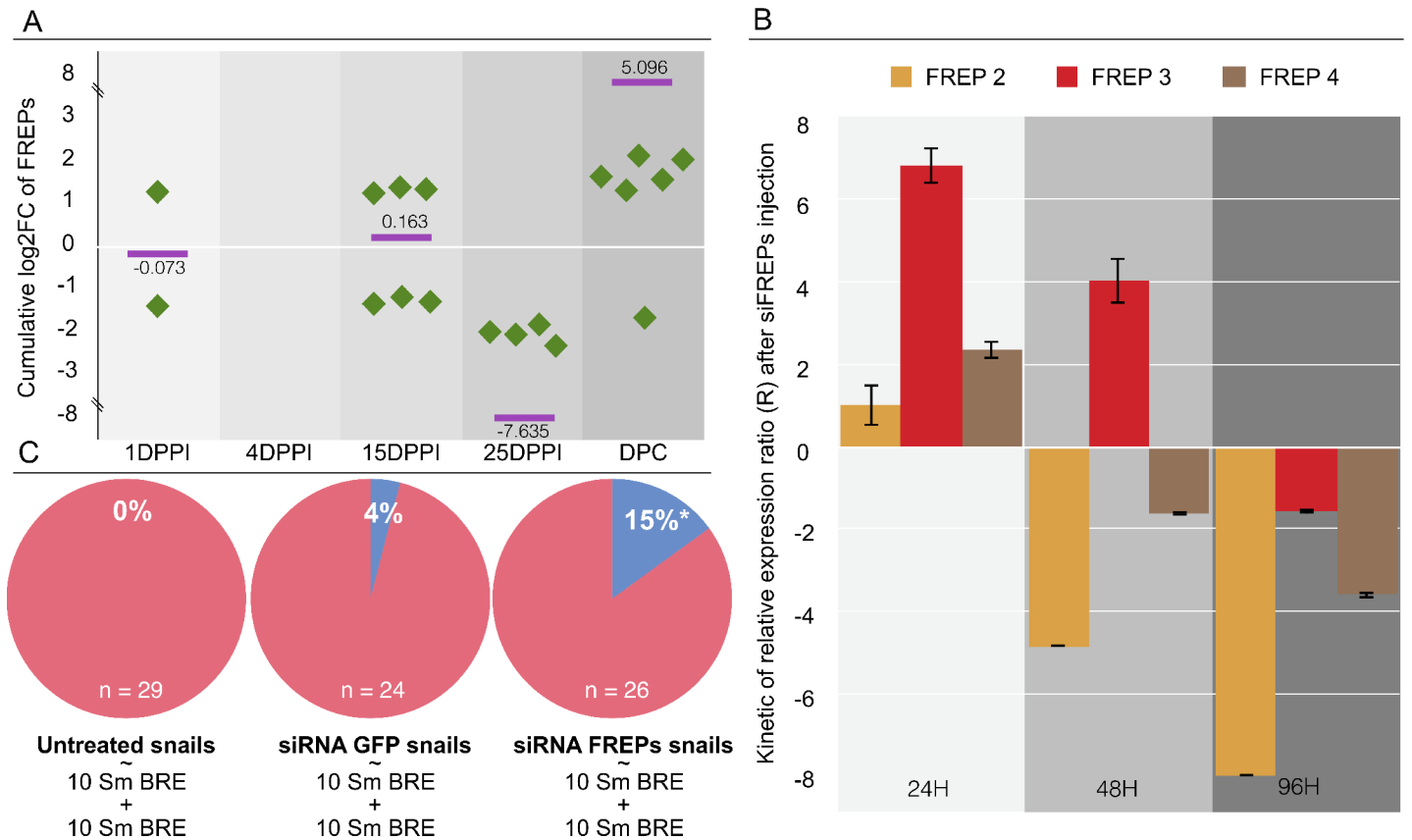


Fig 4. FREPs knock-down mediated by RNA interference. A. Cumulative expression [Log₂FC (fold change) from the DESeq2 analysis] of FREP transcripts showed that FREPs were over-represented after the secondary challenge (DPC; 5.096 log₂ fold change enrichment of FREPs transcripts). Green points corresponded to the differentially represented FREPs transcripts in each samples. Purple bars represent the cumulated Log₂FC of FREP transcripts. At 4DPP no FREP transcripts were differentially expressed, thus no value appeared in the graph. B. siRNA injection against FREP2, FREP3 & FREP4 was carried out and mRNA abundance was monitored during 4 days by Q-RT-PCR. Snails were injected with siRNAs against FREP 2, 3, and 4 or GFP (control), the relevant mRNA levels were assessed following normalization with respect to the S19 gene in siGFP injected snails versus siFREPs injected snails. Knock-down of the three FREPs tested was confirmed at 96h. C. Naïve *B. glabrata* and siFREPs-injected snails were subjected to a typical priming experiment: Snails were infected with 10 miracidia of *S. mansoni* as a primo-infection, 21 days later they were injected with siGFP, or siFREPs or not treated and 4 days later they were infected with another 10 miracidia as a secondary challenge. FREP siRNA-injected snails show a significant proportion of non-primed snails (15%; *, binomial test, P < 0.05).

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regulated following the secondary challenge; those in cluster 2 were up-regulated after the primo-infection and remained highly expressed throughout infection (sustained response); those in cluster 3 were up-regulated after the primo-infection and down-regulated following secondary challenge those of cluster 4 were down-regulated. The differentially expressed proteins were also subjected to functional classification based on their putative functions and/or structural features. Five functional groups were identified: innate immune response proteins (receptors, effectors, and regulators), scavengers of reactive oxygen species, cell signaling proteins, gametogenesis-related proteins, and glycolysis-related proteins. Thirty seven percent of the identified molecules belonged to the innate immune response group (23 of 62 proteins) (Fig 5B). Consistent with our RNAseq results, we observed an important enrichment of potential humoral immune candidates in the plasma of primed snails. The molecules involved in immune recognition and opsonization included numerous isoforms belonging to different families of circulating immune receptors that were also identified in our transcriptomic analysis (e.g., C-type lectins, β -1,3-glucan binding protein, and hemagglutinin/amebocyte

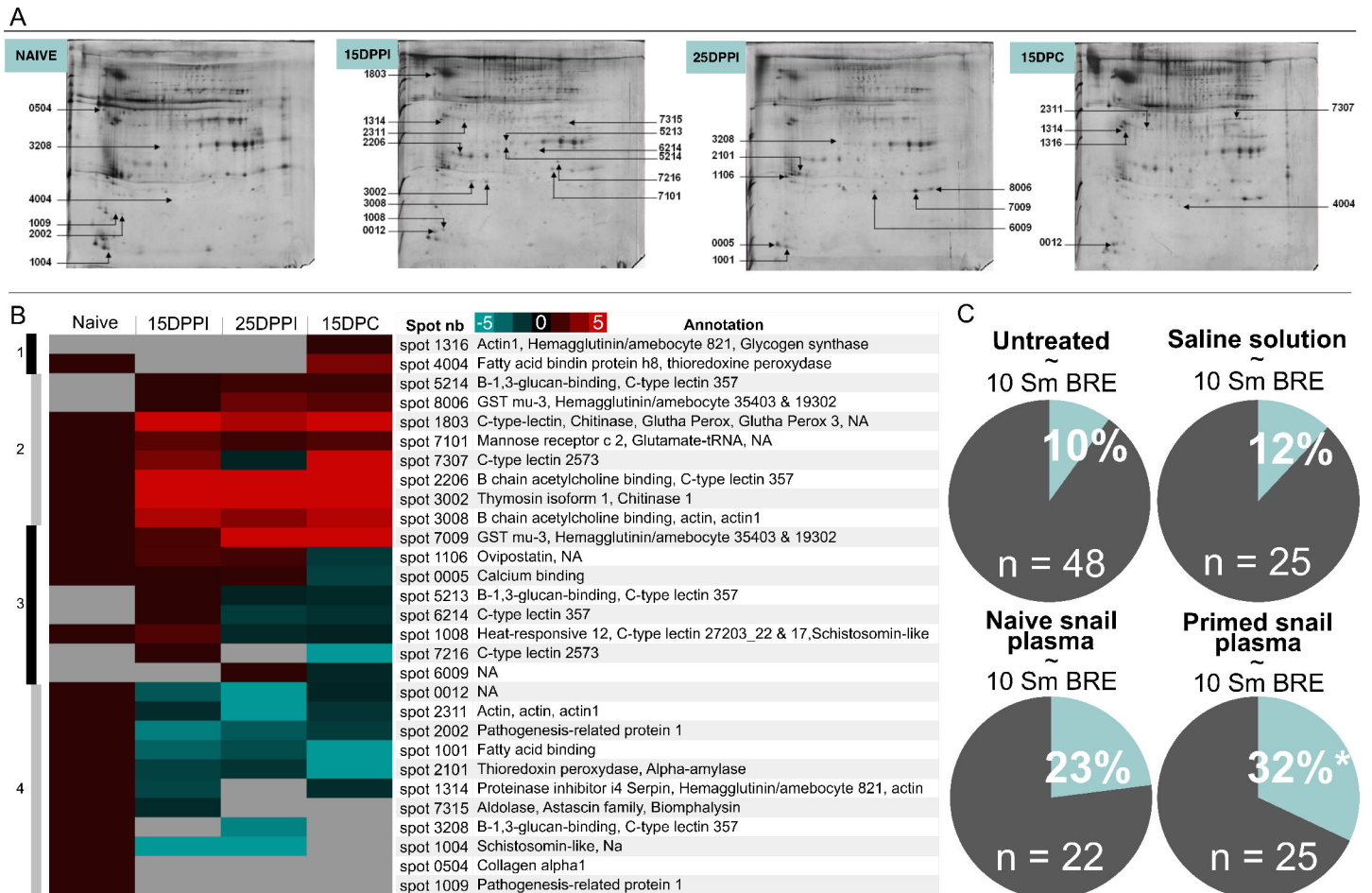


Fig 5. Role of *B. glabrata* plasmatic factors in innate immune memory response. A. 2D gel electrophoresis of plasma proteins. One gel of each plasma sample analysed was shown. Spot numbers of qualitative and quantitative differences were indicated. Four plasma samples were analysed from naïve (uninfected snails), 15DPPI and 25DPPI (recovered at 15 and 25 days after primo-infection) and 15DPC (recovered at 15 days after secondary challenge). B. Heat-Map of the qualitative and quantitative ratio versus naïve sample. Ratios were calculated using PDQuest software between all differentially regulated spots. Blue to red scale indicate ratio values from lower to higher represented spots. Four clusters are identified: Cluster 1: higher-represented proteins exclusively following secondary challenge (15 DPC). Cluster 2: sustained response: higher-represented proteins after the primo-infection and secondary infection. Cluster 3: higher-represented proteins at 15DPPI and thereafter down regulated at 25DPPI and 15DPC. Cluster 4: lower-represented proteins. C. Plasma transfer and effect on prevalence of *S. mansoni* infection. Four conditions were tested: untreated snails (Control group, n = 48); saline injected snails (control of injection, n = 25); naïve-plasma injected snails (n = 22); and primed-plasma injected snails (n = 25). For all the experimental groups, 15 days following injection, snails were infected with 10 miracidia of SmBRE. * indicated significant differences (P < 0.05).

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aggregation factors). Most of these isoforms were found in clusters 1 and 2, suggesting that they may actively contribute to the innate immune memory response (Fig 5B). Among the immune effector molecules, chitinase (spot 3002, Fig 5B), fatty acid binding protein (spots 4004 and 1001, Fig 5B), and biomphalysin (spot 7315, Fig 5B) were found in clusters 1, 2 and 4, respectively. Biomphalysin was over-represented following primo-infection and secondary challenge in the transcriptomic data, but down regulated at 15DPPI and 15DPC in our proteomic data (cluster 4, spot 7315; Fig 5B). Thus, it seems that biomphalysin is highly transcribed during the innate immune memory response against *S. mansoni* parasites, but is thereafter consumed at the protein level. Some known regulators of the immune response (e.g., thymosin, spot 3002; serpin, spot 1314; and β chain acetylcholine binding protein, spots 2206 and 3008) were associated with protein clusters 2 and 4 (Fig 5B). In addition, other proteins known to be

involved in host/parasite interactions were identified as being differentially expressed, including some anti-oxidant molecules, which were identified in protein clusters 1 and 2 (e.g., glutathione peroxidase 3 precursor, spot 1803; glutathione-S-transferase mu 3-like, spots 7009 and 8006; and thioredoxin peroxidase, spot 4004; Fig 5B).

Proteins involved in gametogenesis were also identified by our global proteomic analysis. They included schistosomin (spot 1004), which is involved in the regulation of gametogenesis [32]; pathogen-related protein (spots 2002 and 1009), which is a seminal fluid protein [33,34]; and ovipostatin (spot 1106), which is involved in oviposition and hatching [35]. These proteins were found in clusters 3 and 4, and were thus down-regulated following primo-infection and/or secondary challenge (Fig 5B) (see S2 Appendix for details).

Plasma transfer

A plasma experiment was carried out to confirm the role played by humoral factors in innate immune memory. Plasmas were recovered from naïve or 15DPPI snails and injected into naïve snails. The snails were subjected to infection 15 days post-plasma-injection, and analyzed for phenotypes at 15 days post-infection (Fig 5C). Injection of saline solution has no significant effect on the prevalence of parasitic infection compared to the untreated control (88% and 90%, respectively) (Fig 5C). Transfer of naïve snail plasma also failed to have any significant effect (prevalence of 77%) (Fig 5C). However, a statistically significant decrease in the prevalence of infection was observed for snails injected with primed snail plasma (from 90% to 68%; $P = 0.04$). This protection remained efficient at 15 days post-transfer, confirming that plasma humoral factors function to activate the immune system and prepare the snail to answer a subsequent encounter with the parasite.

Discussion

Innate immune memory, which is a process through which an organism acquires a (more or less) specific and long-lasting protection against later challenges that persists even after the pathogen is neutralized, has been described in diverse invertebrate phyla [2,3,5,6,14,36–39]. This suggests that innate immune memory exists in a broad range of invertebrates.

A form of innate immune memory was previously demonstrated in *B. glabrata* in response to *S. mansoni* infection, and shown to be dependent on the genotype of the pathogen [4]. Here, we provide evidence that innate immune memory in *B. glabrata* is not associated with an enhancement of the cellular immune response, as has been suggested in other invertebrate species [5,12,13]. Instead, our histological analysis (Fig 2) revealed that in primed snails, the parasites of a secondary challenge fail to develop into sporocysts and are killed by the host, without any observable cellular immune response/hemocytic reaction. Together, these findings show that, after a primo-infection, each successive encounter with a similar parasite initiates an exclusive humoral immune defense response in *B. glabrata*.

Accordingly, we set out to identify the humoral factors through which primed snails are able to neutralize the sporocysts of a secondary challenge. A whole-snail RNA sequencing approach was conducted to identify molecular candidates that might be involved in the innate immune memory response. For RNAseq approach the cluster 1 (sustained-response transcripts) and cluster 3 (secondary challenge-specific transcripts) contained the most promising candidates (Fig 3). Numerous circulating or cell-surface PRRs were found. These immune receptors, which recognize terminal sugar residues on the glycans that are attached to the surface proteins of some microorganisms, act in the pathogen recognition and clearance processes of innate immunity. The identified PRRs belong to various families, including the macrophage mannose receptors, selectins, C-type lectins and fibrinogen-related proteins (FREPs).

Cytotoxic and cytolytic molecules were also identified, as were anti-oxidant molecules. The latter finding suggests that the reactive oxygen species pathway may be activated as part of the humoral innate immune memory response of *B. glabrata*. Moreover, RNAseq cluster 4 (molecules down-regulated upon secondary challenge) included transcripts whose products are involved in the epithelial and cellular immune response, such as macrophage mannose receptors, extracellular matrix compounds and mucins. This strengthens our contention that there is a shift from cellular or mucosal immunity to humoral immune response during the innate immune memory response in *B. glabrata* snails.

An unexpected result of this study was the identification of RNAseq cluster 3 and proteomic cluster 1. Until now, innate immune memory was believed to be the result of two potential regulatory processes: (i) a sustained immune response consisting of the long-lasting up-regulation of immune molecules after a primo-infection (as observed in RNAseq cluster 1 and proteomic cluster 2); or (ii) a recalled response that consists of the ability to store information and recall it later for a faster and more powerful response against subsequent pathogenic exposure (never been observed in any of the RNAseq or proteomic clusters) [36,39]. Here, we provide the first report of a novel unexpected regulatory process for invertebrate innate immune memory, in which transcripts are specifically up regulated following the secondary challenge (RNAseq cluster 3, proteomic cluster 1) without having been previously activated by the primo-infection. This new observation warrants further study, such as through the identification of its potential molecular hallmarks, including the genetic (activation of transcription factors) and/or epigenetic (DNA methylation, chromatin markers, lncRNA, miRNA, etc.) factors that form the basis for this exclusive secondary challenge innate immune memory response.

To validate the potential roles played by the identified molecular candidates in the observed innate immune memory phenotype, we performed three sets of experiments: we used RNAi to knockdown certain FREPs and examined the priming response; we characterized the snail plasma proteome; and we examined the effect of injecting naïve snails with plasma from primed snails.

As FREP lectins showed extensive differential representation following the secondary challenge (see Fig 4A), and they are known to play key roles in the immunobiological interactions between *B. glabrata* and *S. mansoni*, they were chosen as candidates for RNAi invalidation. The FREPs comprise one or two amino-terminal immunoglobulin domains (IgSF) and a carboxyl-terminal fibrinogen domain (FBG). They belong to a multigene family of at least 14 members, and undergo somatic rearrangements leading to remarkable diversification [40,41]. FREPs can precipitate soluble antigens derived from trematodes. Their expression levels increase in response to infection with *S. mansoni* [42,43], and they form immune complexes with similarly highly polymorphic and individually variable mucins (the *SmPoMucs*) that act as antigens of *S. mansoni* [44]. The high level of diversification within FREP family members results in a huge proportion of partial FREP transcript sequences generated by the RNAseq *de novo* assembly [22]. We were unable to precisely identify the FREP variants/isoforms present in the various RNAseq clusters. Thus, to confirm the role of FREPs in innate immune memory, we invalidated FREP 2, 3, and 4, as they had previously been demonstrated to be involved in the response of *B. glabrata* to trematodes. More specifically, FREP 2 is involved in immune complexes [44]; FREP 3 knock-down reverts the snail resistance status to trematodes [45]; and microarray analysis showed that FREP 4 is over-expressed following infection by *S. mansoni* [43]. SiRNA-mediated knock-down of FREP 2, 3 and 4 (Fig 4B) was found to reduce the innate immune memory phenotype by 15% (Fig 4C). These results indicate that these FREPs are involved in *B. glabrata* innate immune memory, but demonstrate also that additional molecular partners (and/or other FREP variants) also play roles in the pathogen recognition and innate immune memory of this snail.

Our characterization of the plasmatic proteome confirmed that most of the molecules identified in the RNAseq data were present and differentially expressed at the protein level. The presence of these molecules in snail plasma also confirms that they act as circulating humoral factors. The most promising candidates for participation in innate immune memory were found in protein cluster 1 (secondary challenge-specific proteins) and protein cluster 2 (sustained-response proteins) (Fig 5). Numerous PRRs were identified (e.g., macrophage mannose receptors, C-type lectin, hemagglutinin, and β -1, 3-glucan binding proteins). Interestingly, we did not recover any FREP in this proteomic analysis of primed snail plasma. We speculate that the FREPs were lost from our analysis due to their precipitation with parasitic antigens [44]. Alternatively, and as suggested previously for other families of highly variable molecules [46], the different variants may be expressed at such low levels that they are not visible on 2D gel electrophoresis. Indeed, we recently demonstrated that FREPs are expressed at a low level compared to other PRRs in naive *B. glabrata* snails [22]. Plasmatic proteome was also composed of cytotoxic and cytolytic molecules, including antimicrobial peptides [AMPs; e.g., hydramicin, mytimacin and lipopolysaccharide binding protein/bactericidal permeability-increasing protein (LBP/BPI)], and the biomphalysin. We recently reported the molecular cloning and functional characterization of the *B. glabrata* biomphalysin and its involvement in the killing of *S. mansoni* [47]. Biomphalysin, which shares a common architecture with proteins belonging to the aerolysin superfamily, is strictly expressed in immune-competent cells. Recombinant biomphalysin was shown to bind to parasitic membranes and exhibit cytotoxic activity toward *S. mansoni* sporocysts. Our RNAseq and proteomic analyses primarily recovered immune recognition factors, many of which had multiple isoforms. This suggests the involvement of both translational and post-translational regulation. Additional work is now needed to clarify the functions of these molecules in the humoral innate immune memory response of *B. glabrata*. For example, C-type lectins are known to be involved in the innate immune memory responses of mollusks; their expression levels increased in the scallop, *Chlamys farreri*, following vaccination with heat-killed *Vibrio anguillarum*, and successive challenges with *V. anguillarum* or *Micrococcus luteus* enhanced this protection [11]. In addition to their roles in pathogen recognition and opsonization, lectins also possess direct cytotoxic activities. Examples of cytotoxic lectins include plant-derived ricin from *Ricinus communis* beans [48], the fungus-derived N-acetyl-D-galactosamine-specific lectin from *Schizophyllum commune* [49], and the invertebrate-derived hemolytic lectin, CELIII, from *Cucumaria echinata* [50]. Thus, it seems reasonable to hypothesize that some of the lectins identified in the present study may participate in the humoral innate immune memory response both as recognition receptors and as cytotoxic/cytolytic molecules involved in killing the *S. mansoni* SpIs of the secondary challenge.

Lastly, we explored whether the transfer of plasma from primo-infected snails could provide recipient naïve snails with enhanced immunity. In a previous study, plasma transfer from *S. mansoni*-resistant *Biomphalaria tenagophila* to susceptible snails was associated with a transfer of resistance [51]. Here, we found that transfer of primed snail plasma to naive snails significantly reduced the prevalence of *S. mansoni* infection by more than 20% compared to controls (Fig 5). A comparable experiment was previously performed using the mosquito, *Anopheles gambiae*, and its bacterial pathogens [5]. The transfer of cell-free hemolymph from infected mosquitoes into healthy mosquitoes triggered increases in the hemocyte populations of transferred mosquitoes, indicating that humoral factors could promote a cellular immune response that protected mosquitoes against subsequent bacterial challenges [5]. In *Biomphalaria*, plasma transfer experiments demonstrated that soluble humoral factors are released into the hemolymph of primed snails, and that the transfer of such factors activates/regulates the humoral immune response in recipients and confers enhanced antischistosomal immunity against subsequent encounters with the pathogen.

To conclude, we evidence for the first time the molecular basis of innate immune memory in an invertebrate model, and we demonstrate the role of humoral factors in such phenomenon. The previous studies addressing the molecular mechanisms of innate immune memory in invertebrates have mainly suggested the involvement of elevated hemocyte phagocytosis [9,10]. In *B. glabrata*, innate immune memory seems to be supported by humoral factors that trigger the degeneration/death of the parasite. Moreover the innate immune memory protection was previously shown to decrease with increasing neutral genetic distance between the parasite used for the primo-infection and the one used for the secondary challenge [4]. We hypothesized that better protection against a homologous (vs. heterologous) secondary infection involves the mobilization of specific repertoires of *B. glabrata* immune receptors to target certain subsets of *S. mansoni* genotypes. Our present findings prompt us to speculate that the genotype-dependent innate immune memory of *B. glabrata* may be supported by a diverse repertoire of FREPs and other PRRs (Figs 4 and 5). As previously suggested [22], PRRs might serve as collaborative recognition factors that can be processed as homologous or heterologous multimers, which then act as immune recognition complexes to increase the host's PRR repertoire and mediating anti-pathogen responses. Once recognized, the pathogen is neutralized by the release of cytotoxic/cytolytic circulating factors. Here, we demonstrate that biomphalysin is highly transcribed following secondary challenge and consumed at the protein level during innate immune memory response, suggesting that biomphalysin may play a major role in neutralizing the pathogen following immune recognition. Clearly, future studies are needed to fully understand how these molecules mediate and regulate the specificity of the innate immune memory defense seen in *B. glabrata*.

To reconcile mechanisms with phenomena a molecular characterisation of innate immune memory in *Biomphalaria/Schistosoma* model was undertaken. Transcriptomic, proteomic, and functional validation lead us to a new molecular comprehension of innate immune memory processes and prompted us to revisit the artificial dichotomy between innate and memory immunity in invertebrate systems.

Supporting Information

S1 Fig. Correlation between RNAseq and Q-RT-PCR. The correlations between the RNAseq reads per kilobase per million (log₂ RPKM) values and the corresponding Q-RT-PCR cycle threshold (Ct) values were calculated and shown for 36 randomly selected transcripts. RPKM values were calculated using a local alignment with Bowtie2 on *de novo* assembled *B. glabrata* transcriptome. Cts were calculated by Q-RT-PCR using the Naive1/Naive2 biological samples previously used for RNAseq. Log₂ RPKM values were an average of two biological replicates of two independent snail pools and represented in log₂ scale. Horizontal lines indicate the standard error between these two points. Non-normalized Ct values were an average of three technical replicates of Q-RT-PCR. The vertical lines indicate the standard error between these three points. A dependency between variables, increasing of RPKM and decreasing of Ct is shown by least-square regression analysis and its correlation coefficient $R^2 = 0,76804$. (TIF)

S1 Table. List of differentially represented transcripts in RNAseq clusters. Quality reads (Phred score >29) were aligned on the transcriptome assembly using the C++ script Bowtie2 (v2.0.2) (255 score) running thanks local engine using Galaxy Project server (Giardine, Riemer et al. 2005). The DESeq2 software (Love, Huber et al. 2014) (v2.12;<http://www.bioconductor.org/packages/release/bioc/html/DESeq2.html>) (defaults settings) allows for quantifying the differential gene expression with comparing two biological duplicates from uninfected snails sample (Bre1 and Bre2) against infected samples (Pvalue<0.1). For each cluster transcript ID,

Blast2GO annotation and Log2FC results were indicated.
(XLSX)

S2 Table. All cluster identified in Fig 5. Ratio of expression and mapping annotation were indicated for all the sequences identified as differentially regulated in the plasma comparative proteomic approach.

(XLSX)

S3 Table. Primers used for Q-RT-PCR. Identification numbers (iD), Blast2GO annotation and sequences of forward and reverse primers were indicated for 36 randomly selected transcripts of Naive1/Naive2 RNAseq biological samples. These sequences were used to analyse the correlation between the RNAseq reads per kilobase per million (log2 RPKM) values and the corresponding Q-RT-PCR cycle threshold (Ct) see S1 Fig.

(XLSX)

S1 Appendix. Expanded Materials and Methods section.

(DOCX)

S2 Appendix. Trade-off between reproduction and immunity.

(DOCX)

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This paper is dedicated to our colleague, Bernard Dejean.

Author Contributions

Conceived and designed the experiments: SP JP DD BG. Performed the experiments: SP JP DD FCN MAO JFA RG NMD SKJ BG. Analyzed the data: SP JP DD RG GM AT BG. Wrote the paper: SP JP DD RG NMD GM AT BG.

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Chapitre 2

Résister à l'opresseur

➤ A shift from Cellular to Humoral Responses Contributes to Innate Immune Memory in the Vector Snail *Biomphalaria glabrata*.

Pinaud S, Portela J, Duval D, Nowacki FC, Olive MA, Allienne JF, Galinier R, Dheilly NM, Kieffer-Jaquinod S, Mitta G, Théron A, Gourbal B.

PLoS Pathogens, 2016 doi: 10.1371/journal.ppat.1005361

↻ Polymorphic sets of immune receptors support specificity in the innate immune memory of the Schistosomiasis vector snail, *Biomphalaria glabrata*.

Pinaud S, Portet A, Portela J, Allienne JF, Saint-Beat C, Arancibia N, Galinier R, Duval D, Gourbal B.

En preparation pour Molecular Ecology, Été 2017

↻ Diversified aerolysin toxins in the vector snail *Biomphalaria glabrata*: an innovative family of immune weapons acquired by horizontal.

Pinaud S, Tetreau G, Portet A, Chaparro C, Galinier R, Gourbal B, Duval D.

Soumis dans Genome Biology, Automne 2017

➤ article publié ↻ article soumis

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 $500\mu\text{L}$ 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 $360\mu\text{L}$ 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 $0.05\mu\text{g}$ 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. Clusters 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).

HemocYTE flow cytometry 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.

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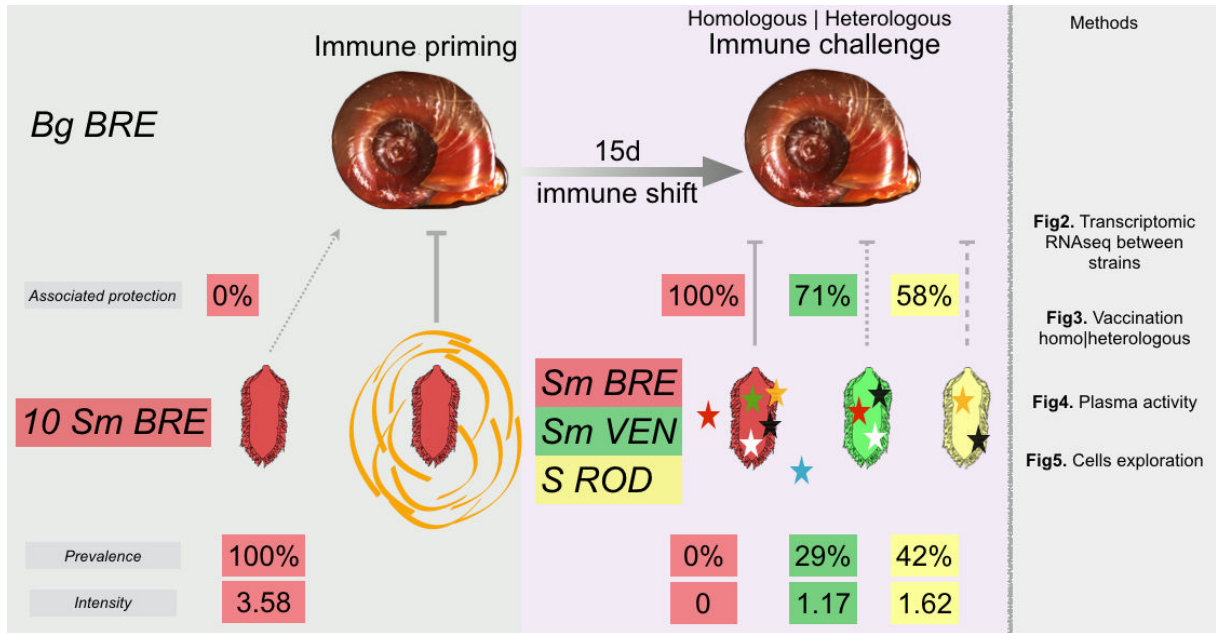


Figure 1. Overview of different analyses

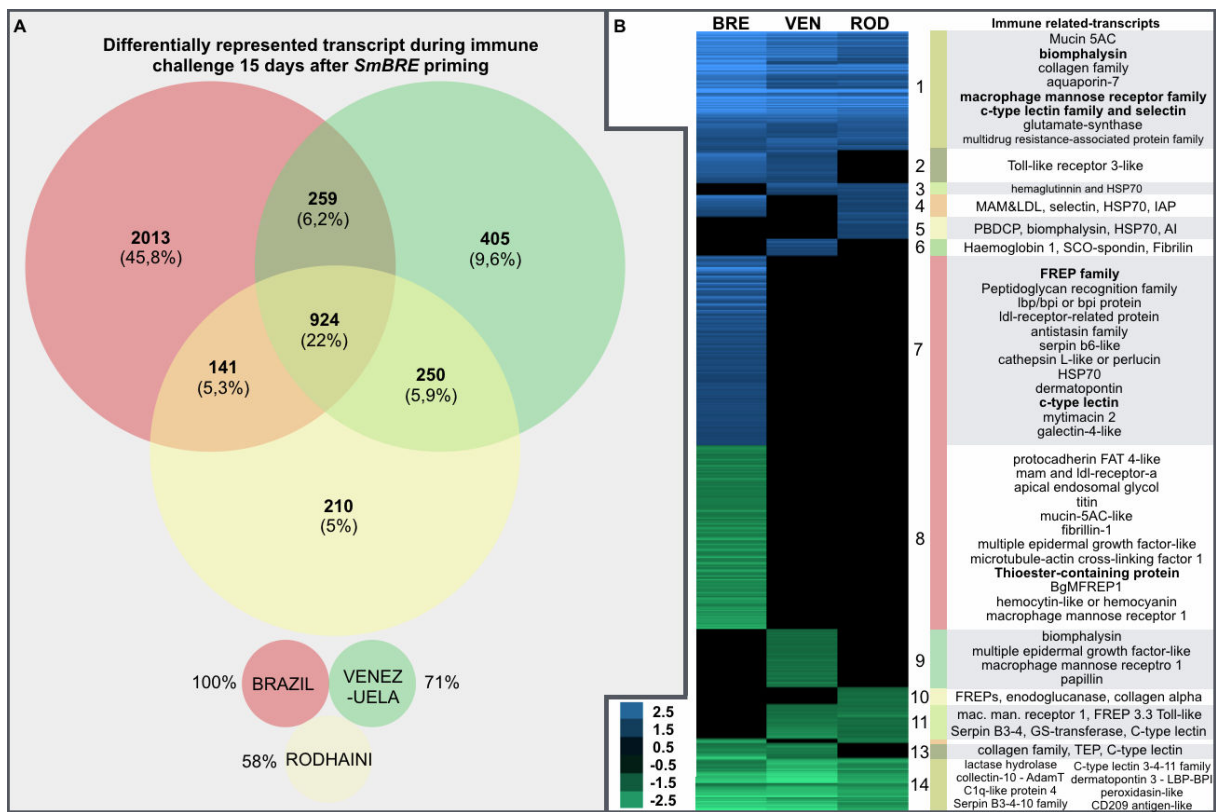


Figure 2 Transcriptomic analyses. A. Venn diagram of differentially represented transcripts depending of strain used for immune challenge. B.

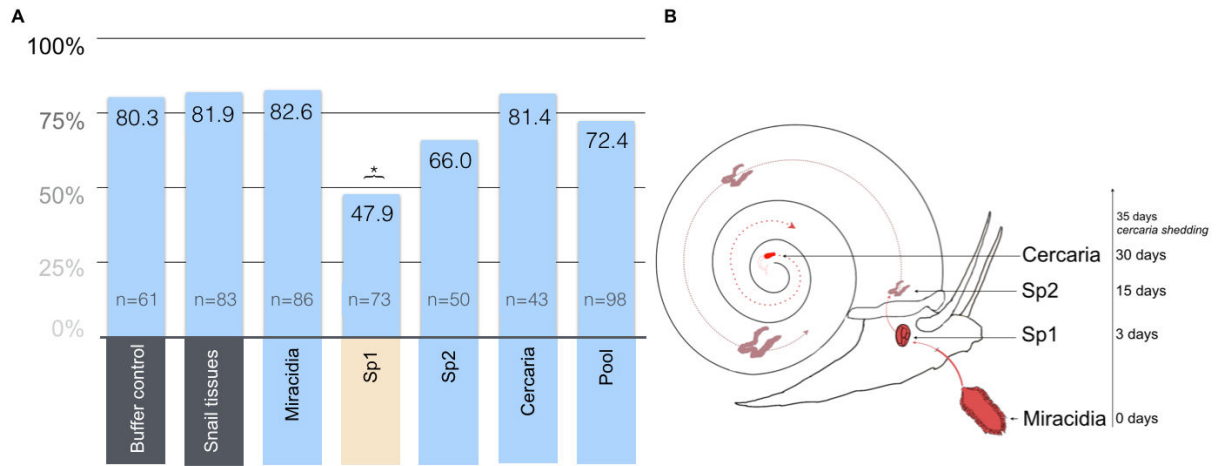


Figure 3. Vaccination A. Developmental stage of parasite used for immunisation B. Dynamic of development of parasite into the snail

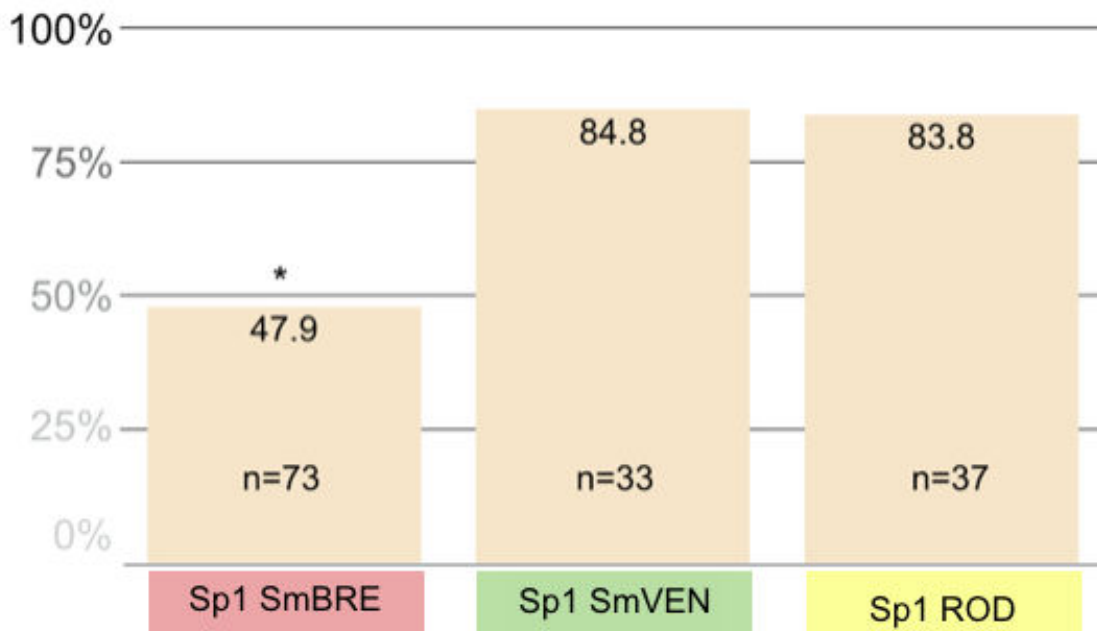


Figure 4 Heterologous vaccination with Sp1 extract

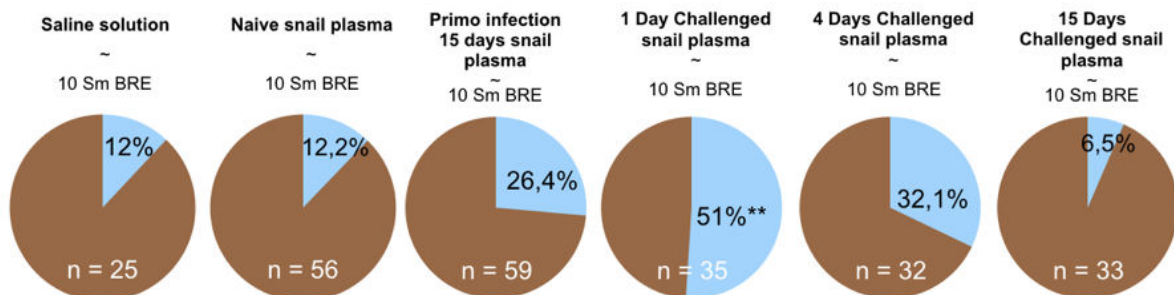


Figure 5. Hemolymph transfer

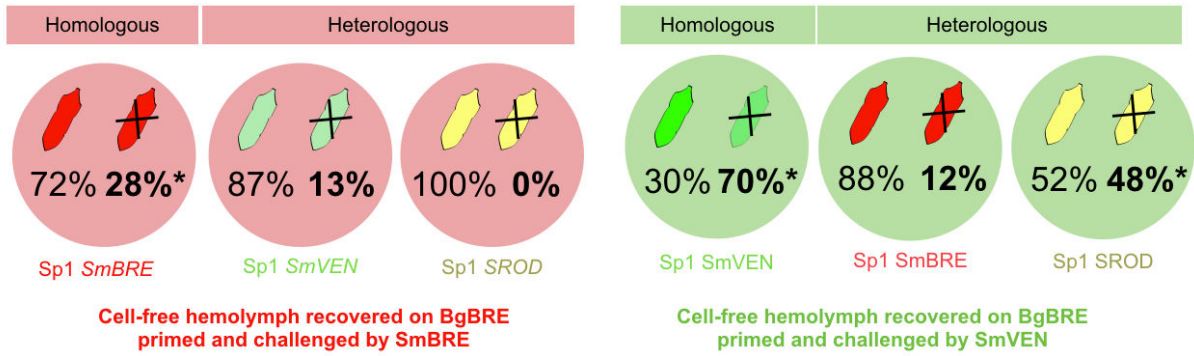
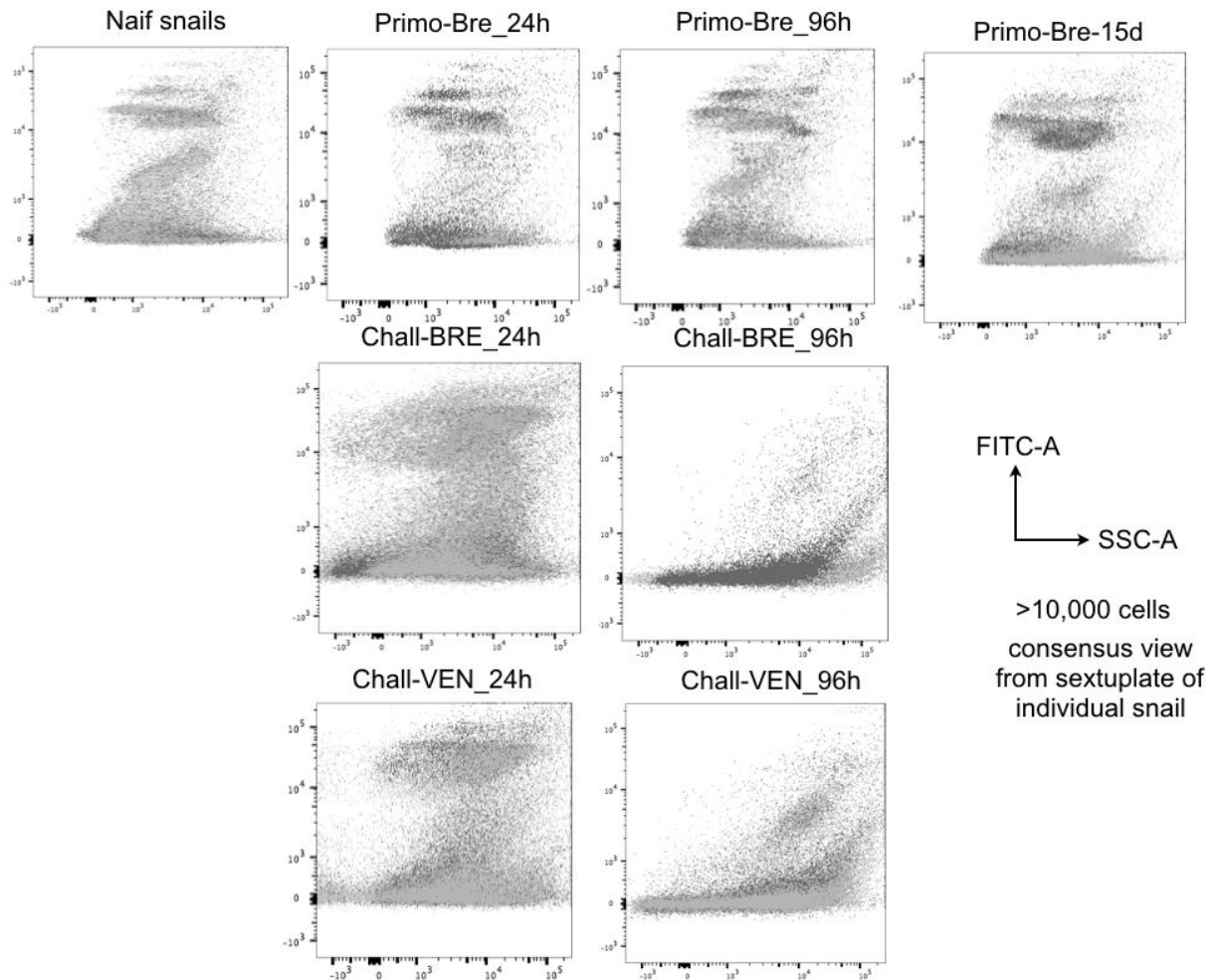
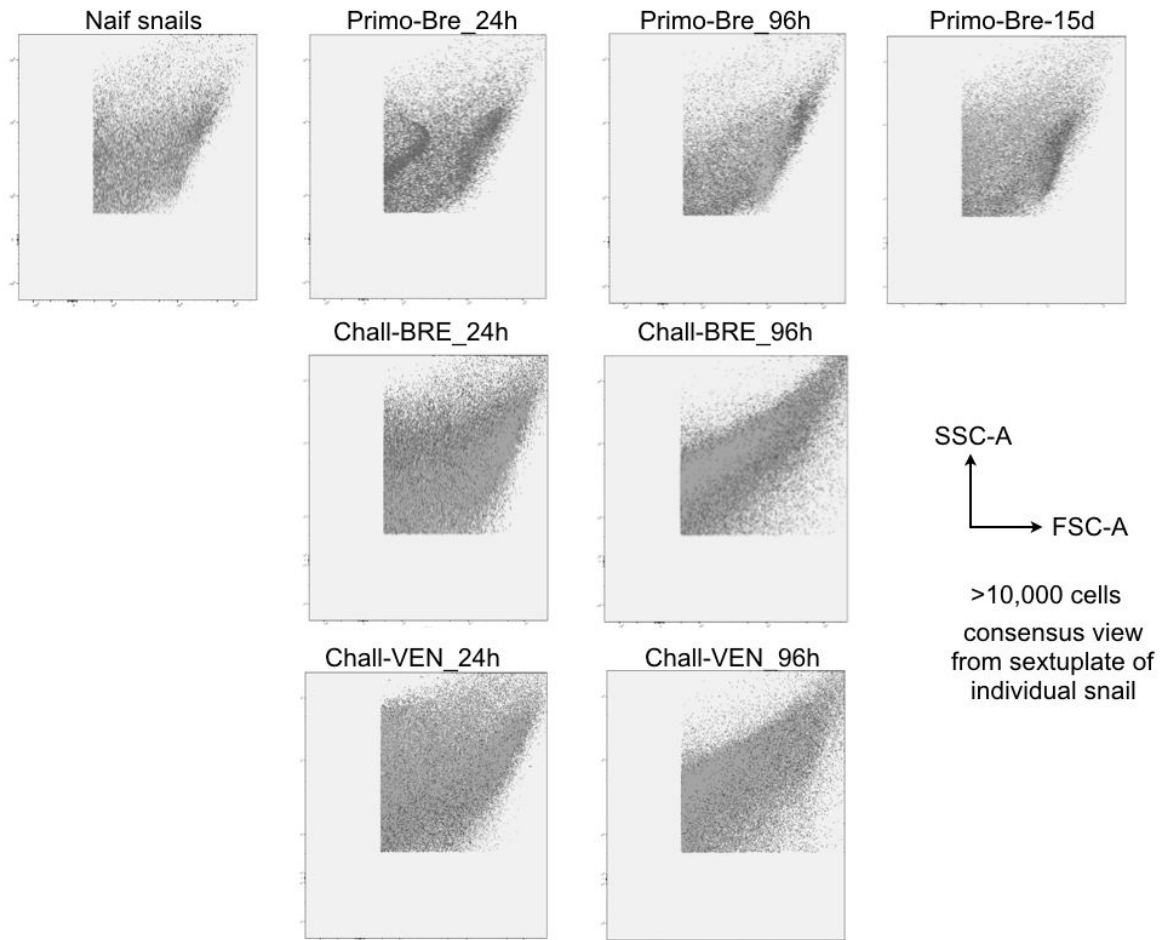


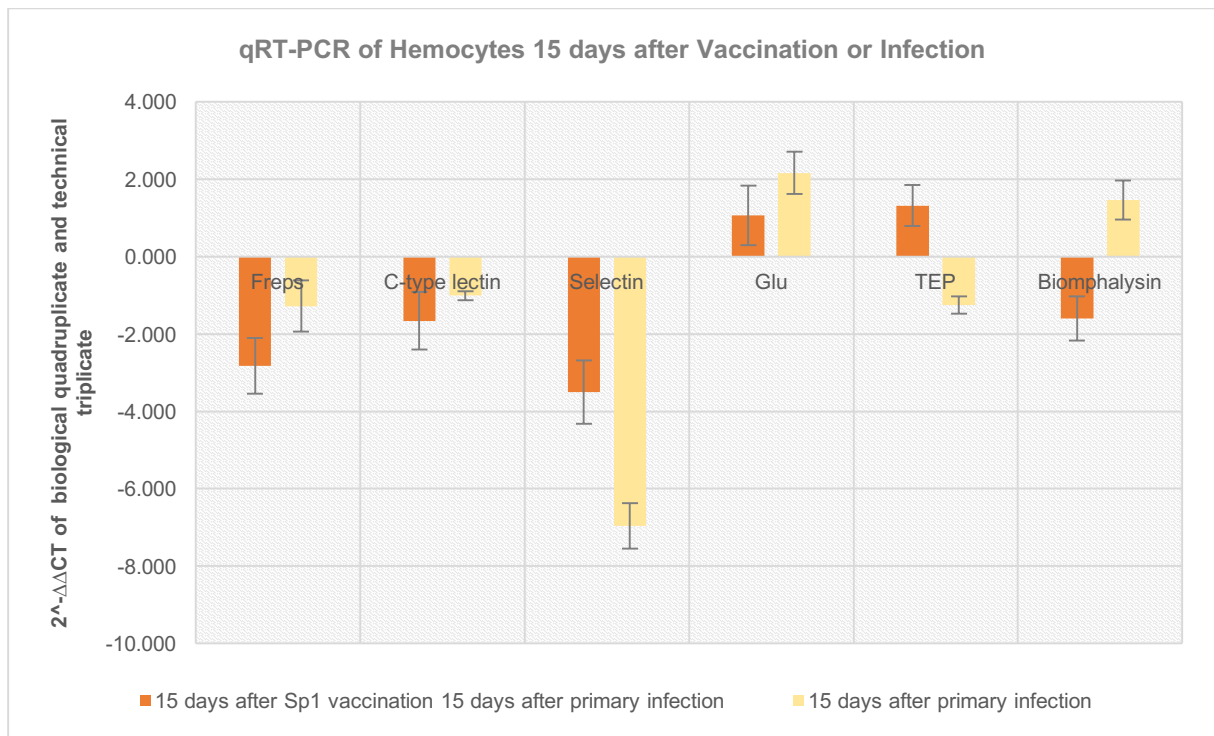
Figure 6. Specificity of *in-vitro* toxicity Red-left part present homologous SmBRE experiment while the green-right part the SmVEN homologous experiment. All tests were realised on BgBRE.



Supplementary 1. Cytometry essay in fluorescent-granularity



Supplementary 2. Cytometry assay in Size-granularity



Supplementary 3. Q-rtpCR assay on priming candidate between natural infection and vaccination

Chapitre 2

Résister à l'oppresseur

➤ **A shift from Cellular to Humoral Responses Contributes to Innate Immune Memory in the Vector Snail *Biomphalaria glabrata*.**

Pinaud S, Portela J, Duval D, Nowacki FC, Olive MA, Allienne JF, Galinier R, Dheilly NM, Kieffer-Jaquinod S, Mitta G, Théron A, Gourbal B.
PLoS Pathogens, 2016 doi: 10.1371/journal.ppat.1005361

↻ **Polymorphic sets of immune receptors support specificity in the innate immune memory of the Schistosomiasis vector snail, *Biomphalaria glabrata*.**

Pinaud S, Portet A, Portela J, Allienne JF, Saint-Beat C, Arancibia N, Galinier R, Duval D, Gourbal B.
En preparation pour Molecular Ecology, Été 2017

↻ **Diversified aerolysin toxins in the vector snail *Biomphalaria glabrata*: an innovative family of immune weapons acquired by horizontal.**

Pinaud S, Tetreau G, Portet A, Chaparro C, Galinier R, Gourbal B, Duval D.
Soumis dans Genome Biology, Automne 2017

➤ article publié ↻ article soumis

1 **Diversified aerolysin toxins in the vector snail *Biomphalaria glabrata*:**
2 **an innovative family of immune weapons acquired by horizontal**
3 **transfer.**

4

5 **Silvain PINAUD¹, Guillaume TETREAU^{1,2}, Anaïs PORTET¹, Cristian CHAPARRO¹,**
6 **Richard GALINIER¹, Damien LASSALLE¹, Benjamin GOUBAL¹, David DUVAL¹**

7

8 ¹Univ. Perpignan Via Domitia, IHPE UMR 5244, CNRS, IFREMER, Univ. Montpellier, F-66860
9 Perpignan, France.

10 ²Current address: Univ. Grenoble Alpes, CNRS, CEA, IBS, F-38000 Grenoble.

11

12

13 SP and GT have contributed equally to this work.

14 Correspondence should be addressed to: D.D. (david.duval@univ-perp.fr).

15

16 E-mail addresses:

17 S.P. silvain.pinaud@univ-perp.fr,

18 G.T. guillaume.tetreau@gmail.com,

19 A.P. anais.portet@univ-perp.fr,

20 C.C. cristian.chaparro@univ-perp.fr,

21 R.G. richard.galinier@univ-perp.fr,

22 D.L. damienlassalle@me.com,

23 B.G. benjamin.goubal@univ-perp.fr,

24 D.D. david.duval@univ-perp.fr.

25

26 **Abstract**

27 **Background.**

28 Horizontal gene transfers (HGTs) are a major force of genome diversification and evolution.
29 While HGT events between prokaryotes or from prokaryotes to plants and animals are
30 increasingly reported, evidences for their functionalization within the recipient species are
31 scarce. Here, we present the acquisition by HGT of bacterial aerolysin toxins, named
32 biomphalysins, maintained as an immunological weapon in the vector snail, *Biomphalaria*
33 *glabrata*.

34 **Results.**

35 Snail genome exploration led to the identification of a total of 23 intron-less biomphalysin
36 genes. By phylogenetic-dependent and -independent methods, we identified at least two HGTs
37 from bacterial microbiota that were at the origin of this new family. Deduced amino acid
38 sequences revealed an important range of sequence identity between members while a high
39 degree of structural homology was predicted. Contrasting patterns of tissue-specific
40 expression and different recognition domains were evidenced in their sequence. This suggests
41 potential non-redundant functions, including the recognition of diverse pathogens, connected
42 to different regulatory networks such as microRNA pathway.

43 **Conclusion.**

44 After the transfer of aerolysin-like ancestor bacterial genes, multiplication and domestication
45 in the snail genome led to the acquisition of the biomphalysin genes family. A strong selective
46 pressure occurred at the structural level rather than at the sequence level with the conservation
47 of the big lobe to maintain the lytic activity. In contrast, the high variation in the small lobe
48 containing a potential pathogen-recognition domain supports the involvement of different
49 biomphalysins in the recognition of a different set of pathogens. Altogether, these results
50 support the central role of biomphalysins as key weapons in the snail immune system.

51 **Keywords :** Horizontal gene transfer (HGT); aerolysin-related protein; neofunctionalization;
52 invertebrate immunity; evolution; genomic; phylogenetic analyses; tridimensional structure;
53 pore forming toxin (PFT)

54 **Background**

55 Horizontal (or lateral) gene transfer (HGT) refers to the transmission of DNA between
56 different individuals independent from vertical gene inheritance (from parents to offspring). The
57 high prevalence of HGT in prokaryotes highlights their major role in their adaptation strategy
58 and genome evolution [1-4]. However, HGT is not restricted to prokaryotes. Inter-kingdom
59 gene transfers have been reported, from prokaryotes to plants and to animals, but also from
60 metazoans to prokaryotes and even between different metazoans [5, 6]. The development and
61 democratization of massive genome sequencing of non-model organisms has led to the
62 consistent rise in the number of studies reporting genes presumably laterally acquired [6, 7].
63 Conservation of new genes acquired by HGT is expected to happen when these genes confer
64 a selective advantage for the recipient species in the adaptation to abiotic and/or biotic
65 environmental conditions [8-10]. Nevertheless, evidences are scarce for multiple recurrent
66 HGTs [11-14] and even scarcer when it comes to their functional diversification within the
67 receiver species [15, 16].

68 In general, the HGT events are favored by an intimate relationship between the
69 recipient and donor organisms, such as the microbiota that represents a major source of gene
70 transfer to their host [17]. In the case of host-pathogen interactions, some acquisition of genes
71 from the host to the pathogens have been reported, which illustrates one pathogens' evolution
72 strategy to circumvent and overcome host immunity [18, 19]. Notably, the acquired genes
73 coopted by viruses to facilitate pathogen evasion by the subversion of innate immune signaling
74 are the most characterized [20]. Transfer of genes also occurs from pathogens to the host.
75 Bacterial toxins that exhibit antibacterial activities are good candidates of such genes
76 potentially providing direct advantage in the recipient species by complementing their immune
77 system, even if no direct evidence are available to date [11, 21]. Several members of the
78 aerolysin family, which are β -pore-forming toxins (PFTs) named after the bacterium genus
79 *Aeromonas*, have been identified in many metazoan genomes [11]. In plants, production of
80 cytolytic enterolobin in seeds seems to protect it from herbivore grazing and/or insect attack

81 [22]. In Cnidarians, hydralysin and nylysin-1b have been suspected to play a role in host
82 defense against predators and for killing preys [11, 23].

83 Recently, another aerolysin-related protein, named biomphalysin, was described in the
84 Schistosomiasis vector snail *Biomphalaria glabrata* [24]. This toxin was able to bind to the
85 membranes of the metazoan parasite *Schistosoma mansoni* and to participate in its elimination
86 through its cytotoxic activity [24]. Moreover, a recent genome-wide study revealed that the
87 biomphalysin locus was strongly correlated with resistance to *S. mansoni* [25] and
88 transcriptomic experiments indicated that re-exposure to *S. mansoni* triggered an increased
89 representation of biomphalysin transcript in *B. glabrata* [26]. In this study, we report the
90 expansion of a large repertoire of immune molecules originally acquired by HGT belonging to
91 the aerolysin family. Interestingly, data from the newly released genome of *B. glabrata* provide
92 evidence for the presence of multiple loci coding for aerolysin analogues in this snail [26, 27].
93 We have focused on the comprehensive characterization of the expansion of this β -PFT
94 biomphalysin family by combining genomic, transcriptomic, phylogenetic, structural and
95 functional analyses in *B. glabrata*. Altogether, our results provide evidence that these toxin
96 genes acquired from bacteria were diversified and functionalized to become a major weapon
97 of the snail immune system.

98

99 **Results**

100 **From one biomphalysin to a family of 23 genes**

101 To identify all the genes encoding analogues of aerolysin toxins, we conducted an
102 exhaustive sequence search of the genome and transcriptomes of *B. glabrata*. Using the gene
103 sequence of the biomphalysin previously identified [24], only one partial sequence of 395
104 bases (Locus_25218_Transcript_19/20) was identified from a local BLAST search of RNA-seq
105 data from uninfected snails [28]. However, a tBLASTn search using the same query and
106 database allowed finding 10 additional hits with potential homology to biomphalysin with an E-
107 value score ranging from 1e-79 to 5e-38. As most of them harbored an aerolysin or epsilon
108 toxin signature in their sequence (IPR005830 and IPR004991, respectively), an InterProScan

109 and sequence similarity searches were conducted to investigate the presence of additional
110 aerolysin-like genes in the genome of *B. glabrata* (preliminary assembly v4.3) [27] and the
111 RNA-seq data from *S. mansoni* infected snails [26]. A total of 23 unique loci were identified on
112 20 different genomic scaffolds (Additional file 1). This included the biomphalysin gene
113 previously identified, which was renamed biomphalysin 1 and the others were named from 2
114 to 23. Interestingly, all the biomphalysin genes identified were devoid of intron, which
115 represents an evolutionary conserved characteristic of this family.

116 Several biomphalysins were found in the same scaffold. Biomphalysins 1 & 2, 11 & 22 and 20
117 & 21 were located in tandem in large scaffolds that are scaffold 10 (1 Mb), 2594 (72 kb) and
118 2201 (88 kb), respectively. This suggests that these gene pairs resulted from a duplication
119 event. Interestingly, although present on different scaffolds, biomphalysins 5 and 6 exhibited
120 a high sequence homology of the two gene parts located in the 5' and 3' regions of the genes,
121 as evidenced by comparative sequence dot blot analyses performed with Gepard (Additional
122 file 2, Figure S1). A similar observation was made for biomphalysins 11 and 22 in the 5' part
123 of the sequence. No other highly conserved regions were detected by Gepard analysis
124 (Additional file 2, Figure S1). This is supported by the absence of significant signatures of
125 transposable elements in the 20 Kb vicinity of biomphalysin genes by using RepeatMasker
126 software and by performing BLAST searches against the repeat database (Dfam and
127 Repbase). Repetitive elements such as transposable elements do not seem to have been
128 involved in gene duplication and expansion of this novel family. This further supports that
129 biomphalysin gene family expansion likely occurred through gene duplication.

130

131 **Biomphalysins represent a highly diverse family in *B. glabrata***

132 To rule out eventual errors in genome sequencing and assembly, each biomphalysin
133 gene was amplified and sequenced by Sanger sequencing. PCRs were performed on snail
134 genomic DNA using specific primer pairs flanking the predicted coding DNA sequence of each
135 biomphalysin gene. The putative translated regions were 1725-bp long on average (1698 -
136 1758 bp). The new biomphalysins identified exhibited a 43.2 to 94.7% and 33.1 to 95.1%

137 identity as compared to biomphalysin 1 based on the nucleic and deduced amino acid
138 sequences, respectively (Additional file 3). Moreover, protein sequence similarity to aerolysin
139 toxin (Accession number: AAA21938) ranged from 23.6 to 30.2% (Additional file 4). The
140 lowest identity was found between biomphalysins 9 and 22 (29.2%), while the highest was
141 observed for biomphalysins 1 and 2 (95.1%). Their level of pairwise identity was also very
142 dissimilar, ranging from 32.6% between biomphalysins 5 and 23 to 97.7% between
143 biomphalysins 1 and 2.

144 The deduced amino-acid sequences of the 23 biomphalysins were compared by
145 performing a phylogenetic analysis (Figure 1). Analyses performed with gene and protein
146 sequences generated similar results. At least three clusters of biomphalysins could be
147 observed and they were supported by high bootstrap values (Figure 1). The first cluster
148 included biomphalysins from 1 to 10 (except biomphalysin 9), the second one biomphalysins
149 from 11 to 18 and the last one biomphalysins from 19 to 23. Biomphalysin 9 was isolated from
150 the 22 other biomphalysins and it was the closest biomphalysin to the aerolysin sequence used
151 as an outgroup (Figure 1).

152

153 **Biomphalysins exhibit common domains and cluster-specific features**

154 Biomphalysin proteins are composed of 575 residues on average (from 565 to 585
155 amino acids). They all harbored a predicted N-terminal signal peptide for secretion composed
156 of 17-23 amino acids that adopt an alpha-helical conformation (Figure 2). A structural feature
157 common to all aerolysin-like toxins is the presence of many β -sheet elements that represent
158 approximately 40% of total sequence. Most of them are present in the common core, also
159 called aerolysin domain, consisting of five β -strands with an insertion loop corresponding to
160 the pore-forming transmembrane domain (TMD) (Figure 2). This TMD is found in all
161 biomphalysins and is shared by members of the β -PFTs as it plays a crucial role in the
162 formation of amphipathic barrels by oligomerization for membrane insertion [29, 30]. This
163 predicted membrane spanning domain flanked by two polar residue regions is found in all
164 biomphalysin sequences (Additional file 5, Figure S2-A). Moreover, an alternating pattern of

165 hydrophobic and hydrophilic residues located in this beta-hairpin loop has been conserved in
166 all biomphalysins, highlighting their ability to perforate the lipid layer of target organisms
167 (Additional file 5, Figure S2-B, S2-C) [31]. Interestingly, the amino acids involved in
168 oligomerization and cytolytic activity were highly conserved in all biomphalysin genes.
169 Compared to biomphalysin 1, Asp₂₃₅ and Cys₂₅₅ mutations display a crucial role in the
170 oligomeric assembly into a ring like structure [32, 33]. Tryp₄₆₆ and Tryp₄₆₈ mutations modify in
171 the aerolysin toxin its ability to oligomerize [34] whereas Tryp₄₂₀ and His₄₂₈ could be involved
172 in binding to a membrane receptor [34, 35]. The C terminal part tends to be divergent and
173 could be associated to a differential lytic activation [36].

174

175 **Species-specific diversity of aerolysin-like proteins among invertebrates**

176 To determine whether such an expansion and/or diversification of the aerolysin-like
177 genes occurred in other invertebrates, we also searched in several Sequence Databases of
178 NCBI (Nucleotide, Protein, EST). Then, a phylogenetic analysis was performed using
179 sequences of the aerolysin domain of aerolysin-like proteins from 3 different bacteria genera
180 (*Aeromonas*, *Clostridium* and *Vibrio*) and different invertebrates, including the Gastropoda *B.*
181 *glabrata* and *Aplysia californica*, the Hydrozoa *Hydra vulgaris* and *H. viridissima*, the Anthozoa
182 *Nematostella vectensis* and *Exaiptasia pallida*, and the Arachnida *Ixodes scapularis* (Figure
183 3). Bacterial toxins all clustered together, with three different sub-clusters corresponding to the
184 three bacterial genera used, and they were clearly separated from invertebrate aerolysin-like
185 sequences (Figure 3). Increasing the number of bacterial sequences used in the analysis did
186 not modify the clustering of bacteria in the tree (Additional file 6, Figure S3).

187 For all invertebrate species analyzed, with the exception of *B. glabrata*, all aerolysin-
188 like proteins identified in the same species were clustered together, with relatively high
189 bootstrap support (ranging from 60 to 100%) (Figure 3). Interestingly, the phylogenetic
190 closeness of species did not induce a higher phylogenetic similarity in the sequences of
191 aerolysin-like proteins. Indeed, aerolysin-like proteins from the Gastropoda *A. californica* were
192 more similar to those from the Anthozoa *E. pallida* and *N. vectensis* than to most of the

193 biomphalysins from the Gastropoda *B. glabrata* (Figure 3). Similarly, Anthozoa and Hydra,
194 which are two classes from the Cnidarian phylum, were found at the opposite of the tree
195 generated with sequences of aerolysin domains from aerolysin-like proteins. This suggests
196 that the multiple aerolysin-like proteins identified in these invertebrate species resulted from a
197 species-specific diversification.

198 In *B. glabrata*, only the biomphalysin 9 was not clustered with the 22 other
199 biomphalysins and rather grouped with aerolysin-like proteins from Anthozoa (Figure 3),
200 supporting the hypothesis that biomphalysins originated from at least two distinct HGT events.

201

202 **Biomphalysins result from an old domestication of aerolysin-like genes**

203 Recent horizontal transfer events can generally be determined by a significant
204 difference in the GC content and in the codon usage between the sequence of the gene
205 supposedly acquired by HGT (here, biomphalysin) and gene sequences from the recipient
206 species (here, other genes from *B. glabrata*). The GC content (GC%) of biomphalysin
207 nucleotide sequences (N = 23) was calculated and compared to the one of *B. glabrata*
208 transcripts sequences (N = 25) of similar size (± 1700 pb) from RNA-seq data and of bacterial
209 toxin sequences (N = 13) retrieved from NCBI (Figure 4-A). *B. glabrata* transcripts exhibited a
210 GC% ranging from 21% to 56% with a peak of normal distribution at 39%. The GC content of
211 biomphalysin genes (41.0 %) was similar to *B. glabrata* transcripts but was significantly lower
212 than the GC% of bacterial toxin nucleic sequences (55.8 %, Fisher's exact test, p-value =
213 0.0232). Concerning the codon composition, a principal component analysis (PCA) was
214 performed with mean codon usage values for each triplet in *B. glabrata* transcripts,
215 biomphalysin gene sequences and toxin sequences from bacteria (Figure 4-B). Most of the
216 variance (78.1%) was explained by the first axis, which opposed bacterial sequences to snail
217 ones, while the second axis, explaining 21.9% of variance, separated the biomphalysins from
218 other genes from *B. glabrata* (Figure 4-B). GC% and codon usage of biomphalysin and
219 bacterial toxin genes have distinct signatures and their analysis indicates that biomphalysins

220 are closer to endogenous *Biomphalaria* transcripts than bacterial toxin genes, which suggests
221 an old domestication of biomphalysin genes by the snail.

222 HGT is known to be enhanced by an immediate proximity between the potential donor
223 of gene and its recipient [3, 37]. To find the potential bacterial origin of the laterally-acquired
224 aerolysin-like coding gene, we explored the microbiota bacterial diversity by 16S rRNA-
225 targeted Next-Gen sequencing. A total of 324 different families of OTUs (Operational
226 Taxonomic Units) were identified in naive snails (Additional file 7). Among them, several key
227 families of bacteria known to produce β -PFTs were identified, including *Aeromonadaceae*,
228 *Clostridiaceae* and *Pseudomonadaceae*, which corresponds to 2.03% of total OTUs observed.
229 The presence of these taxa in the snail microbiota nowadays suggests that they might have
230 been associated with the *Biomphalaria* genus at the time it acquired biomphalysins and it could
231 support their origin by horizontal transfer from microbiota.

232

233 **Highly conserved three-dimensional structures despite a high variation in sequence** 234 **conservation**

235 Despite the apparent divergence and the low sequence homology between the 23
236 biomphalysins, their structure conserves high levels of similarity. We previously reported that
237 the anti-parasitic biomphalysin 1 displayed the same structural features as the bacterial
238 aerolysin toxin [24]. A structural modeling for each biomphalysin protein was performed using
239 the best aligned template against the Protein Data Bank (PDB) database (<http://rcsb.org/>) by
240 using the I-Tasser server [38, 39]. Structural similarities are indicated by a TM score ranging
241 from 0 (totally dissimilar proteins) to 1 (exact same structure). All biomphalysins exhibited a
242 structure close to the aerolysin one with a TM score ranging from 0.66 to 0.91 (Additional file
243 4). The analysis allowed predicting two distinct lobes: a small lobe named Domain I and a large
244 lobe, also qualified as an aerolysin domain, constituted of three structural domains (Domain II,
245 III and IV). Despite differences in the primary structure, highly similar conformations were
246 observed between the different members, with an average pairwise TM score of 0.73. Analysis
247 of the conservation of residue positions among the biomphalysin family was performed with

248 the ConSurf software (Figure 5) [40]. Conservation scores mapped onto the 3D structure of
249 biomphalysin 1 revealed that most conserved residues are located in the Domain II, which is
250 involved in oligomerization [32] and receptor binding in aerolysin [41, 42], and in the Domain
251 III, which is necessary for the stabilization of the channel [43] and for toxin insertion into lipid
252 bilayers via the transmembrane α -hairpin loop (Figure 5) [29]. Conserved residues are
253 strategically located to maintain the highly organized typical topological arrangement of a β -
254 PFT. Indeed, the most conserved residues shape the two α -helices and the different β -strands
255 from Domains II and III. The structure of this aerolysin domain appears to be under selection
256 despite a divergent sequence with an average TM score of 0.93 ± 0.02 (Additional file 8).
257 Surprisingly, many variable amino acids were found in loop regions and particularly in the loop
258 from TMD (Figure 5). Nevertheless, the stretches of alternating hydrophobic and hydrophilic
259 residues, which are key elements for the transmembrane feature, are conserved (Additional
260 file 5, Figure S2).

261 Considering that structural information revealed the presence of variable loops mostly
262 located in the Domain II, which could contribute to the specificity of interaction with its target,
263 protein-ligand binding sites were predicted based on biomphalysin three-dimensional
264 structures. A mannose-6-phosphate binding domain was identified in the Domain II of all
265 biomphalysins (Figure 6). This carbohydrate binding pocket is located in the vicinity of the two
266 α -helices, a well-characterized site in aerolysin toxin involved in the binding of the mannose
267 residue from glycosyl phosphatidylinositol (GPI)-anchored receptors [44]. It contains several
268 key residues for its binding activity, such as arginine, glycine and tryptophan (Figure 6) [30,
269 45, 46].

270

271 **Small lobe shares high structure similarity with recognition domains**

272 The small lobe (Domain I) of the aerolysin toxin presents a binding site similar to the
273 carbohydrate-binding domain of C-type lectins, also called Aerolysin Pertussis Toxin (APT)
274 domain (IPR005138) [47]. No lectin motif was found in the amino acids sequence from all the
275 biomphalysins. As most residues were variable in the small lobe of biomphalysin proteins, a

276 similarity structural search was performed using the I-TASSER software against proteins
277 whose tridimensional structure has been resolved by crystallography [38]. TM scores ranged
278 from 0.45 to 0.83 and were therefore much lower than those of the aerolysin lobe (0.89 to 0.96)
279 (Additional file 8). No significant prediction was obtained for seven of them as their similarity
280 score was below the threshold of 0.5 (Additional file 8) [38]. The analysis of the small lobes of
281 the 15 other biomphalysins gave relevant structural alignments to 12 different crystallized
282 proteins or domain of crystallized proteins (Table 1). Despite an overall similar architecture,
283 the small lobe of the different biomphalysins were predicted to fold into different states (Figure
284 7). For example, the small lobes of the biomphalysin 8 and 23 were similar to the Domain II of
285 a mosquitocidal Cry toxin, a domain involved in receptor recognition and binding [48]. Eight of
286 them matched with a high TM score (higher than 0.6) to members of the complement system,
287 which is part of the innate immune system that enhance the immune response against
288 pathogens through their interacting features [49]. This indicates that the structural
289 diversification of the small lobe might have led to a functional diversification of biomphalysins.
290 Domain I presents the highest variability of folding units that are independent from the aerolysin
291 lobe, leading to a potential broad spectrum of targeted organisms. This unit module could be
292 considered as a diversified receptor-binding domain associated with the aerolysin domain
293 responsible for lytic function.

294

295 **Each biomphalysin is expressed and exhibits a specific pattern of tissue-specific gene** 296 **expression**

297 High quality reads from a previous RNA-seq experiment using two pools of 20 naïve
298 snails were used [26]. They were aligned with high-stringency to the 23 biomphalysin genes
299 using Bowtie2, counted and normalized by upper-quartile for each library. Every biomphalysin
300 was detected in whole naïve snails with different levels of expression (Figure 8-A).
301 Biomphalysins 1, 2, 4, 20 and 21 were the most represented whereas biomphalysins 5, 14 and
302 16 were the least abundant. Presence of all biomphalysin transcripts in snail highlights that all

303 biomphalysins seem to be assimilated in the host genome and suggest that none of them are
304 pseudogenes.

305 To further explore the patterns of expression of the biomphalysin genes, we analyzed
306 their transcript representation by RT-PCR in seven different tissues (stomach, albumen gland,
307 ovotestis, hepatopancreas, hemocytes, mantel, head-foot). This revealed a mosaic pattern of
308 expression of these genes in naïve snails (Figure 8-B). While some biomphalysins, such as
309 biomphalysins 2, 10, 14, 18 and 23, were ubiquitously expressed in all tested tissues, others
310 were specifically expressed in some tissues, such as biomphalysin 7 that was restricted to the
311 hepatopancreas and stomach or the biomphalysin 17 only located in the mantel and stomach
312 (Figure 8-B). Moreover, a high variation in the number of biomphalysins expressed could be
313 observed between the tissues (Figure 8-B). The highest number of biomphalysins expressed
314 was observed in the stomach, containing transcripts for 22 out of the 23 biomphalysins, while
315 albumen gland and hemocytes solely displayed 11 and 12 biomphalysins expressed,
316 respectively (Figure 8-B). Such high variability in tissue expression suggests that the different
317 biomphalysins might have different functions. Many transcriptional factors binding sites were
318 identified in the promoter regions of the biomphalysins but none was found conserved for all
319 the 23 biomphalysins (Additional files 9 and 10). The diversity of the *cis* regulatory elements
320 found in the promoter regions suggest that each biomphalysin gene could be regulated
321 spatially and temporally by specific transcription factors, supporting the hypothesis of a
322 functional divergence in this family (Additional file 10). Among the most represented
323 transcription factors, Sox5 and AT-rich interactive domain-containing protein 3A (Arid3a) were
324 found. They are both involved in the activation of immune gene transcription [50-52] and in the
325 differentiation of lymphocytes B and T in vertebrates [53, 54]. Consequently, expansion of the
326 biomphalysin gene family could have been associated with a functional diversification and sub-
327 functionalization of the snail innate immune system to mount an efficient response to different
328 pathogens.

329

330 **Different biomphalysins are involved in the recognition of a large diversity of pathogens**

331 A recent interactome experiment was performed, which consisted in putting in contact
332 circulating proteins from the snail cell-free hemolymph with outer membrane proteins from
333 living pathogens [55]. This approach allowed the identification of biomphalysin as a potential
334 pathogen-interacting protein [55]. It was interacting *in vivo* with gram-negative (*Escherichia*
335 *coli*) and gram-positive bacteria (*Micrococcus luteus*), yeast (*Saccharomyces cerevisiae*) and
336 two metazoan parasites, *Echinostoma caproni* and *S. mansoni*) [55]. From the 2D gels
337 performed, we collected the peptide list obtained by the MS/MS analysis of the spots identified
338 as biomphalysin and we mapped them on the deduced amino acid sequence of the 23
339 biomphalysins (Table 2). While some peptides were identified in 4 out of 5 interactions, some
340 were only present in 1 pathogen interaction. By comparing all the biomphalysin genes to which
341 each peptide could refer to, we managed to calculate the minimum number of biomphalysins
342 involved in the interaction with each pathogen. While *E. coli* and *E. caproni* were recognized
343 by at least one biomphalysin, a minimum of two biomphalysins were required to sense the
344 presence of *M. luteus*, *S. cerevisiae* and *S. mansoni* in the snail hemolymph (Table 2). This is
345 consistent with the fact that three different spots were identified in the gels for these three
346 pathogens, which seem to be different biomphalysins and not different isoforms of the same
347 biomphalysin as previously thought [55].

348

349 ***B. glabrata* produces miRNA that target biomphalysin genes**

350 To integrate an horizontally acquired gene into a larger genetic network, the recipient
351 organism must not only reformat the gene to use it in function of its own codon usage but also
352 set up a fine regulatory mechanism. As biomphalysins could display a key role in immunity as
353 a pathogen receptor but also as an effector, we investigated whether their transcripts could be
354 targeted by endogenous miRNAs, a key component of immune regulation [56]. Sequences of
355 5'-UTR, CDS and 3'-UTR of all biomphalysins were examined using miRanda software to
356 determine if the recently characterized snail miRNAs repertoire do possess high binding
357 enthalpy for the biomphalysins UTR or CDS regions [27]. All biomphalysin transcripts were
358 identified as potential targets by at least one small non-coding RNA molecule (Figure 9). One

359 transcript could be affected by several miRNAs, such as the 3'UTR of biomphalysin 3 targeted
360 by the two miRNAs bgl-miR-1992-3p and bgl-miR-34-5p. The same miRNA could also interact
361 with different biomphalysin transcripts, such as bgl-miR-133-5p that can interact with 7 sites
362 located on transcripts from biomphalysins 1 to 7 (Additional file 11). This finding of potential
363 miRNA-mediated biomphalysin gene expression regulation underlies their genomic
364 assimilation but also their functional integration into well-established regulatory networks
365 including immune response.

366

367 **Discussion**

368 Horizontal gene transfers are considered as a major source of innovation for a recipient
369 genome if the acquired gene confers a gain of fitness and/or a better adaptation in a changing
370 environment. Genetic material exchange has been known for many years between prokaryotes
371 and allowed them acquiring new phenotypes. The gene transfer must not be considered as an
372 epiphenomenon since massive gene acquisition has been reported in numerous organisms.
373 For example, 18% of total genes in *E. coli* were acquired horizontally and the hyperthermophilic
374 bacteria *Aquifex aleolicus* would herit about 40% of genes from archaeal families making it a
375 true chimera. If the mechanisms of HGT between prokaryotes are well characterized, the
376 question of gene transfer between prokaryote and metazoan still requires further investigation.
377 Considered as exogenous DNA genomic contamination or important evolutionary force
378 involved in the acquisition of selective advantages by some pluricellular organisms, unknown
379 transfer mechanisms and no evidence for a functional bona fide gene makes it difficult to
380 validate most of them and to highlight the great importance of HGT in genome evolution.

381 In this work, we report the characterization of 23 aerolysin-like genes in the snail *B.*
382 *glabrata*. Diversification of this family to obtain the 23 biomphalysins likely occurred through
383 multiple gene duplications events, unrelated to transposable elements. Nevertheless, not all
384 biomphalysin genes were clustered together. Biomphalysin 9 displayed more similarity with
385 the sequences of aerolysin-related toxins from the cnidarians *Axaiptasia pallida* and
386 *Nematostella vectensis* than with the other biomphalysins, which supports at least two HGT

387 events. Considering that all biomphalysin genes are intron-less, it was expected that
388 biomphalysins were acquired from bacteria. Although phylogeny failed at identifying the donor
389 bacterial species, the presence of several bacterial taxa known to produce aerolysin-like toxins
390 in the snail's microbiota suggests that biomphalysin acquisition might have happened through
391 a microbiota-to-snail gene transfer. Indeed, this ecological proximity could facilitate the trans-
392 kingdom DNA transfer as it has been reported between *Wolbachia* and several invertebrate
393 [2]. The high similarity in GC content and codon usage between the biomphalysins and other
394 genes from *B. glabrata* further indicate an ancient transfer leading to genes domestication.

395 Diversification of aerolysin-like families by gene multiplication seems to be a shared
396 feature among all invertebrates containing such proteins, with the exception of the sea
397 anemone *E. pallida* for which genomic and transcriptomic resources are limited. This species-
398 specific diversity of aerolysin-related toxins observed among different invertebrates strongly
399 highlights that these acquired genes must confer an adaptive advantage for each recipient
400 organism. In cnidarian, hydralysins are suspected to play a major role in protecting against
401 predators or killing prey [11, 23]. In *Aplysia*, the presence of toxin-related proteins could be
402 used to escape predators as a defensive mechanism [57]. The success for aerolysin-related
403 gene retention by metazoan organisms could be linked to the absence of preexisting similar
404 metabolic network in the recipient organism [3, 8, 58]. Thus, the acquisition of new functions
405 can easily be integrated into the recipient's metabolome to provide an immediate benefit [21,
406 59]. These acquired protein-encoding genes can be described as self-contained units [11].
407 Aerolysin-related proteins are composed of two structurally independent modules, one being
408 involved in substrate interaction and the other in lytic activity. As such, biomphalysin 1 has
409 been shown to directly interact with the surface of *Schistosoma* sporocysts as well as that its
410 lytic activity was promoted by additional plasmatic factors [24].

411 These horizontally transferred genes are more likely to be transmitted vertically in the
412 case of a selective advantage for the recipient organism. They can be the starting genetic
413 support for domestication and diversification by providing a beneficial phenotype and/or novel
414 traits. In the snail, all biomphalysins were expressed and they all exhibited a tissue-specific

415 pattern of expression. Some tissues such as the stomach expressed almost all biomphalysin
416 genes while some others like the albumen gland only expressed half of them. Moreover,
417 miRNA able to target each of the 23 biomphalysin transcripts produced by the snail were
418 identified. Altogether, this shows that biomphalysin transcription and/or translation are well
419 integrated and finely regulated in snail and that each biomphalysin might display a different
420 function in the snail.

421 The structure of the big lobe is highly conserved in all biomphalysins, which supports
422 the conservation of their lytic activity. Conversely, structural predictions of the isolated small
423 lobes revealed a high divergence between biomphalysin's and bacterial aerolysin's one. While
424 the small domain of bacterial aerolysin is a carbohydrate-binding site (APT domain) [47], the
425 one of biomphalysins matched to various domains involved in receptor recognition. For
426 example, the small lobe of biomphalysin 8 is similar to the Domain II of Cry β -PFT, which is
427 critical for specific targeting of insect membrane receptors [60, 61] and its directed
428 mutagenesis can generate a shift in their recognition spectrum [62]. The small lobe of
429 biomphalysin 2 is structurally close to the complement factor H that has been involved in self
430 surface recognition to control complement-mediated immunity by binding to the mycobacterial
431 surface in vertebrates [63]. The module similar to ApoH, present in biomphalysin 13, could
432 also be involved in pathogen binding [64]. The Decay-Accelerating Factor (DAF)-like motif
433 harbored by biomphalysin 1, 11 and 18 is known to interact with the bacterial protein Dr
434 fimbriae expressed by an uropathogenic *E. coli* [65]. All these evidences suggest that ligand-
435 binding specificity of biomphalysins could be related to the structural nature of their small lobe.
436 Indeed, the interactome results show that the repertoire of biomphalysins required for
437 pathogen sensing depends on the nature of the pathogen encountered. Thus, it appears that
438 biomphalysin acquisition, expansion and domestication underline a neo- and sub-
439 functionalization to provide obvious benefits to the snail immune system. A systematical
440 investigation on each biomphalysin should now be conducted to unravel their function in
441 response to exposure to different pathogens.

442

443 **Conclusions**

444 Evidences are scarce for multiple recurrent HGTs and data are often missing about
445 their potential functional diversification within the receiver species. Here, we provide evidence
446 that aerolysin-like proteins were acquired from multiple HGTs and that they diversified to form
447 an entire family of 23 different members in the snail *B. glabrata*. All biomphalysin genes were
448 expressed and exhibited a tissue-specific expression pattern, indicating that they were all
449 potentially playing a role in the snail. The high structural conservation of their large lobes
450 indicates that the conservation of the lytic function of aerolysins constrained the evolution of
451 the biomphalysin family while the high variation in the small lobes, presumably involved in
452 pathogen recognition, rather suggests their potential to recognize a wide range of
453 antigens/pathogens. In line with this, different biomphalysins were involved in the recognition
454 of different pathogens from different kingdoms. Altogether, our results provide evidence that
455 toxin genes from bacteria were acquired, diversified and functionalized to become a major
456 weapons family in *B. glabrata*'s immune system.

457

458 **Methods**

459 **Phylogenetic analysis**

460 Gene and protein sequences were retrieved from the genome of *B. glabrata* for each of the 23
461 biomphalysins. To rule out eventual errors in genome assembly, PCRs were performed on
462 snail genomic DNA using specific primer pairs flanking the predicted coding DNA sequence of
463 each biomphalysin gene. The gene and protein sequences corresponding to the crystallized
464 aerolysin from *Aeromonas hydrophila* was used as an outgroup. Sequences were aligned
465 using ClustalW and then a phylogenetic tree was generated using the neighbor joining method
466 (Poisson substitution model; uniform substitution rate; gaps/missing data treatment: pairwise
467 deletion) with MEGA 6.06 software [66]. A bootstrap analysis of 2000 replications was carried
468 out to assess the robustness of the branches on the trees inferred from the neighbor joining
469 method. Bootstrap values are shown on each branch of the trees generated.

470 The aerolysin and MTX2 domain (IPR005830 and IPR004991) were identified in the protein
471 sequence of each biomphalysin by searching against the Conserved Domains Database
472 (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) [67]. These sequences were used as a
473 query for a BLAST search against the NCBI database to identify similar sequences in other
474 species. A total of 87 sequences were used for the phylogenetic analysis performed as
475 described previously. They included 39 sequences from Gastropoda, 16 from Hydrozoa &
476 Anthozoa, 18 from Arachnida, and 14 from bacteria. The accession numbers and
477 corresponding names of each sequence used are indicated in Additional file 12 for all
478 phylogenetic trees performed.

479

480 **Bioinformatic analysis,**

481 Whole genome and transcriptome screening by use of the Interproscan software were
482 performed with the Interproscan database to find IPR005830 and IPR004991 signature
483 characteristic to aerolysin and aerolysin-like toxins. The genome of *B. glabrata* (preliminary
484 assembly v4.3) and the RNA-seq data from naïve and infected snails were used as data
485 sources [26, 28].

486 The presence of a signal peptide was determined by a primary structure analysis
487 performed using SignalP 3.0. Protein domain searches were performed using SMART
488 (<http://smart.embl-heidelberg.de/>) and Motif Scan (<http://hits.isb-sib.ch/cgi-bin/PFSCAN>)
489 software. α -Helical and β -sheet regions were identified by secondary structure prediction using
490 Jpred3 and HHpred servers (<http://toolkit.tuebingen.mpg.de/hhpred>). Sequences were
491 searched for putative transmembrane domains (TMDs) using the PRED-TMBB server.

492 3D Prediction of biomphalysin and their isolated small lobe structures were performed
493 using the I-Tasser and TM align servers [38, 68]. The 3D structure was obtained by multiple
494 threading using the I-Tasser server (available online), which combines two protein structure
495 prediction methods: threading and *ab initio* prediction [69]. Structural similarities between
496 bacterial aerolysin and biomphalysin proteins were determined by calculating a TM-score. A

497 TM-score greater than 0.5 reveals significant alignment, whereas a TM-score less than 0.17
498 indicates a random similarity.

499 Multiple structural alignment were performed using the ConSurfWeb from the predicted
500 structure of different biomphalysins. The conservation scores are projected onto the protein
501 sequence of biomphalysin 1.

502

503 **Search for transposable elements (TE)**

504 Up to a 20Kb region surrounding each biomphalysin (when size and position on the scaffold
505 allowed) was screened for the presence of TEs through a similarity search using BLAST with
506 the *Biomphalaria* specific database of repeat elements as well as using RepeatMasker. We
507 performed a visual inspection for conserved sequences in these 10Kb regions surrounding the
508 biomphalysin genes by producing a dotplot using gepard v. 1.4 [70].

509

510 **Transcription factor motif search**

511 Transcription factor motifs were searched using the 2016 version of the JASPAR [71] database
512 of transcription factors and the Motif Occurrence Detection Suite MOODS v. 1.9.2 [72] by using
513 the provided script (moods_dna.py) and the data was parsed, filtered and summarized by in
514 house developed scripts. The search for conserved domains was carried out by using MEME
515 [73] and searching for motifs between 12 and 50 bases long. Shorter motifs did not produce
516 significant results except for TATA boxes and a poly-C stretch.

517

518 **Relative transcript abundance in whole snails using RNAseq experiment.**

519 High quality reads were recovered from previously published RNA-seq data [26] and they were
520 aligned on nucleic sequences of the 23 biomphalysins. Briefly, two pools of 20 uninfected
521 snails were sampled and RNA was extracted using Trizol® Reagent (Sigma Aldrich) prior to
522 Illumina sequencing. Best quality reads (Phred >29) from both libraries (Naive1 & Naive2) were
523 aligned as previously described [26] on each of the 23 biomphalysins using Bowtie2 (v2.2.4)
524 and samtools (v0.1.18), which were run on a local Galaxy Project server. Raw counts were

525 normalized with upper-quartile division per libraries [74].

526

527 **Tissue representation of biomphalysin transcripts**

528 Seven organs (mantel, head-foot, hemocytes, albumen gland, stomach, hepatopancreas and
529 ovotestis) from 5 individual *B. glabrata* snails (± 8 mm) were dissected and immediately
530 transferred in liquid nitrogen for mRNA total extraction using Norgen Biotek (Ontario, Canada)
531 RNA extraction kit following manufacturer's protocol. DNase treatment (ThermoFischer, USA)
532 was carried on to eliminate potential traces of contaminant genomic DNA. Total RNA (500 ng)
533 was reverse transcribed (RT) using Maxima H Minus First Strand cDNA synthesis kit
534 (ThermoFischer, USA) following manufacturer's protocol. Equimolar random hexamer primer
535 and oligo (dT)₁₈ primer were used. Absence of genomic DNA and quality of cDNA synthesis
536 was verified by using myoglobin primers (forward: 5'-GAT GTT CGC CAA TGT TCC C-3' and
537 reverse: 5'-AGC GAT CAA GTT TCC CCA G-3') targeting intron/exon junctions. All primer
538 couples targeting biomphalysin genes were designed with perlprimer software. Specificity of
539 amplification was validated sanger sequencing of amplicons (Additional file 13). For tissue-
540 specific expression, PCR amplifications were performed on the 7 organs in biological
541 quintuplicate on cDNAs (diluted 5-fold with nuclease-free water). PCR reactions were
542 performed with GoTaq® G2 Hot Start Polymerase kit (Promega, Madison, USA) and
543 performed on a thermocycler (Eppendorf, Hamburg, Germany). PCR protocol was as follows:
544 4 min at 95°C for initial polymerase activation then 35 cycles constituted of 20s denaturation
545 at 95°C, 30s annealing at 48°C and 30s extension at 72°C with 5 min at 72°C of final extension.
546 PCR products were separated by electrophoresis on 1.5% agarose gels.

547

548 **Codon Usage and GC content**

549 Codon usage were compared between sequences from the 23 biomphalysins, 13
550 aerolysins/Pertussis Toxin for bacteria and 25 randomly selected *B. glabrata* predicted
551 transcript (1600-1800 pb) (Additional file 14). The size of *B. glabrata* transcripts was chosen
552 to be in the range of biomphalysin genes (1600-1800 pb) to avoid biases due to differences in

553 gene length. Nucleotide sequence were compared using codon usage tools of CAIcal server
554 (<http://genomes.urv.es/CAIcal/>) [75]. Mean codon usage was compared for each triplet
555 between biomphalysin transcripts, *Biomphalaria* transcripts and bacteria transcripts by PCA
556 (Principal Component Analysis) using ClustVis web tool (<http://biit.cs.ut.ee/clustvis/>) [76]. Local
557 transcriptome of *B. glabrata* [26], the 23 nucleic sequences of biomphalysins and 13 aerolysin-
558 like sequences were used to calculate the GC content using geecee tools (v5.0.0) on local
559 Galaxy server.

560

561 ***In-silico* biomphalysin-targets of miRNA**

562 All the miRNA described in the *B. glabrata* genome [27] were used to predict miRNA/mRNA
563 interactions and decipher putative miRNA targeting of biomphalysin transcripts. Genomic
564 portions surrounding biomphalysin positions up to 500 nucleotides were recovered as putative
565 5' and 3' UTRs. UTRs were cleaned by confronting the putative sequences to RNAseq reads
566 to delimit Transcription Starting Sites (TSS) and detect polyadenylation signals when available.
567 Sequences of 5' UTR, CDS and 3'UTR are used to predict miRNAs targets using Miranda
568 software [77, 78] with high strictness (-en -20 -sc 150). Details of prediction are included in
569 Additional file 11.

570

571 **Microbiota diversity**

572 Twelve naive snails were recovered without shell and ground individually in liquid nitrogen.
573 Total DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen) following
574 manufacturer's instructions. Pair-end sequencing with 250 bp read length was performed on
575 the Illumina MiSeq PE (Genome Québec, Montréal, Québec, Canada) using the v2 chemistry
576 according to the manufacturer's protocol. The FROGS pipeline (Find Rapidly OTU with Galaxy
577 Solution) implemented on a galaxy instance was used for data processing ([http://sigenae-
578 workbench.toulouse.inra.fr/galaxy/](http://sigenae-workbench.toulouse.inra.fr/galaxy/)).

579

580 **List of abbreviations**

581 Arid3a, AT-rich interactive domain-containing protein 3A; β -PFT, Beta Pore Forming Toxin;
582 CDS, Coding DNA Sequence; GPI, Glycosyl Phosphatidyl-Inositol; HGT, Horizontal Gene
583 Transfer; M6P, Mannose-6-Phosphate; OTU, Operational Taxonomic Unit; PCA, Principal
584 Component Analysis; PDB, Protein Data Bank; TMD, Trans-Membrane Domain; TSS,
585 Transcription Starting Sites; UTR, Untranslated Transcribed Region.

586

587 **Declarations**

588 **Ethics approval and consent to participate.** The laboratory and experimenters possessed
589 an official certificate from the French Ministry of National Education, Research, and
590 Technology, CNRS and DRAAF Languedoc Roussillon for experiments on animals, animal
591 housing, and animal breeding (# A66040; decree # 87-848, October 19, 1987; and
592 authorization # 007083).

593 **Consent for publication.** Not applicable

594 **Availability of data and materials.** All relevant data are within the paper and its Supporting
595 Information files. Transcriptome is available on IHPE website bioinformatics resources
596 (http://2ei.univ-perp.fr/?page_id=89).

597 **Competing interests.** The authors declare that they have no competing interests

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601

602 **Authors' contributions.** DD designed the research. SP, GT, BG substantially participated in
603 conception and improvement of research. GT performed the phylogenetic analysis. DD
604 performed structural predictions. CC performed global analysis on promoter and search on
605 duplication events on whole genome. SP performed gene expression analysis. AP performed
606 microbiota analysis. SP, GT, AP, performed interaction experiments. DL and SP performed
607 the microRNA network analysis. GT, SP, DD leded the manuscript writing. All authors

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610

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613

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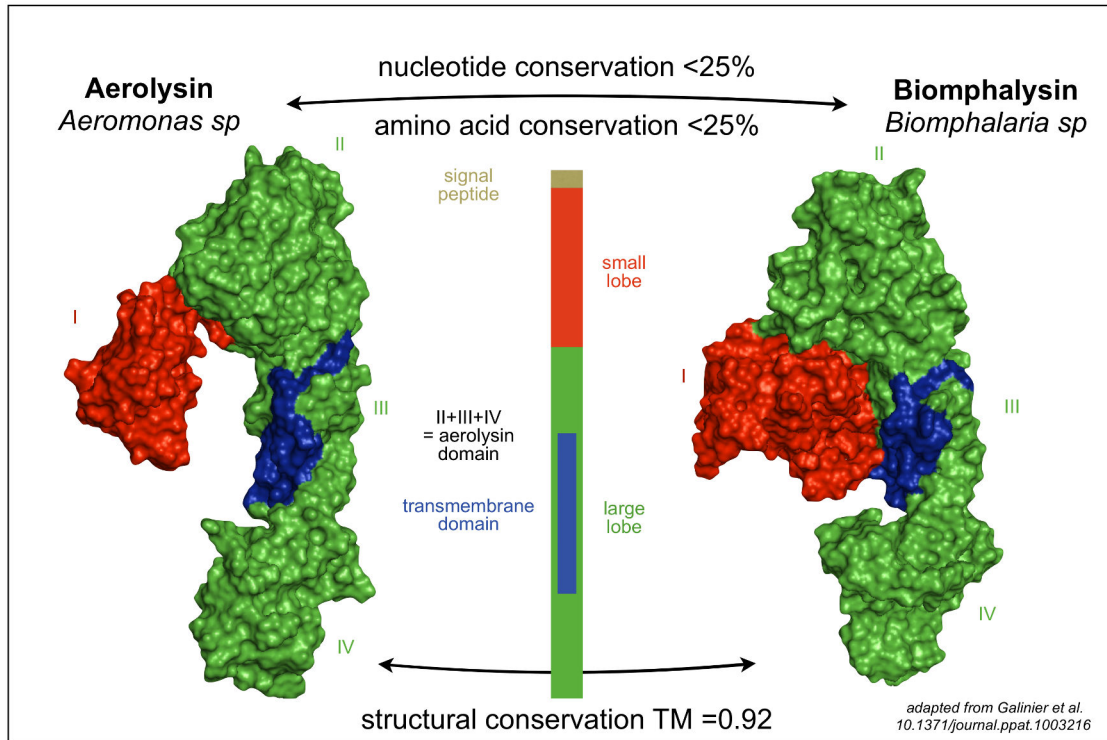
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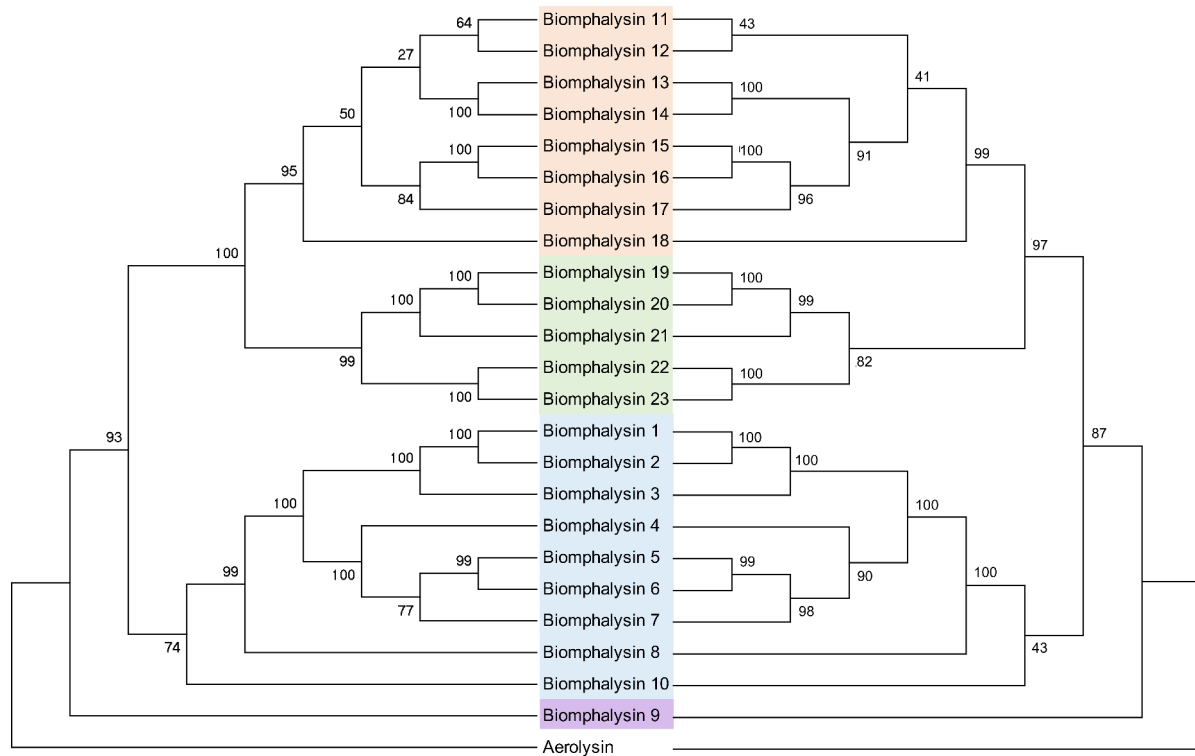
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840 **Figure legends.**



842 **Figure Abstract. Biomphalysin: an aerolysin-like protein from *Aeromonas sp.*** All typical
843 domain features from aerolysins can be identified in biomphalysin, which includes the aerolysin
844 domain (IPR005830) in one large lobe (green box), one small lobe (red box) and a signal
845 peptide (brown box).



846

847 **Figure 1. Phylogenetic analysis of the gene (left) and protein (right) sequences from the**
 848 **23 biomphalysins from *B. glabrata*.**

849 The three clusters identified in this analysis are highlighted in blue, red and green for the
 850 clusters I, II and III that contain biomphalysins 1 to 10 (excluding 9), 11 to 18 and 19 to 23,
 851 respectively. The aerolysin sequence from *A. hydrophila* was used as an outgroup. A bootstrap
 852 analysis of 2000 replications was carried out on the trees inferred from the neighbor joining
 853 method. Bootstrap values are shown at each branch of the trees. The accession number of
 854 each gene and protein used is listed in Table S9.

10 20 30 40 50 60 70 80 90 100 110 120 130
B1 ...
B2 ...
B3 ...
B4 ...
B5 ...
B6 ...
B7 ...
B8 ...
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B12 ...
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SIGNAL PEPTIDE SMALL DOMAIN

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B8 ...
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B10 ...
B11 ...
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B21 ...
B22 ...
B23 ...
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SMALL DOMAIN AEROLYSIN DOMAIN

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B1 ...
B2 ...
B3 ...
B4 ...
B5 ...
B6 ...
B7 ...
B8 ...
B9 ...
B10 ...
B11 ...
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AEROLYSIN DOMAIN

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B4 ...
B5 ...
B6 ...
B7 ...
B8 ...
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AEROLYSIN DOMAIN

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B2 ...
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B5 ...
B6 ...
B7 ...
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AEROLYSIN DOMAIN

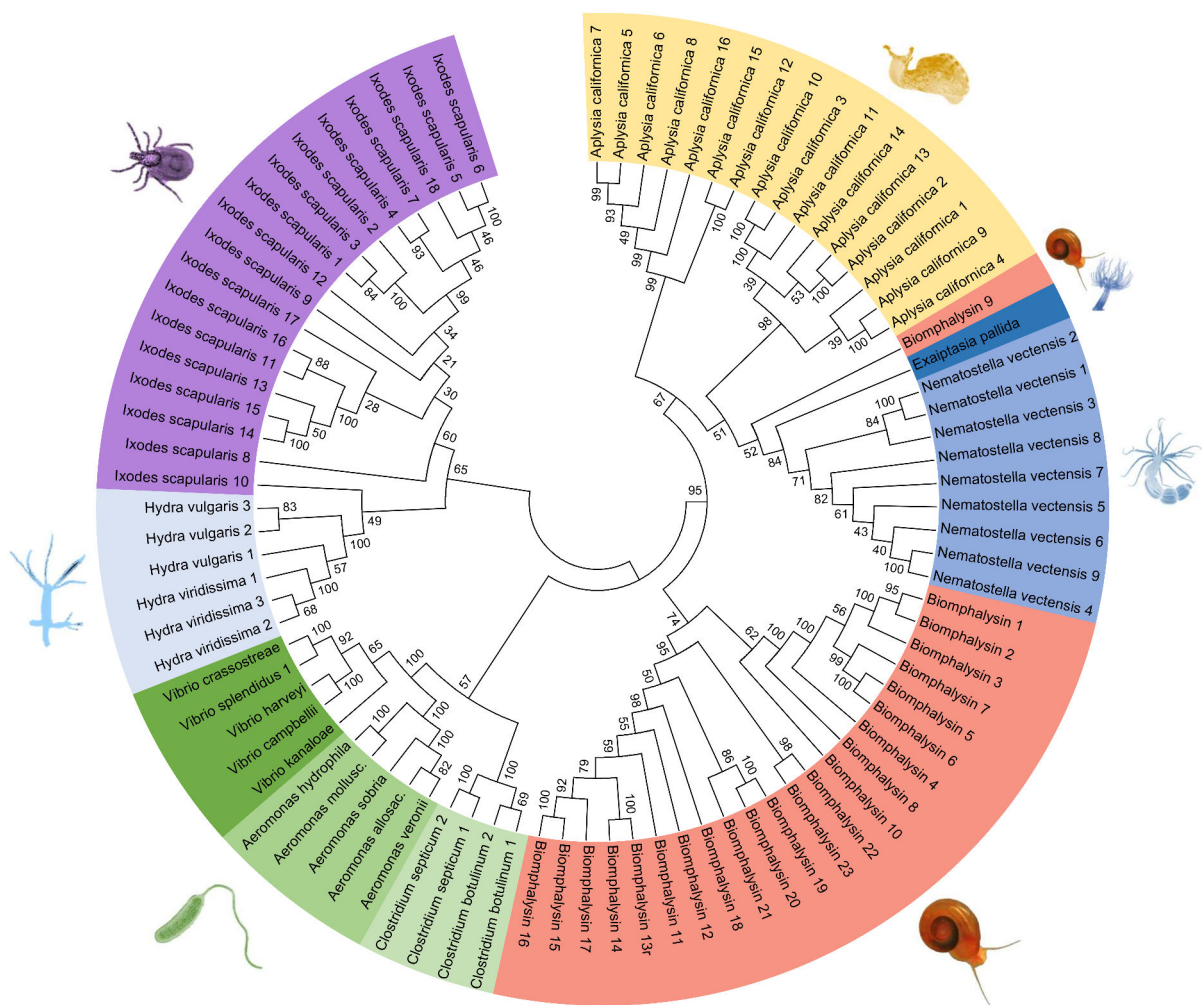
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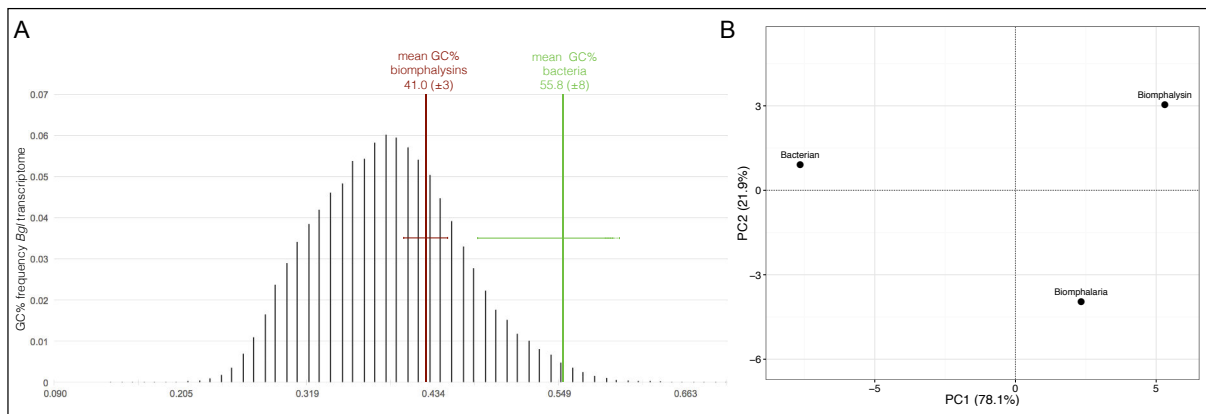
858 **Figure 2. Sequence alignment and predicted topology of biomphalysin proteins**

859 Multiple alignment of the biomphalysin proteins were aligned using CLUSTALO. Conserved
860 amino acid residues identified in 75% of all sequences are highlighted with a black box. Amino
861 acids containing similar chemical properties are shown in a gray box. Key amino acids whose
862 roles are described as important for oligomerization and lytic activity are indicated in white on
863 a red background. The positions of secondary structure elements were predicted using the
864 server PROMALS3D. α -helices and β -strands are indicated by “h” and “e”, respectively. The
865 putative peptide signal indicated in brown were predicted on SignalP 4.1 server [79]. The
866 Transmembrane Domain boxed with a blue dashed line was predicted using the PRED –TMBB
867 server. The structural domains are represented as black boxes. The signal peptide is in brown,
868 the small domain in red and the aerolysin domain containing the aerolysin motif (IPR005830)
869 in green.

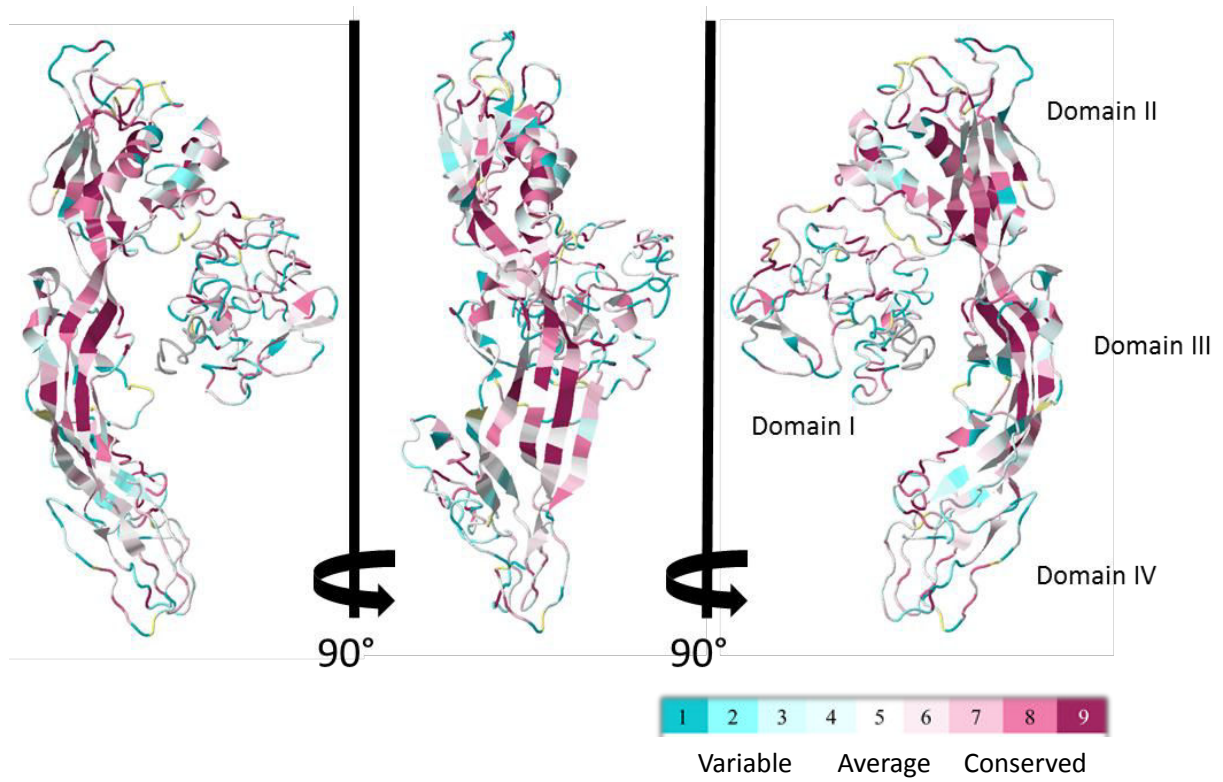


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871 **Figure 3. Phylogenetic analysis of the aerolysin domain from 19 different species.**
 872 Arthropoda (*Ixodes scapularis*) is highlighted in purple, Gastropoda in red (*Biomphalaria*
 873 *glabrata*) and yellow (*Aplysia californica*), Cnidaria in dark blue (Anthozoa – *Exaiptasia pallida*),
 874 medium blue (Anthozoa – *Nematostella vectensis*) and in light blue (Hydrozoa – *Hydra*
 875 *viridissima* and *H. vulgaris*) and bacteria in dark green (*Vibrio sp.*), medium green (*Aeromonas*
 876 *sp.*) and light green (*Clostridium sp.*). A bootstrap analysis of 2000 replications was carried out
 877 on the trees inferred from the neighbor joining method. Bootstrap values are shown at each
 878 branch of the trees. The accession number of each gene and protein used is listed in Table
 879 S9.



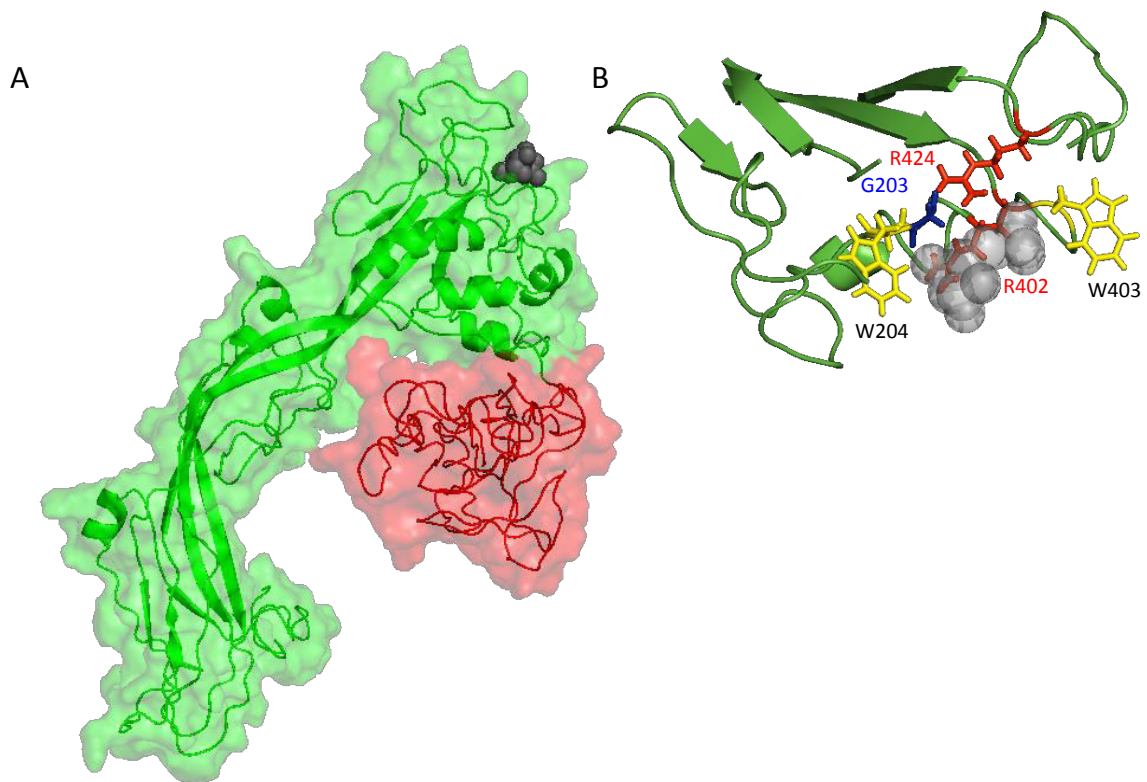
880
 881 **Figure 4. Nucleotide support of domestication**
 882 A. Box-plot representation of GC content and B) principal component analysis on codon usage
 883 comparing nucleotide sequences of biomphalysins (N = 23) with other *B. glabrata* sequences
 884 (± 1700 bp; N = 25) and bacterial toxin sequences (N = 13). Distribution of GC% was indicated
 885 for *B. glabrata* transcripts while only the mean and standard deviation were indicated for
 886 biomphalysin and bacterial toxin sequences.



887

888 **Figure 5. ConSurf sequence conservation score of biomphalysin proteins.**

889 ConSurf image was generated using alignment of all 23 biomphalysin proteins and by plotting
 890 alignment scores on the predicted structure of biomphalysin 1. Variable residues are shown in
 891 cyan while the most conserved ones are in dark red. Images are rotated 90° around the y axis.

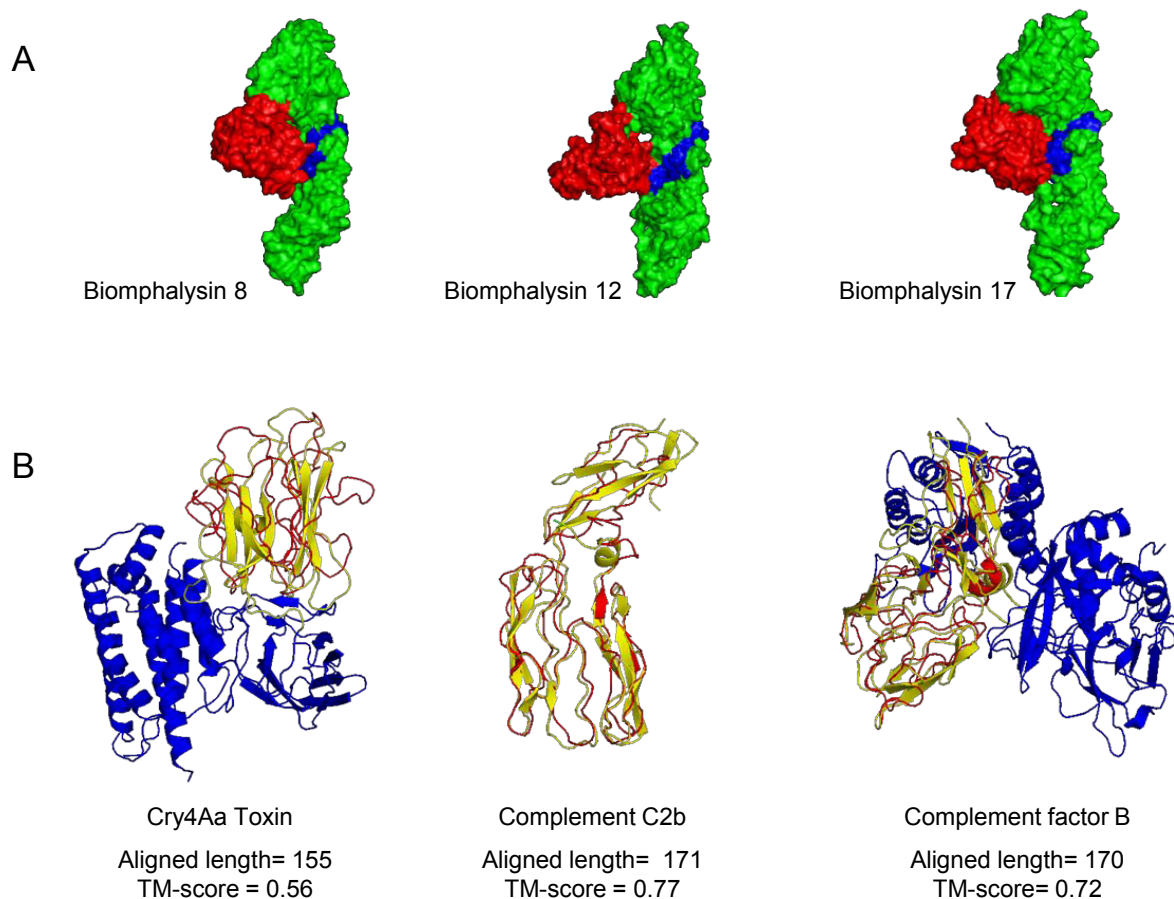


892

893 **Figure 6. Carbohydrate binding site prediction on biomphalysin proteins.**

894 A. Surface representation of biomphalysin 16 protein interacting with alpha-D mannose-6-
 895 phosphate (PDB: M6P). M6P are shown as spherical structures with gray carbon, phosphate
 896 and oxygen atoms. Ribbon representation of biomphalysin 16 was superimposed on a
 897 transparent surface map.

898 B. Closeup of the binding site. Alpha-D mannose-6-phosphate bound to the predicted pocket
 899 is located in the domain II of biomphalysin protein. The predicted residues involved in mannose
 900 binding are shown.

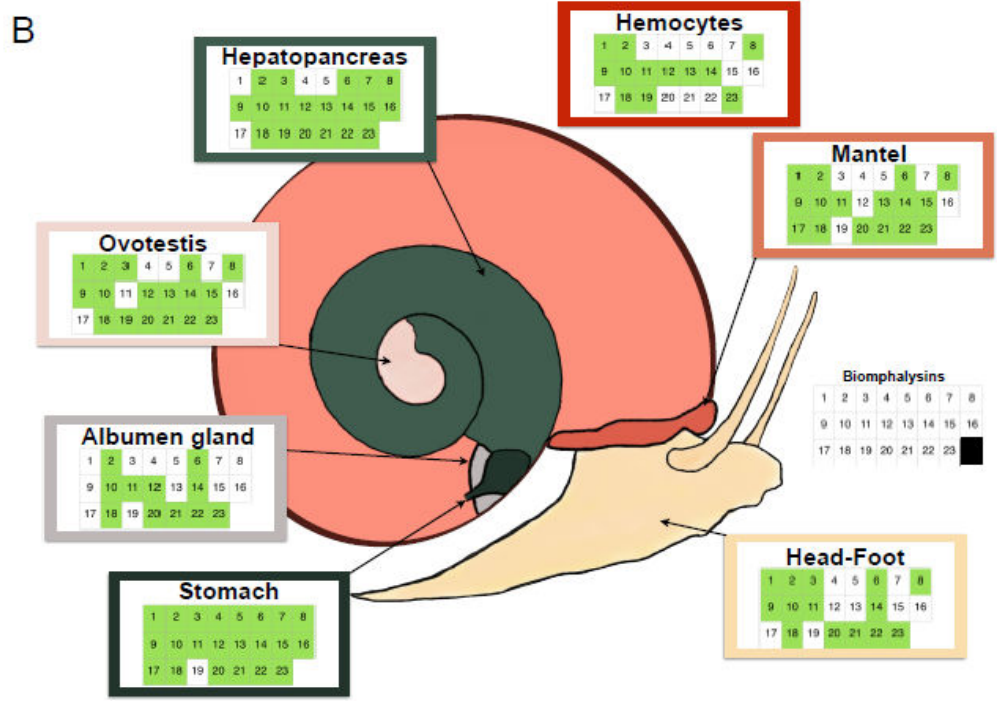
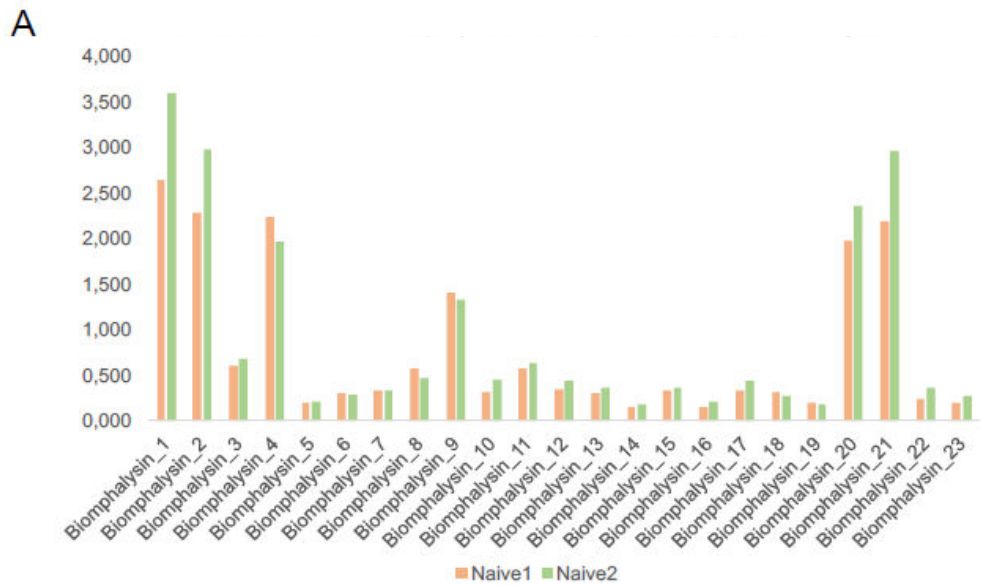


901

902 **Figure 7. Structural prediction of the biomphalysin small lobes.**

903 Three-dimensional structure prediction of biomphalysins 8, 12 and 17 was performed using I-
 904 Tasser server with the structure of proaerolysin as pattern. A C-score was determined for each
 905 prediction (see Table S5). Surface representation was realized with PyMol software. Small
 906 lobe was shown in red, large lobe in green and TMD domain in blue.

907 The small lobe of biomphalysin proteins 8, 12 and 17 were investigated against RCSB Protein
 908 Data Bank (PDB). A TM-score was calculated between the small lobes and the best hit found
 909 by I-Tasser server. A score greater than 0.5 reveals significant alignment, whereas a TM-score
 910 less than 0.17 indicates random similarity. 3D superposition of each small lobe of biomphalysin
 911 8, 12 and 17 and the respective best hit were performed by TM align and visualized with PyMol.
 912 The best structural alignment was reported between the predicted isolated structure of the
 913 small lobe in red and the significant template hit identified in yellow.



914

915 **Figure 8. Expression analysis of the biomphalysin family.**

916 A. Constitutive expression of biomphalysins in whole snails.

917 Transcripts abundance for all 23 biomphalysins retrieved from RNAseq experiment performed
 918 on two pools of 20 naive snails (Naive1/Naive2). Libraries from Naive1 and Naive2 samples
 919 are highlighted in orange and green, respectively. Counts were normalized by upper-quartile
 920 (UQ) division for each library.

921 B. Patterns of expression of biomphalysins in different snail tissues.

922 Each table represents the presence of the 23 biomphalysin transcripts in each tissue by RT-
 923 PCR, from biomphalysin 1 in the upper left case to biomphalysin 23 in the lower right case of
 924 each table. A green-colored case corresponds to a biomphalysin expressed in the tissue in at
 925 least one of the 5 snails tested while an uncolored case indicates that the biomphalysin was
 926 not expressed in any biological replicate tested.



927

928 **Figure 9. Biomphalysin transcripts targeted by several miRNAs.**

929 Prediction of miRNA targets were performed using miRanda software from snail miRNAs
 930 previously characterized. All the 23 members of biomphalysin can be potentially targeted by
 931 *Biomphalaria glabrata* miRNAs. miRNA target sites are located within CDS and UTR regions.

932

933 **Table 1. Best hit predicted for a structural alignment with the small lobes of each**
 934 **biomphalysin.** Prediction structure was performed using the I-Tasser server and the structure
 935 alignment was realized between the first I-Tasser prediction and the most similar structure
 936 templates in PDB.

	Top hit of I-TASSER structure prediction generated from the small biomphalysin lobe
Biomphalysin 1	Human decay-accelerating factor
Biomphalysin 2	Human Complement Factor H
Biomphalysin 3	TM-score < 0.5
Biomphalysin 4	TM-score < 0.5
Biomphalysin 5	TM-score < 0.5
Biomphalysin 6	TM-score < 0.5
Biomphalysin 7	TM-score < 0.5
Biomphalysin 8	CRY4Aa toxin
Biomphalysin 9	TM-score < 0.5
Biomphalysin 10	Human Complement factor B
Biomphalysin 11	Human decay-accelerating factor
Biomphalysin 12	Complement Component C2b
Biomphalysin 13	Apolipoprotein H
Biomphalysin 14	Prediction score < 0.5
Biomphalysin 15	Human Complement factor B
Biomphalysin 16	Human Complement factor B
Biomphalysin 17	Human Complement factor B
Biomphalysin 18	Human decay-accelerating factor
Biomphalysin 19	Complement Component C2b
Biomphalysin 20	Complement Component C2b
Biomphalysin 21	Complement Component C2b
Biomphalysin 22	TM-score < 0.5
Biomphalysin 23	CRY4Aa toxin

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944 **Table 2. Biomphalysin identification revealed by an interactome approach between**

945 **pathogens and cell-free hemolymph.**

Peptide List	<i>M. luteus</i>	<i>E. coli</i>	<i>S. cerevisiae</i>	<i>S. mansoni</i>	<i>E. caproni</i>
LTDQYQFTLTGK	B2, B3, B4, B5, B6, B7		B2, B3, B4, B5, B6, B7	B2, B3, B4, B5, B6, B7	B2, B3, B4, B5, B6, B7
FGDSSVPFYK	B1, B2		B1, B2	B1, B2	B1, B2
ADGDDLYFLK	B1, B2, B3, B4, B7		B1, B2, B3, B4, B7		B1, B2, B3, B4, B7

ADGDDLYFLKK	B1, B2, B3, B4, B7		B1, B2, B3, B4, B7		B1, B2, B3, B4, B7
QSSITLGPMEAAK	B1, B3	B1, B3			
ASSPVTESIER	B1, B3, B5, B6			B1, B3, B5, B6	
LEKVEGTSVNVK			B1, B2		B1, B2
TTVPYTAITTK			B1, B2, B3		B1, B2, B3
SVIEDLQAESVDSGVLYNR			B1, B2		B1, B2
SVIEDLQAESVDSG			B1, B2, B3		
CCTPAAKPLEMDEK			B1, B2, B5		
SISSETQGFTK			B1		
FEYSTSTTNSK			B1		
TRFEYSTSTTNSK			B1		
YQVIMSK		B1, B3			
SISQTTGFTK					B2, B7
# spots identified as biomphalysin	3	1	3	3	3
Min. # of biomphalysins involved	2	1	2	2	1

946

947 **Additional material**

948 **Additional file 1. Accession number and genomic localization of biomphalysin genes**
949 **family**

950 **Additional file 2, Figure S1. Dot plot of the different biomphalysins and their**
951 **surrounding genomic regions.** The first row and column represent all the biomphalysins
952 ordered from 1 to 23, followed by the (up to 20 kbp) genomic regions surrounding and including
953 the biomphalysin. Blue circle, conserved structure between biomphalysins 5 and 6. Green
954 circle, similarity region near biomphalysins 11 and 22.

955 **Additional file 3. Identity score of nucleic and proteic alignment between all**
956 **biomphalysin members**

957 **Additional file 4. Structural and protein sequence similarity between all biomphalysin**
958 **members and aerolysin toxin.**

959 **Additional file 5, Figure S2. TMD characterization in biomphalysin proteins**

960 A. Multiple alignment of the region surrounding the transmembrane domain of all Biomphalysin
961 proteins was performed using the HHpred server. The Transmembrane Domain boxed in blue
962 was predicted using the PRED –TMBB server and confronted to the TMD of aerolysin protein

963 (AFP82959) from *Aeromonas hydrophyla*. Hydrophylics (Ser and Thr) and Hydrophobics (Val,
964 Ile, Leu and Ala) are shown in red and blue respectively.

965 B. A hydropathy plot of the predicted TMD from all Biomphalysin proteins was done by the
966 method of Kyte and Doolittle (1982). An alternation of hydrophilic and hydrophobic residues
967 was observed for all predicted TMD. For example, TMD sequence of biomphalysin 2 is given
968 in this plot.

969 C. Structure of the TMD of biomphalysin 2 was predicted by the I Tasser server from the
970 complete sequence of the protein. Close view on TMD was generated by PyMOLWin. Beta
971 sheets represented by arrows forms a beta hairpin similar to the loop region in domain III of
972 aerolysin toxin. All Biomphalysins contains a transmembrane beta hairpin required to drive
973 membrane insertion.

974 **Additional file 6, Figure S3. Phylogenetic analysis of the aerolysin domain.** Expansion of
975 the analysis performed in Figure 3 but containing 246 additional bacterial sequences.

976 **Additional file 7. Number of sequences in 324 OTU's families recovered in 16S**
977 **metabarcoding analysis of naive *B. glabrata* microbiota.**

978 **Additional file 8. Scores of structural alignment and prediction of each domain**
979 **separately (small lobe, aerolysin domain and whole sequence) and full list of the domain**
980 **matched by the analysis with the small lobe as a query.**

981 **Additional file 9. Total number of transcription factor motifs found in the upstream**
982 **regions of biomphalysins.**

983 **Additional file 10. Number of each individual transcription factor motif found in the**
984 **upstream regions of biomphalysins.**

985 **Additional file 11. miRanda output detailing the miRNA, the targeted gene and the**
986 **associated scores.**

987 **Additional file 12. Accession numbers of the genes and proteins used for the**
988 **phylogenetic analysis shown in Figure 5 & Figure S3.**

989 **Additional file 13. Primers used to evaluate biomphalysin genes expression.**

990 **Additional file 14. Accession number of bacterial toxin used for GC% determination.**

Résister à l'opresseur : Discussion

Que savons-nous sur le phénotype de résistance décrit chez *Biomphalaria* ? Lorsqu'on expose un escargot *Biomphalaria glabrata* du Brésil (BgBRE) à 10 miracidia (le stade infestant pour le mollusque du parasite) de souche sympatrique *Schistosoma mansoni* du Brésil (SmBRE), 100 % des mollusques sont infestés par 3,5 parasites environ. Ainsi, ces données montrent également que 6,5 parasites en moyenne sont éliminés par la réponse immunitaire de l'hôte. Une quinzaine de jours après, si l'on propose à nouveau aux mollusques infestés 10 miracidia de la même souche de parasite, aucun mollusque n'est ré-infesté et tous les parasites sont éliminés.

Deux hypothèses ont été proposées pour expliquer cette apparente résistance, la première repose sur une résistance acquise par l'hôte à la suite de la première rencontre ; la seconde se base sur un antagonisme larvaire, le premier parasite entré essayant de protéger sa niche écologique contre les nouveaux entrants (Sire, Rognon et al. 1998).

Afin de caractériser au mieux cette résistance, le temps nécessaire à sa mise en place a été étudié. Après une première infestation, des parasites ont été alors proposés lors d'un challenge au cours de la même journée et jusqu'à 56 jours après (Sire, Rognon et al. 1998). Le pourcentage de prévalence diminue dès le premier jour de réexposition puis cette baisse est continue les jours qui suivent (Sire, Rognon et al. 1998). À partir de 14 jours, plus aucun parasite ne s'implante à nouveau, et ce jusqu'à 140 jours (Pinaud, Portet et al. Soumis). Ce délai observé dans la mise en place de cette résistance pourrait être dû soit à : (i) une maturation progressive d'un certain nombre de déterminants immunitaires, comme cela peut être observé dans le cadre de la réponse adaptative des vertébrés, (ii) une libération tardive et/ou progressive de facteurs parasitaires dans l'hôte, facteurs nécessaires à la compétition larvaire bloquant alors la ré-infestation. Ces expériences de résistance acquise ont été également réalisées sur différents couples hôte/parasite et conduisent aux mêmes résultats (BgBRE/SmBRE, BgBAR/SmLe (Portela, Duval et al. 2013), BgVen/SmVEN (Pinaud, Portet et al, soumis). Les différentes combinaisons hôte/parasite testées jusqu'à présent permettent également de mettre en évidence l'existence d'une résistance totale lors d'une infestation et ré-infestation homologue. Cette résistance diminue en condition hétérologue lorsqu'on augmente la diversité génétique entre le premier et le deuxième parasite utilisé (Portela, Duval et al. 2013). Cette observation phénotypique a généré alors beaucoup de questionnement sur le support à la fois de cette résistance acquise, mais également de cette grande spécificité.

Pour essayer de comprendre l'immunité mémoire innée de *Biomphalaria glabrata*, une étude intégrative de la réponse immunitaire induite par l'interaction avec

Schistosoma a été conduite. Celle-ci a été partagée en deux volets : (i) les bases moléculaires du priming (Pinaud, Portela et al. 2016) et (ii) les bases moléculaires de la spécificité du priming (Pinaud, Portet et al, en préparation pour soumission dans Molecular Ecology).

Bases Moléculaires de l'immunité mémoire innée chez *Biomphalaria glabrata*

Des analyses histologiques sur des coupes de mollusques parasités lors d'une primo-rencontre indiquent la présence d'encapsulation du pathogène par des hémocytes. Aucune trace d'encapsulation hémocytaire n'est par contre observée lors d'une seconde exposition aux mêmes parasites. Ces deux résultats suggèrent que le parasite est détruit par une immunité non-dépendante des hémocytes mais par une immunité humorale plasmatique. Cette bascule « shift », d'une réponse cellulaire innée (encapsulation) vers une réponse humorale a donc été explorée pour comprendre sa mise en place et son fonctionnement.

Un séquençage du transcriptome a été réalisé lors d'une cinétique d'infection et de réinfection pour appréhender les gènes clés de cette résistance. Outre la caractérisation des déterminants moléculaires, la dynamique de leur régulation semble être révélatrice de ce support moléculaire. En effet à la suite de la première rencontre, très peu de transcrits liés à une fonction immunitaire sont sur-représentés. Au contraire, une immuno-modulation pouvant être induite par le parasite est observée. Par contre lors de la deuxième rencontre, deux clusters spécifiques du challenge sont observables, représentant les transcrits sur et sous-représentés. Ces transcrits pourraient donc être à l'origine du changement de phénotype observé dans ce contexte de mémoire immunitaire. De manière plus surprenante, une biomphalysine (Galinier, Portela et al. 2013) est présente dans le cluster des transcrits sur-représentés. La présence de ce transcrit spécifique lors du challenge est très cohérente avec le phénotype de shift vers une réponse humorale. De la même manière, un très grand nombre de récepteurs comme des lectines et d'autres effecteurs immunitaires sont augmentés que ce soit en nombre de variant ou en quantité totale, l'hôte augmentant ainsi son potentiel de reconnaissance et/ou d'armes à sa disposition pour réaliser la réponse la plus efficace possible (Pinaud, Portela et al. 2016). Pour confirmer le rôle de certains de ces récepteurs diversifiés, une invalidation par interférence ARN (siRNA) a été réalisée en ciblant les FREP 2, 3 et 4 avant le challenge immunitaire. Ainsi, 15 % des mollusques perdent leur résistance acquise. Les FREP pourraient donc jouer un rôle dans ce phénotype de résistance, mais forcément d'autres facteurs et récepteurs doivent être également impliqués au vu du phénotype partiel de réversion obtenu. Ces différents résultats nous ont permis de poser les premières bases concernant la réponse immunitaire mémoire innée de *Biomphalaria* : (i) un shift d'une immunité cellulaire d'encapsulation vers une réponse humorale impliquant des facteurs plasmatiques ; (ii) un shift au niveau transcriptomique qui prépare une réponse spécifique au

challenge immunitaire ; (iii) de l'implication d'un répertoire complexe de récepteurs immunitaires de type lectine ; et (iv) de la présence de biomphalysine, un candidat clé permettant d'expliquer la dégénérescence observée des parasites issu du challenge dans les tissus du mollusque.

Cet article et ce travail ont valu un coup de projecteur publiés en mai 2016 dans « Trends in Parasitology » (Figure 6, (Coustau, Kurtz et al. 2016)). Les auteurs suggèrent que l'immunité mémoire chez les invertébrés, et les mécanismes sous-jacent peuvent être considérés comme une mosaïque de deux répertoires d'armes, d'un côté des processus partagés par l'ensemble des invertébrés (Imd, Toll, lectines), de l'autre des innovations spécifiques de chaque phylum (comme les biomphalysines). L'exploration des mécanismes moléculaires chez d'autres organismes phylogénétiquement éloignés de *Biomphalaria* permettrait de découvrir quels sont les modèles consensuels et le support de l'immunité mémoire chez les invertébrés (Figure 6).

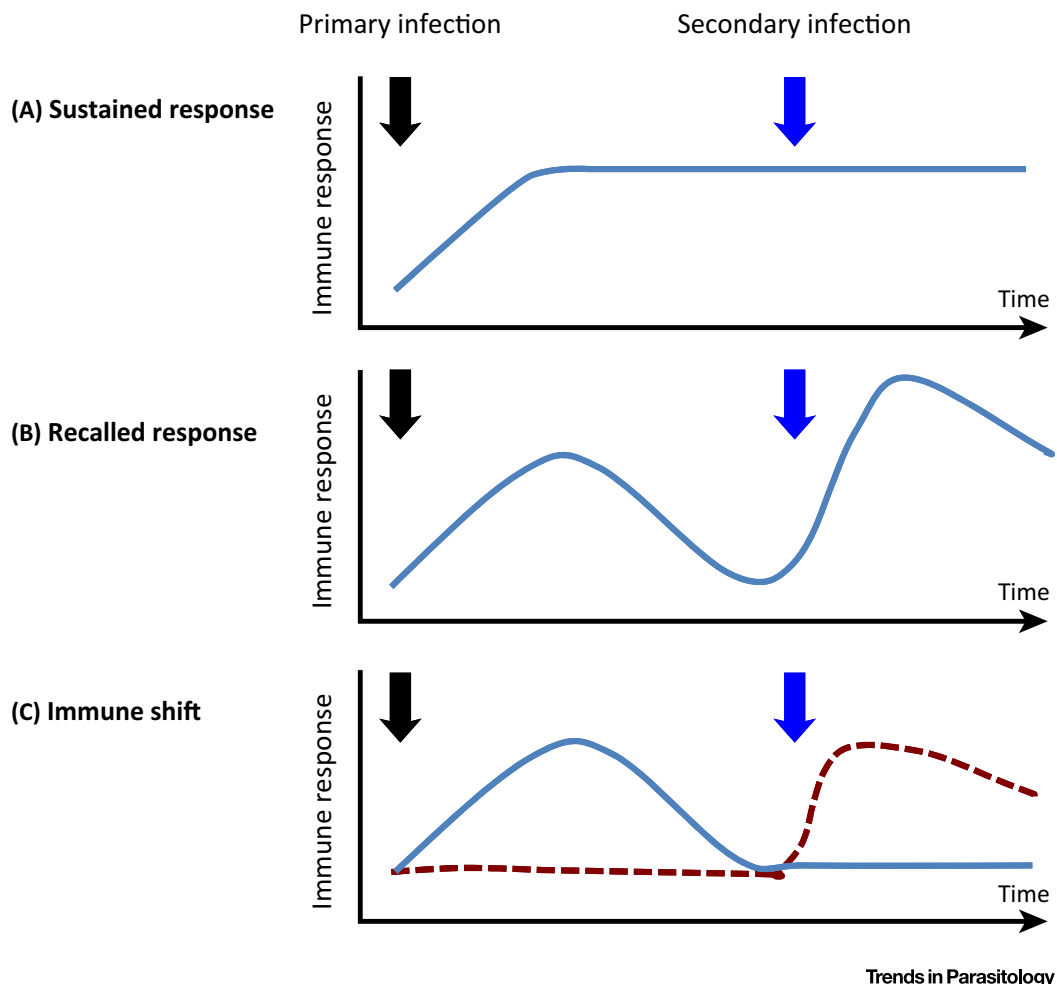


Figure 6. **Modèles simplifiés de l'immunité mémoire chez les invertébrés.**

D'après Coustau, Kurtz et Moret dans Trends in Parasitology 2016

La ligne bleue présente la réponse immunitaire issue de la première rencontre lorsque la ligne marron présente la réponse humorale de la seconde rencontre.

Bases moléculaire et cellulaire de la spécificité de l'immunité mémoire innée chez *Biomphalaria glabrata*

La spécificité de l'immunité mémoire innée a été explorée par l'étude de cette réponse d'un hôte issu du Brésil (BgBRE) face à une interaction homologue BgBRE/SmBRE ou deux interactions hétérologues BgBRE/SmVEN et BgBRE/SmROD. Le parasite SmVEN (pour Venezuela) montre des scores d'infectivité très similaire à ceux de SmBRE lors d'une première rencontre chez BgBRE (100 % de prévalence pour les deux souches et 3,6 et 3,2 d'intensité pour SmBRE et SmVEN respectivement). SROD est par contre une autre espèce, *Schistosoma rodhaini* échantillonnée au Burundi. Cette souche ne possède aucune histoire évolutive avec BgBRE, pourtant elle montre de hauts scores d'infectivité (prévalence de 75 % et intensité égale à 2). Des expériences de résistance acquise ont donc été réalisées en exposant à des escargots BgBRE naïfs une souche SmBRE sympatrique. Après 25 jours, les hôtes infestés sont à-nouveau exposés à la même quantité de parasites, soit de type SmBRE, soit SmVEN, ou encore SROD. Puis un jour, deux, jours et quinze jours après le challenge, des mollusques sont échantillonnés. Suite à un challenge homologue, une résistance totale lors de la deuxième rencontre est observée (0 % des animaux peuvent être ré-infestés), en ce qui concerne le challenge hétérologue avec le parasite SmVEN (le moins dissimilaire de SmBRE), ce dernier parvient à ré-infester 29 % des animaux. Enfin, la souche la plus dissimilaire, SROD est capable de ré-infester 42 % des animaux (Portela, Duval et al. 2013).

Ces trois interactions ont donc servi de support pour éprouver la spécificité de cette résistance acquise et cela, à différentes échelles par une approche comparative, au niveau : (i) des transcriptomes lors d'un challenge immunitaire, (ii) des effets de la vaccination, (iii) de la toxicité du plasma et (iv) des populations cellulaires par cytométrie en flux.

Grâce à la transcriptomique comparative, le shift immunitaire est également retrouvé dans les interactions hétérologues ; même si la réponse homologue induit d'avantage de modifications quantitatives et qualitatives. La présence de clusters de transcrits commun quelle que soit la souche de parasite présentée, suggère avec insistance la recherche de candidats capables d'expliquer le niveau basal de résistance observé lors d'une seconde infestation (Portela, Duval et al. 2013).

La biomphalysine, est également retrouvée dans un cluster de transcrits sur-régulés quel que soit le pathogène utilisé pour le challenge. Sa présence suggère fortement la potentielle fonction de cette molécule dans le support humoral de la résistance chez *Biomphalaria* contre *Schistosoma*.

Les clusters partagés sont également constitués de nombreux membres appartenant à la famille des lectines : C-type lectin, Selectin, Macrophage mannose-receptor, CD209, etc. Enfin, deux clusters spécifiques apparaissent uniquement dans la réinfestation homologue. Ils sont constitués de molécules de reconnaissances en grande quantité et en nombre de variants importants (FREP,

Peptidoglycan recognition receptor, galectin, etc). À l'inverse, les clusters spécifiques des interactions hétérologues, ne présentent pas de nouvelles lectines dans les clusters de transcrits surreprésentés.

Ces résultats indiquent que la diversité ainsi que la quantité des variants des différentes familles de lectines pourraient permettre à *Biomphalaria* de reconnaître, plus efficacement le parasite lors d'une deuxième rencontre. Pour autant, il est intéressant de noter que la réponse immunitaire observée lors de cette seconde rencontre est dépendante de la souche du parasite utilisée pour le challenge indiquant une reconnaissance spécifique lors de chaque interaction avec un nouveau parasite.

Afin de caractériser plus précisément cette spécificité de reconnaissance chez *Biomphalaria*, des expériences de vaccination ont été réalisées (Pinaud, Portet, et al. en préparation). Les différents stades de développement intra-molluscaux du parasite SmBRE ont été échantillonnés avant qu'une extraction native (TBS-Tween 0.05 %) soit réalisée. Les miracidia et les cercaires sont des stades larvaires nageurs directement accessibles dans le milieu extérieur après émission par leur hôte ; les sporocystes primaires (Sp1) sont produits par transformation *in-vitro* de miracidia ; les sporocystes secondaires (Sp2) quant à eux, sont récupérés dans la glande digestive d'un escargot infesté depuis 25 jours. À cette date, la glande est occupée majoritairement par des parasites qui n'ont pas encore démarré le processus de transformation vers le dernier stade parasitaire, la cercaire. Ces extraits antigéniques de parasite sont alors injectés à des mollusques sains. Quinze jours après cette vaccination, les animaux sont individuellement exposés à 10 miracidia de SmBRE. Enfin 15 jours après infection les animaux sont disséqués pour estimer la prévalence et l'intensité parasitaire pour chaque condition de vaccination. Des expériences complémentaires de RT-qPCR montrent que des gènes candidats (variant de FREP ou de c-type lectine) sont affectés de la même manière qu'il s'agisse d'une infection naturelle ou une injection d'extraits protéiques (vaccination, immunisation).

Seule la vaccination avec les extraits protéiques issus du stade Sp1 entraîne une protection. Ce stade correspond au premier stade de développement intramolluscal. Les extraits protéiques de miracidium n'induisent pas de protection, sûrement parce qu'il ne s'agit que du stade impliqué dans la recherche et la pénétration chez l'hôte intermédiaire mollusque. En effet immédiatement après pénétration, le miracidium perd ses plaques ciliées et devient alors un Sp1 qui exprime à sa surface un profil d'antigènes spécifiques et fondamentalement différent de celui porté par des plaques ciliées du miracidium. Des études protéomiques ont d'ailleurs montré une transformation majeure des protéines de surfaces et précisément des glycoprotéines de surface, confirmant une antigénicité très différente entre ces deux stades parasitaires (Peterson, Hokke et al. 2009, Yoshino, Wu et al. 2012).

L'utilisation d'un pool constitué de l'ensemble des extraits protéiques issus de l'ensemble des stades parasitaires n'entraîne pas de vaccination significativement

protectrice. Ainsi, l'hypothèse proposant que le développement du parasite, et la succession des différents stades parasitaires exprimant chacun un profil différent de déterminants moléculaires de surface soit nécessaire à l'acquisition de la résistance, est à remettre en cause. Ainsi, pour définitivement conclure sur le phénomène de vaccination continue au court du développement intra-molluscal, des extraits parasitaires pourraient être injectés en respectant le rythme du développement du parasite dans les tissus de l'hôte : t0 : miracidia, t12h : Sp1, t10j : Sp2, t30j : cercaire.

La résistance induite par la vaccination avec les extraits de Sp1 n'est pour autant pas totale. Si la précision de la vaccination n'est pas testée pour chaque animal injecté (plus de mille escargots ont été vaccinés) on peut considérer que la marge d'amélioration technique n'est pas immense. Il serait par exemple possible d'utiliser un micro-injecteur pour limiter les réponses inflammatoires liées à l'injection et mieux contrôler l'exactitude du volume injecté. Toutefois, les contrôles réalisés avec l'injection d'un tampon ou les tests d'un effet dose de nos extraits protéiques n'ont pour autant apporté aucune protection significative, suggérant respectivement que les conséquences physiques de l'injection et l'inflammation qui devrait en découler n'induisent pas une protection contre *Schistosoma* ; la dose d'antigène ne semble pas améliorer la réponse suggérant qu'une fois franchi un seuil d'activation cette réponse ne peut pas être améliorée.

La même procédure de vaccination a également été réalisée avec des extraits de miracidia et de Sp1 issus des trois souches précédemment présentées (SmBRE, SmVEN et SROD) afin d'observer si la protection génotype-dépendante pouvait être retrouvée avec des injections vaccinales. Quinze jours après l'injection, les mollusques vaccinés sont exposés à 10 miracidia de SmBRE. Nous avons observé que seuls les animaux vaccinés avec un extrait issu d'un parasite SmBRE (homologue) sont protégés, la vaccination avec les autres extraits parasitaires (hétérologues) n'apportant aucune protection. Seule la confrontation à un extrait parasitaire homologue protège ce qui pourrait indiquer que les récepteurs immunitaires capables de détecter cette souche ont été sélectionnés uniquement dans cette condition homologue, ceci confirmant un certain niveau de spécificité de la vaccination. Des expériences complémentaires utilisant de nouvelles souches d'hôtes et de parasites devront être réalisées afin d'infirmier ou de confirmer ces observations.

Ce résultat est toutefois assez différent de celui obtenu avec l'expérience utilisant des parasites vivants, car on ne retrouve pas un effet de protection proportionnel à la distance génétique entre le parasite utilisé pour vacciner et le parasite du challenge (spécificité génotype-dépendant). Cela indique que la réponse basale et sa pourraient ne pas être uniquement basées sur une reconnaissance antigénique.

La question de la spécificité reste encore ouverte, car un certain nombre de facteurs restent encore inconnus. Néanmoins, nous avons montré que cette

spécificité de réponse antiparasitaire pouvait être présente et véhiculée dans le compartiment hémolympatique. En effet, le plasma récupéré chez des animaux ayant subi un challenge homologue, est capable d'induire une protection significative de 51 % après transfert à des animaux naïfs. Lorsque ce plasma est récupéré chez des animaux infestés 15 jours après une première rencontre, ou 4 et 15 jours après la seconde rencontre, aucune protection significative n'est observée. Il existe donc des molécules dans le plasma des individus challengés qui sont capables : (i) d'induire l'acquisition de la résistance, ou bien (ii) d'agir directement comme récepteurs ou effecteurs immunitaires. La deuxième hypothèse n'est possible que s'il est possible d'envisager que ces molécules soient particulièrement stables chez les mollusques après transfert et qu'elles soient encore présentes et actives chez les animaux 15 jours après injection au moment de l'infestation par le Schistosome. Une injection des protéines plasmatiques préalablement marquées par un fluorochrome (Cy5 par exemple), pourrait nous permettre de suivre leur rémanence dans le plasma par une approche d'électrophorèse à différents temps après leur injection chez les *Biomphalaria* receveurs.

Les résultats obtenus par protéomique comparative sur du plasma d'animaux naïfs, primés ou challengés et décrits dans le shift immunitaire (Pinaud, Portela et al. 2016), semblent converger vers un rôle de la biomphalysine, comme support de cette résistance acquise. La toxicité de ce plasma a d'ailleurs été testée contre des parasites en culture. En utilisant des parasites brésiliens et vénézuéliens infectant le même hôte brésilien (BgBRE /SmBRE, BgBRE/SmVEN), nous avons réalisé des expériences d'infestation et de ré-infestation pour récupérer de l'hémolymphe chez des animaux subissant un challenge homologue ou hétérologue. La toxicité des plasmas récupérés est alors testée sur des parasites Sp1 en culture (des souches SmBRE, SmVEN, SROD) qui sont mis en contact avec de l'hémolymphe récupérée 24 heures après le challenge. Les parasites les plus sensibles sont toujours les homologues, la même souche qui a servi à générer la résistance chez les mollusques et dont est issu le plasma. Ce compartiment plasmatique contient donc tous les déterminants moléculaires nécessaires pour tuer efficacement le parasite, mais également ceux pour le reconnaître spécifiquement.

Les scores de toxicités pourraient être améliorés en améliorant la culture *in-vitro* des parasites. Ainsi, il pourrait être proposé d'utiliser une co-culture entre les parasites et des cellules Bge (*Biomphalaria glabrata* embryonic cell line) (Vermeire, Boyle et al. 2004) qui augmentent grandement la survie des parasites. De même, cette culture pourrait être réalisée dans du plasma de mollusque naïf (donc pas encore résistant) permettant ainsi d'offrir une situation de test de toxicité bien plus comparable à la réponse humorale naturelle chez le mollusque.

Également, l'utilisation d'anticorps anti-biomphalysine pour les empêcher d'interagir avec des Sp1 pourrait être testée en culture pour estimer la fonction de reconnaissance/binding dans le phénotype de toxicité *in-vitro* des biomphalysines.

Si la composante principale est humorale, la nature des cellules sécrétant ces

facteurs reste encore à déterminer. Afin d'étudier le comportement des hémocytes lors de la seconde infestation une expérience de cytométrie en flux a été réalisée. Comme il n'existe, chez *Biomphalaria glabrata*, aucun marqueur fonctionnel pour identifier les différentes populations hémocytaires, un simple marquage réalisé à l'aide de SybR-green, une molécule montrant une affinité pour les acides nucléiques a été utilisé. Cette expérience préliminaire suggère : (i) que les mêmes populations cellulaires sont présentes dans les deux réponses immunitaires (primo-infection et challenge), et (ii) que la quantité de cellules ne semble pas être modulée lors de l'acquisition de la résistance quel que soit le parasite utilisé.

Cette résistance acquise chez *Biomphalaria* contre *Schistosoma* dépendrait donc d'une population cellulaire morphologiquement identique à celle retrouvée chez des organismes naïfs. Celle-ci serait ensuite responsable de la production de répertoires complexes de récepteurs et d'effecteurs immunitaires spécifiquement dédiés aux parasites précédemment rencontrés. Ce phénotype demeure très similaire à celui observé dans l'entraînement des monocytes chez les vertébrés par exemple. Cette immunité entraînée est portée par une reprogrammation chromatinienne grâce à des facteurs épigénétiques permettant de préparer ces cellules à une meilleure réponse lors d'une seconde exposition, mais ces cellules conservent leurs morphologies (Pinaud, Portet et al ; en préparation). Notre shift phénotypique pourrait ainsi être porté par le même type de reprogrammation pour expliquer cette bascule transcriptomique et phénotypique d'une réponse cellulaire vers une réponse humorale. Pour répondre à cette hypothèse d'immunité entraînée et des modifications épigénétiques qu'elle nécessite, différentes approches expérimentales ont été réalisées et sont présentées dans le Chapitre 3.

L'ensemble de nos travaux montre la présence récurrente de facteurs clés comme les lectines et les biomphalysines à toutes les étapes de l'acquisition de cette mémoire immunitaire innée chez *Biomphalaria*. À la fois présents dans les expériences d'interactome (Chapitre 1), de protéomique et de transcriptomique (Chapitre 2), l'ensemble concourt à confirmer l'implication des lectines et des biomphalysines comme support moléculaire de la résistance et de la spécificité chez *Biomphalaria glabrata* contre *Schistosoma*.

Histoire évolutive des Biomphalysines : armes humorales d'origine bactérienne domestiquées par l'immunité de *Biomphalaria glabrata*

Le shift immunitaire chez *Biomphalaria* semble dépendre de facteurs humoraux relâchés dans le plasma pour induire une dégénérescence spontanée des parasites. Parmi les candidats les plus sérieux, on peut citer les biomphalysines dont le premier membre a été caractérisé par une approche d'interactome global (Mone, Gourbal et al. 2010). La caractérisation du premier membre a été difficile en raison de la difficulté à produire une toxine recombinante (Galinier, Portela et al. 2013). En participant à l'annotation du génome de *Biomphalaria glabrata* (Adema, Hillier et al. 2017), l'équipe a pu identifier 20 autres membres de cette famille par une recherche de motifs protéiques à l'aide d'interproscan. En parallèle, des transcriptomes réalisés par le laboratoire ont permis de révéler la présence de deux membres supplémentaires. Un travail de caractérisation a permis de montrer que : (i) au moins deux transferts horizontaux de biomphalysines depuis des bactéries vers *Biomphalaria* ont eu lieu, (ii) des duplications génomiques ont permis d'augmenter leur nombre de gènes de Biomphalysines à 23, (iii) leurs séquences régulatrices sont très divergentes et aucun rétro-élément n'a été caractérisé pour expliquer ces duplications, (iv) les 23 membres sont fonctionnels avec une expression tissulaire spécifique, (v) leur conformation spatiale tridimensionnelle est l'unité de sélection des biomphalysines et non pas leurs séquences nucléotidiques, (vi) le petit lobe des 23 biomphalysines pourrait avoir été sélectionné dans un but de spécificité afin de cibler des pathogènes différents.

La caractérisation des 23 biomphalysines, en parallèle des expériences concernant l'acquisition de la résistance contre *Schistosoma*, s'est révélée cruciale pour comprendre le support humoral de ce phénotype.

Des travaux complémentaires doivent être réalisés afin de valider les structures des différents membres ainsi que leurs affinités pour différents pathogènes. Un projet spécifique devrait s'intéresser à la compréhension des mécanismes d'actions des biomphalysines et leurs potentielles utilisations en médecine. En effet, il serait particulièrement intéressant de produire ces 23 membres afin de cribler leurs activités contre un panel de pathogènes. Les résultats obtenus en protéomique comparative d'interactomes (Tetreau, Pinaud, Portet et al., *Frontiers in Immunology*) révèlent la présence de différents membres de biomphalysine au contact de différents pathogènes. L'analyse des peptides permet de conclure que certaines biomphalysines semblent spécifiques de certains pathogènes lorsque d'autres sont retrouvées contre plusieurs pathogènes. Leur capacité à générer des hétéromères ou non lors de la formation d'un pore lytique pourrait également apporter des informations sur le support de la spécificité de ciblage d'agent pathogène chez cette famille. Cette capacité pourrait alors étendre la capacité de reconnaissance de l'hôte. La découverte d'un domaine récepteur dans le petit lobe des biomphalysines

suggère que le ciblage des pathogènes soit très spécifique. La modification ciblée du récepteur pourrait alors trouver un rôle dans la lutte contre des bactéries résistantes à des antibiotiques ou des cellules cancéreuses.

Ces travaux apportent pour la première fois des candidats pertinents pour comprendre l'immunité de *Biomphalaria* et les bases moléculaires de son interaction avec le *Schistosome*. À l'heure de la lutte vectorielle utilisant des moustiques transgéniques (Hammond, Galizi et al. 2016), bien que contestable d'un point de vue mécanistique (Volohonsky, Hopp et al. 2017), la modification de l'expression des FREP, de lectines ou des biomphalysines pourrait amener à bloquer la transmission parasitaire dans le milieu naturel si ces phénotypes étaient stables, sélectionnables et non associés à des « trade-off » ou coûts à la résistance affectant la fitness des individus transgéniques.

Ces travaux ont permis la description des premiers éléments permettant de comprendre le support moléculaire de cette résistance acquise à la fois efficace et spécifique contre des souches de parasites précédemment rencontrées. Ce phénotype est très complexe et nécessite une bascule phénotype et moléculaire pour changer la nature de la réponse, d'une immunité cellulaire vers une immunité humorale.

Néanmoins, il reste intéressant d'étudier cette résistance chez d'autres vecteurs, à la fois pour la bilharziose ou d'autres parasitoses. Il serait très intéressant de tester si ce type de résistance peut être retrouvé chez des *Biomphalaria pfeiferi* ou des *Bulinus sp.*, des espèces Africaines de mollusque impliquées dans la transmission de la bilharziose sur le continent africain.

Chapitre 3

Du Priming immunitaire à l'Immunité Entraînée

*La résistance acquise de *Biomphalaria* ressemble fortement à une immunité mémoire particulière des vertébrés, l'immunité entraînée : la résistance acquise de *Biomphalaria* peut-elle être supportée par une forme d'immunité entraînée similaire à celle rencontrée chez les vertébrés ?*

► **Trained Immune Cells Support Innate Immune Memory In Invertebrate.**

Pinaud S, Chaparro C, Lebron R, Cosseau C, Portet A, Grunau C, Netea M, Duval D & Gourbal B.

En preparation pour Cell Host & Microbe

La résistance acquise de *Biomphalaria* à une première infestation a été caractérisée comme dépendante d'un shift immunitaire, à la fois au niveau phénotypique en passant d'une réponse d'encapsulation du pathogène à une élimination humorale de ce dernier, mais également au niveau moléculaire où une réponse transcriptionnelle spécifique de la seconde réponse est constatée.

Cette capacité à exprimer une très grande quantité de facteurs pour mieux répondre lors d'une seconde rencontre, suggère qu'une préparation de la transcription s'est produite avant la seconde rencontre. Le phénotype de l'acquisition de la résistance chez *Biomphalaria* nécessite une quinzaine de jours pour être totale. Durant ce temps, une protection partielle peut être observée. Cette information de la dynamique temporelle suggère également qu'un processus de transformation de l'immunité est en cours. Lors de la seconde rencontre par contre, la réponse immunitaire observée est très rapide, en quelques heures le plasma est suffisamment toxique pour tuer des parasites en cultures. Encore une fois, la différence de dynamique temporelle dans la réponse immunitaire de la première rencontre et la seconde suggère qu'une modification profonde s'est produite pour préparer ces animaux à mieux répondre à une nouvelle infestation. Récemment, une autre réponse immunitaire, connue pour exprimer un comportement mémoriel, spécifique et dépendant d'une modification profonde de la physiologie et du fonctionnement des cellules de l'immunité innée a été démontrée chez les Vertébrés. Cette capacité mémorielle du système innée a été nommée : immunité entraînée ou « trained immunity » et concerne les monocytes, les cellules dendritiques et les NK chez les Vertébrés (Netea, Quintin et al. 2011, Netea and van der Meer 2017).

En effet, chez les vertébrés, à la suite d'une stimulation, certaines cellules de l'immunité innée (monocytes, NK) ne présentent qu'une résistance partielle à ce premier stimulus. Ensuite, ces cellules subissent de profondes modifications et des changements épigénétiques conduisant à l'expression de : (i) molécules de communication pour transmettre l'information d'entraînement aux autres cellules (IL, cytokine, miRNA), (ii) plus de récepteurs immunitaire (Toll) (iii) plus d'effecteurs (Cheng, Quintin et al. 2014, Ifrim, Quintin et al. 2014, Kleinnijenhuis, Quintin et al. 2014, Ifrim, Quintin et al. 2015, Rizzetto, Ifrim et al. 2016, Smith, Kleinnijenhuis et al. 2017). Ces modifications vont véritablement entraîner, préparer ces cellules à produire un nouveau panel d'armes immunitaires nouveau, qui seront nécessaires lors d'une prochaine rencontre (Bekkering, Joosten et al. 2014, Blok, Arts et al. 2015, Ifrim, Quintin et al. 2015, Bekkering, Blok et al. 2016). Un shift métabolique va également supporter les nouveaux besoins énergétiques de ces cellules entraînées en passant d'un métabolisme de phosphorylation vers un processus de glycolyse, qui est beaucoup plus toxique pour l'hôte, mais bien plus efficace (Arts, Carvalho et al. 2016, Arts, Joosten et al. 2016, Arts, Novakovic et al. 2016, Netea, Joosten et al. 2016). Ces cellules dorénavant entraînées, produisent une meilleure réponse

immunitaire contre un pathogène homologue de la première rencontre.

De la même manière que chez les Vertébrés, nous avons réalisé une étude intégrative centrée sur les hémocytes et incluant l'étude de la structure chromatinienne, de la méthylation de l'ADN, de l'expression des mRNA, de l'expression des miRNA et de protéomique plasmatique associée à une analyse de cytométrie en flux des hémocytes afin de tester l'existence d'un processus d'immunité entraînée chez *Biomphalaria glabrata*. Les différents compartiments sont comparés à partir d'animaux « naïfs » (jamais exposé à un parasite), « primés » (15 jours après la première exposition aux parasites) et « challengés » (1 jour après la deuxième exposition homologue).

Modification de l'état chromatinien

L'état chromatinien, défini une position non-binaire, où l'accessibilité à l'ADN est rendue par l'action de facteurs et d'enzymes de la transcription, possible (euchromatine) ou non (hétérochromatine). La modification de cet état pourrait donc moduler l'accessibilité à l'ADN de la machinerie transcriptionnelle et ainsi réguler l'expression de gènes.

Cette accessibilité chromatinienne est régulée dans les cellules des eucaryotes par un répertoire de modifications chimiques de protéines qui entourent la molécule d'ADN et particulièrement les histones. Les histones sont les principaux constituants protéiques des chromosomes et permettent l'assemblage de l'ADN en un octamère d'histone appelé : nucléosomes. La position de ces nucléosomes, mais également le degré de compaction (hétérochromatine vs euchromatine) de l'ADN peuvent être régulés par la modification post-traductionnelle de marques chimiques portées par ces histones. Différents variants protéiques des différentes histones sont nécessaires à la formation de l'octamère d'histone (H2A/B, H3, H4). Associés aux marques chimiques différentes disponibles dans les cellules pour ces protéines (acétylation, méthylation, phosphorylation et ubiquitylation) ; combinés aux nombres de résidus de ces protéines disponibles par marque chimique, cela constitue un véritable support physique d'information qui complète la séquence de nucléotide de l'ADN ; on l'appelle le code des histones (Strahl and Allis 2000). Ce code, par la complexité portée pour chaque protéine d'histone impliquée dans la formation de l'octamère, pourrait apporter autant d'information à la cellule que le propre code nucléotidique. Par convention, ces informations chimiques sont discutées en précisant quelle histone est concernée, puis le résidu de la queue de l'histone portant la ou les marques chimiques observées. Par exemple, la modification H2BK120ac c'est à dire l'acétylation de la lysine 120 de l'histone H2B est particulièrement enrichi autour du site de début de transcription (TSS) (Gatta, Dolfini et al. 2011). Ce code peut être exploré en précipitant la chromatine en utilisant des anticorps ciblant certaines de ces marques chimiques, on parle alors de CHIP-seq (CHromatin Immuno Precipitation sequencing). Malheureusement, cette technique ne permet de se focaliser qu'uniquement sur la marque concernée par l'anticorps. Si la conservation

du code des histones est importante, les conséquences que pourraient avoir telle ou telle marque sur l'accessibilité chromatinienne ne sont suggérées que par ce qui est connu chez d'autres modèles sur lesquels les modifications ont été décrites. Pour se soustraire de ces lacunes qui entourent encore le rôle des marques du code des histones chez les invertébrés et en particulier chez *Biomphalaria glabrata*, nous avons décidé d'utiliser une expérience sans à priori, qui a permis d'observer des modifications d'état chromatinien sans utiliser d'anticorps spécifiques à des marques d'histones.

La potentielle modification de l'état chromatinien a donc été suivie par la méthode d'ATACseq (Assay for Transposase-Accessible Chromatin using Sequencing). Ce protocole développé par Jason Buenrostro et Beijin Wu au sein de laboratoire de Greenleaf et Chang de Stanford University, détourne l'utilisation de la transposase d'origine bactérienne Tn5 afin de préparer une banque à la « tagmentation » de l'ADN. Cette enzyme est très sensible aux variations d'état chromatinien. Elle ne peut pas accéder aux zones fortement condensées, par contre dans les zones moins condensées, elle insère une séquence nucléique directement dans la séquence d'ADN avec laquelle elle interagit. Brièvement, une purification de noyaux cellulaires est fraîchement réalisée, afin de permettre l'action in vitro d'une transposase pour insérer une séquence nucléique connue dans le génome. Cette séquence sera utilisée pour amplifier les portions d'ADN situées entre 2 insertions. Ces portions amplifiées sont séquencées puis alignées contre un génome de référence (Figure 7). Les parties séquencées et alignées en grande quantité correspondent aux zones chromatiniennes accessibles à l'enzyme Tn5.

Une comparaison entre des résultats de CHIPseq d'une part, ciblant un grand nombre de marques du code des histones et des méthodes d'accessibilité chromatinienne, d'autre part, pourraient renseigner sur le rôle et la conservation des marques d'histone chez *Biomphalaria*. Aussi, l'utilisation d'extraction protéiques des histones suivie de spectrométrie de masse, permet à l'heure actuelle d'accéder rapidement à une grande quantité d'informations concernant les marques d'histones dans des populations cellulaires homogènes et apparaît comme une alternative au CHIPseq couteux en développement (Wang, Alekseyenko et al. 2013, Minshull and Dickman 2015).

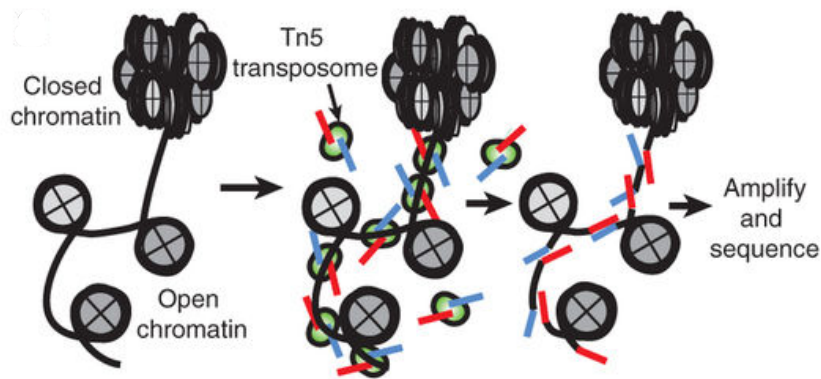


Figure 7. **Protocole d'ATACseq** pour étudier la modification de l'état chromatinien, issu de Buenrostro et al., 2013 dans Nature Methods (10.1038/nmeth.2688). Le complexe Tn5 contenant la transposase est capable de s'insérer dans des portions génomiques peu compactées. Les portions entre deux insertions sont ensuite amplifiées puis séquencées pour être alignées sur un génome de référence.

Analyse de l'ATACseq

L'alignement des lectures est réalisé à l'aide du logiciel Bowtie2 (-X 2000) qui indique rapidement les portions génomiques qui sont accessibles ou non à la transposition (Figure 8). Il reste toutefois à discriminer de manière automatisé les pics représentant le bruit de fond environnant et les véritables modifications de structure chromatinienne.

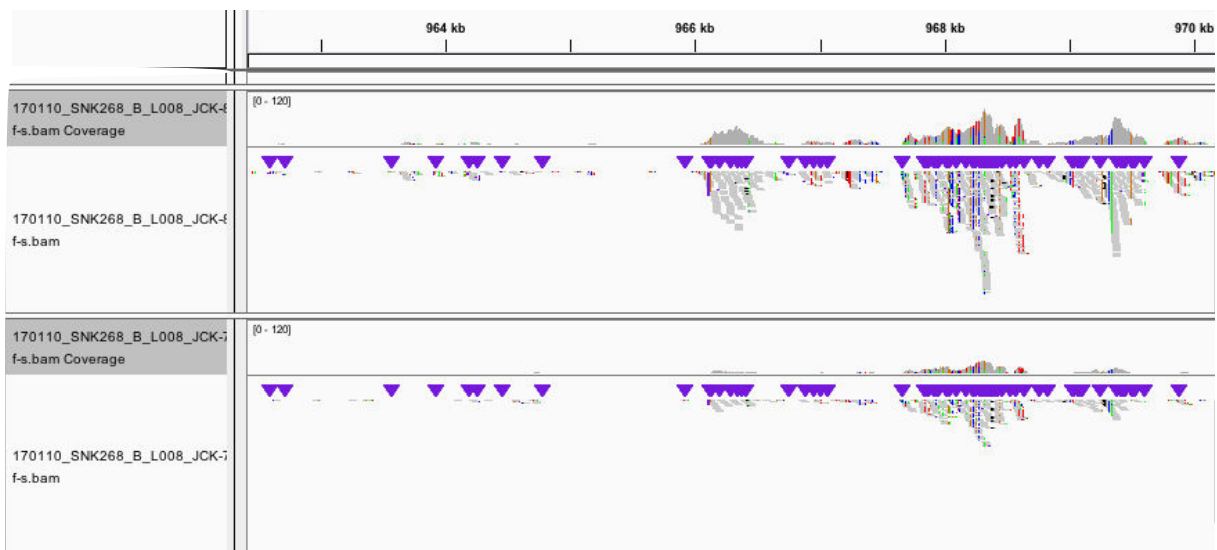


Figure 8. **Alignement des données de séquençage d'ATACseq contre le génome de *Biomphalaria* sur IGV.** Chaque fragment gris représente une lecture de séquençage, l'abondance de lecture est estimée par la hauteur du pic sur le graphe. La première piste correspond à la condition résistance (15 jours après la première rencontre) et la seconde piste à la condition naïve.

Le logiciel MACS2 développé par Yong Zhang et Tao Liu du Laboratoire Xialoe Shirley Liu d'Harvard est actuellement le logiciel le plus répandu pour l'analyse de

pics de séquençage obtenus à partir d'expériences de types ATACseq, CHIPseq ou encore MNase-seq. L'identification des différents pics permet alors, comme pour une analyse différentielle de transcriptomes ou de génomes, d'avoir une valeur de l'abondance normalisée de lecture en fonction de leurs positions sur le génome. Le nombre de lectures pour une zone peut ainsi être comparé aux autres conditions. Deux informations sont ainsi obtenues : (i) la répartition de zones accessibles ou non sur le génome et (ii) la dynamique de ces zones entre différentes conditions.

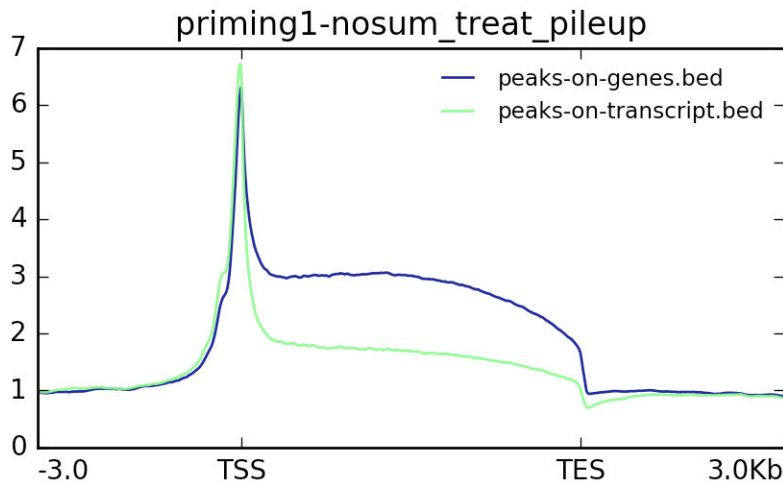


Figure 9. Représentation graphique de l'abondance de lectures alignées par rapport à un gène synthétique. Les informations des différents gènes annotés sur le génome sont positionnées sur un seul gène générique regroupant l'information de l'ensemble du génome. Le TSS signifie « transcription starting site », TES signifie « transcription ending site ». Les -3 et 3 Kb représentent les parties en amont et en aval d'un gène. La ligne violette définit la position des pics de lectures contre le génome lorsque la ligne verte correspond à l'alignement des pics de lectures contre le transcriptome.

Quel que soit la répartition des pics sur le génome ou sur un transcriptome, ce résultat nous indique que la très grande majorité des pics (Figure 9), qu'ils indiquent une ouverture ou une fermeture chromatinienne, se situe d'abord autour du TSS puis dans le corps des gènes et/ou des transcrits.

Tableau 1. Portions génomiques qui voient leurs structures chromatiniennes modifiées significativement

ATACseq	Naïf	Primé	Challengé
Naïf		423	1 869
Primé			774

L'analyse différentielle des pics différentiels et donc de la structure chromatinienne prédite a été réalisée en comparant les différentes conditions (hémocytes naïfs, primé ou challengés) entre elles grâce à DESeq2 (Q.value>0.1). Elle révèle que 423

portions génomiques présentent une structure chromatinienne différente entre hémocytes d'animaux naïfs et les hémocytes d'animaux primés depuis quinze jours, 1 869 entre les hémocytes d'animaux challengés depuis 1 jour (challengés) et les naïfs et 774 entre les hémocytes d'animaux primés et challengés. Le même patron de régulation peut être observé si on aligne les transcrits séquencés (RNAseq des hémocytes) sur le génome : 1 238 portions sont différentiellement représentées entre les naïfs et les primés, 5 296 entre naïfs et challengés et 2 275 entre les primés et les challengés.

Tableau 2. Portions génomiques pour lesquelles le profil de transcriptions est modifié significativement

RNAseq	Naïf	Primé	Challengé
Naïf		1 238	5 296
Primé			2 275

La modification de la compaction chromatinienne, en changeant l'accessibilité d'un gène et particulièrement son TSS, pourrait faciliter ou non l'accès au complexe de la transcription pour ce gène et ainsi réguler son expression.

Avec cette hypothèse, nos résultats suggèrent que 15 jours après une infestation, lorsque les animaux sont résistants, mais qu'ils n'ont pas encore exprimé leur résistance à travers un challenge, les hémocytes ont subis un profond remodelage chromatinien avec plus de 1 800 zones significativement modifiées. On peut alors poser l'hypothèse de la corrélation qui permet alors au niveau transcriptomique à plus de 5 000 portions génomiques d'être différentiellement exprimées suite au challenge et expliquer potentiellement le phénotype de shift immunitaire et l'élimination humorale des parasites.

L'analyse fonctionnelle des portions génomiques doit nous permettre d'identifier la nature biologique de celle-ci et ainsi identifier ou confirmer les candidats de la réponse immunitaire mémoire entraînée chez *Biomphalaria*, ce travail est actuellement en cours.

On peut noter que la quantité de lectures pour une position peut avoir plusieurs significations biologiques en rapport avec le phénotype observé. En effet, dans notre population hétérogène d'hémocytes, un pic plus intense dans une portion génomique peut signifier en comparaison avec une autre condition que cette portion s'est ouverte par rapport à notre contrôle. Mais pour autant, il est difficile de savoir si cette modification, et donc l'abondance de lectures alignées pour une position donnée pour la même proportion de cellules, provient d'une sous-population cellulaire pour laquelle cette portion génomique n'était jusqu'à présent pas accessible, ou si toutes les cellules ont vu leur accessibilité augmenter très légèrement. L'utilisation de tri cellulaire avant les expériences d'accessibilité chromatinienne pour ainsi étudier des populations plus homogènes de cellules,

devraient permettre de combler ces doutes.

Il est également important de noter que la modification chromatinienne pourrait être la conséquence de l'activité de transcription de gènes d'intérêts dû à la présence de facteurs de transcriptions spécifiques et non pas l'inverse, c'est-à-dire que l'ouverture soit nécessaire pour accéder à la chromatine et ainsi transcrire des gènes. Si la cause et la conséquence sont modifiées dans l'exploration de nos résultats, cela ne modifie pas l'hypothèse qu'un changement massif de transcription soit le support de notre résistance acquise et qu'il cause in-fine une modification profonde de l'état chromatinien des hémocytes.

Modification de la méthylation des gènes

Une étude de la méthylation des génomes des hémocytes a été réalisée à la suite d'une première rencontre avec un parasite. Pour cela un pool d'hémocytes a été récupéré chez des animaux 15 jours après leur première infestation. L'ADN des hémocytes est ensuite traité au bisulfite avant séquençage. Ce traitement chimique permet de convertir les cytosines (C) non-méthylées en uracile (U) à l'inverse des cytosines méthylées qui vont rester en cytosines. En comparant un séquençage après traitement d'un séquençage contrôle, il est alors possible d'identifier les sites de méthylation des cytosines. L'expérience d'infestation et la récupération des hémocytes ont été réalisées en 2016 au laboratoire. Le traitement des données est toujours en cours. Si l'analyse des résultats est encore très préliminaire, tout semble indiquer un phénomène de déméthylation générale de l'ADN dans les hémocytes à la suite d'une première exposition, comme ceci est observé dans le cadre de l'immunité entraînée des vertébrés (Arts, Joosten & Netea, 2016). La position de ces marques et les gènes particulièrement impactés par ces modifications devront être plus spécifiquement étudiés.

Modification de l'expression des miRNA

La communication entre cellules est déterminante dans la genèse d'une réponse immunitaire mémoire. En effet, à partir de cellules sentinelles qui auraient détecté la présence d'un pathogène, il faut que l'organisme soit capable de transmettre une réponse appropriée à l'ensemble des cellules immunitaires pour pouvoir dans le futur exprimer un phénotype de résistance. Les microARN (miARN) sont connus pour être des éléments régulateurs majeurs de la physiologie des organismes en étant capables de modifier des états physiologiques, des séquences de développement ou la réponse immunitaire (Lindsay 2008, Vasudevan, Tong et al. 2008, Lampe and Levashina 2017). Chez *Biomphalaria*, la majorité des miARN a été décrite dans deux articles fondateurs que sont : l'annotation du génome de *Biomphalaria* et la description de l'outil utilisé par les mêmes auteurs pour décrire les petit ARNs (Peace, Biggar et al. 2015, Adema, Hillier et al. 2017). Pourtant nos données de séquençage, premières du genre chez *Biomphalaria* ont permis de prédire au moins un nouveau miARN qui a la particularité de se situer dans un intron

d'un ARN ribosomique, le r-miR-Perpignan. Peu de descriptions de la sorte ont été réalisées à ce jour (Son, Kumar et al. 2013, Chak, Mohammed et al. 2015, Yoshikawa and Fujii 2016).

Le suivi des miARNs de *Biomphalaria* au cours de l'acquisition de la résistance contre *Schistosoma* (Figure 10), et en particulier les miARN circulants dans l'hémolymphe, révèle que 19 microARN sont différentiellement régulés en réponse à la première infestation dont 10 uniquement lors de cette première rencontre. À 15 jours, lorsque les animaux sont résistants, sur 10 miRNA exprimés chez les animaux résistants, seul 3 (miR-193-3p, 252b-5p, 981-3p) sont spécifiques de cette date. Enfin, 10 miARN sont différentiellement représentés lors de la réponse de challenge, dont 5 spécifiquement et uniquement lors de la deuxième rencontre avec le parasite.

L'analyse de l'expression des miARN suggère qu'à la suite d'une première rencontre certains d'entre eux pourraient participer soit à la régulation de la première réponse immunitaire soit à l'activation de l'acquisition de la résistance. À 15 jours, les individus ayant acquis la résistance, expriment une diversité de miARN qui pourrait servir à maintenir ce potentiel de résistance. Enfin, lors de la seconde rencontre, de nouveaux miRNA vont être surreprésentés et pourraient intervenir dans la régulation de l'expression de gènes d'intérêts propres au phénotype de shift immunitaire, notamment en ciblant certains variants de récepteurs immuns pour ainsi favoriser l'expression de variants spécifiquement mémorisés. Ces miARNs pourraient participer directement dans la balance que nous avons observée entre surexpression et sous-expression des familles de variants d'intérêt (Pinaud, Portela et al 2016 et Pinaud, Portet et al, en préparation, Chapitre 2) en réprimant l'expression de certains variants et permettant l'expression d'autres impliqués dans le phénotype de résistance au sein d'une même famille fonctionnelle.

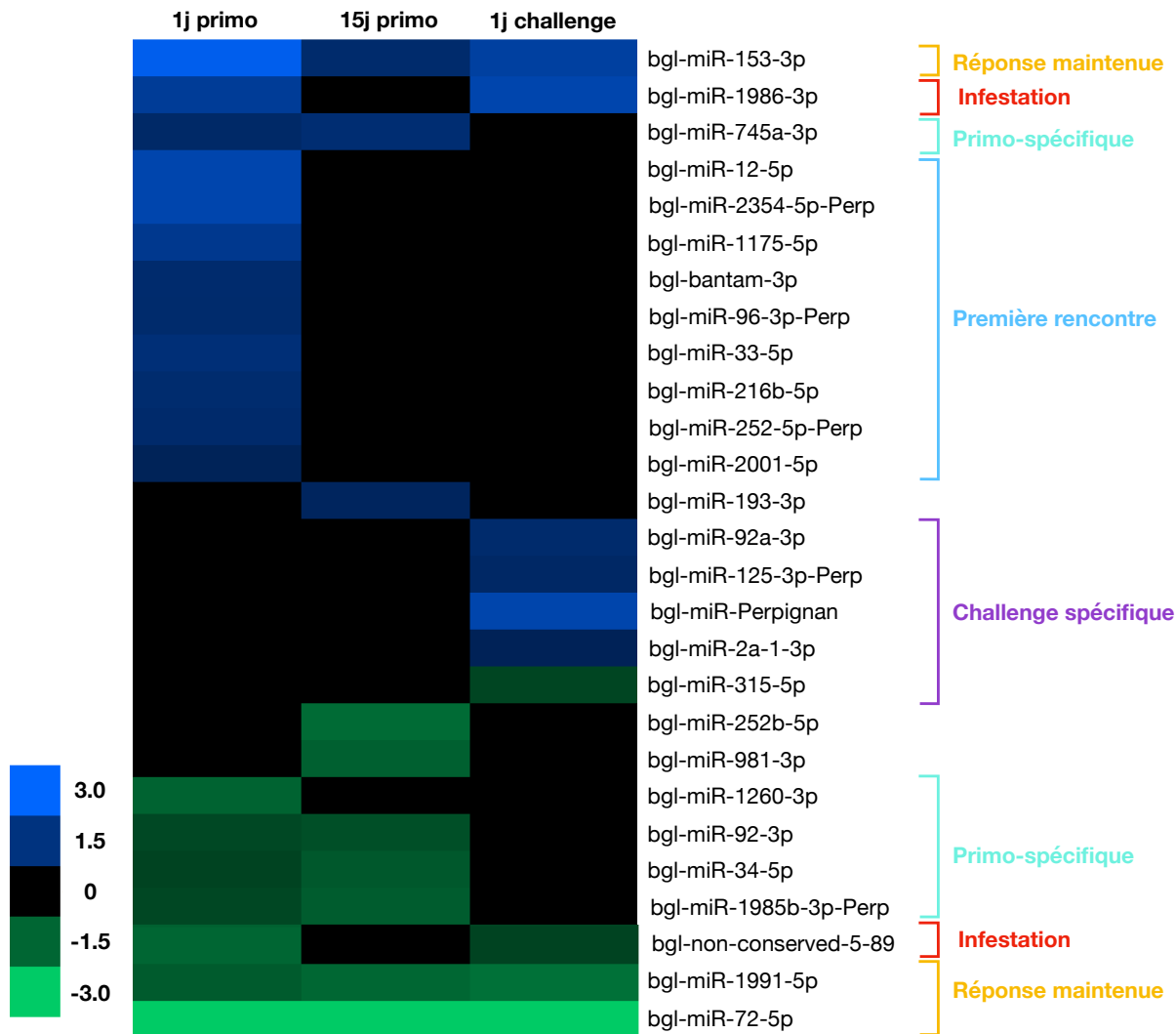


Figure 10. Heatmap de la représentation des miARN de *Biomphalaria* régulés lors de l'acquisition de l'immunité mémoire innée. Chaque colonne correspond à un temps d'infestation ou de réinfestation d'escargot BgBRE par le parasite SmbRE. La première colonne correspond à 1 jour après la première rencontre avec le parasite, la deuxième colonne à 15 jours après la première rencontre, lorsque l'hôte est résistant mais n'a pas encore exprimé ce phénotype par une seconde rencontre et enfin la troisième colonne correspond à des escargots 1 jour après la seconde rencontre avec le parasite. Chaque ligne représente un miRNA de *Biomphalaria* différent significativement par rapport à des animaux naïfs. La couleur verte correspond à une sous-représentation par rapport au contrôle quand le bleu correspond à une surreprésentation par rapport au contrôle.

Shift transcriptomique des hémocytes

L'analyse du comportement des transcrits au cours d'une cinétique d'infestation et de ré-infestation produit par les hémocytes, apporte deux informations majeures (Figure 11) : (i) le shift transcriptomique révélé par l'analyse des transcriptomes de l'animal en réponse à *Schistosoma* est également observé chez les hémocytes ; (ii) ce shift semble soutenu par une réponse transcriptionnelle très rapide et intense

précocement après le challenge (6h et 12h après le challenge). L'analyse préliminaire de l'annotation des transcrits révèle les mêmes familles de gènes que celles caractérisées lors de la première étude du shift chez *Biomphalaria* (Pinaud, Portela et al. 2016). Pour pouvoir comparer la régulation potentielle de portion génomique définie par l'analyse différentielle précédente (Tableau 2), l'analyse de l'expression des gènes doit être réalisée à présent sur le génome. Des résultats préliminaires montrent toutefois que la combinaison de récepteurs immunitaires potentiels (FREP, C-type lectin, macrophage mannose récepteurs, PGRP, etc.), des effecteurs comme la biomphalysine (sur-réglée uniquement au challenge) ou la TEP (sous-réglée au challenge) sont encore présentes.

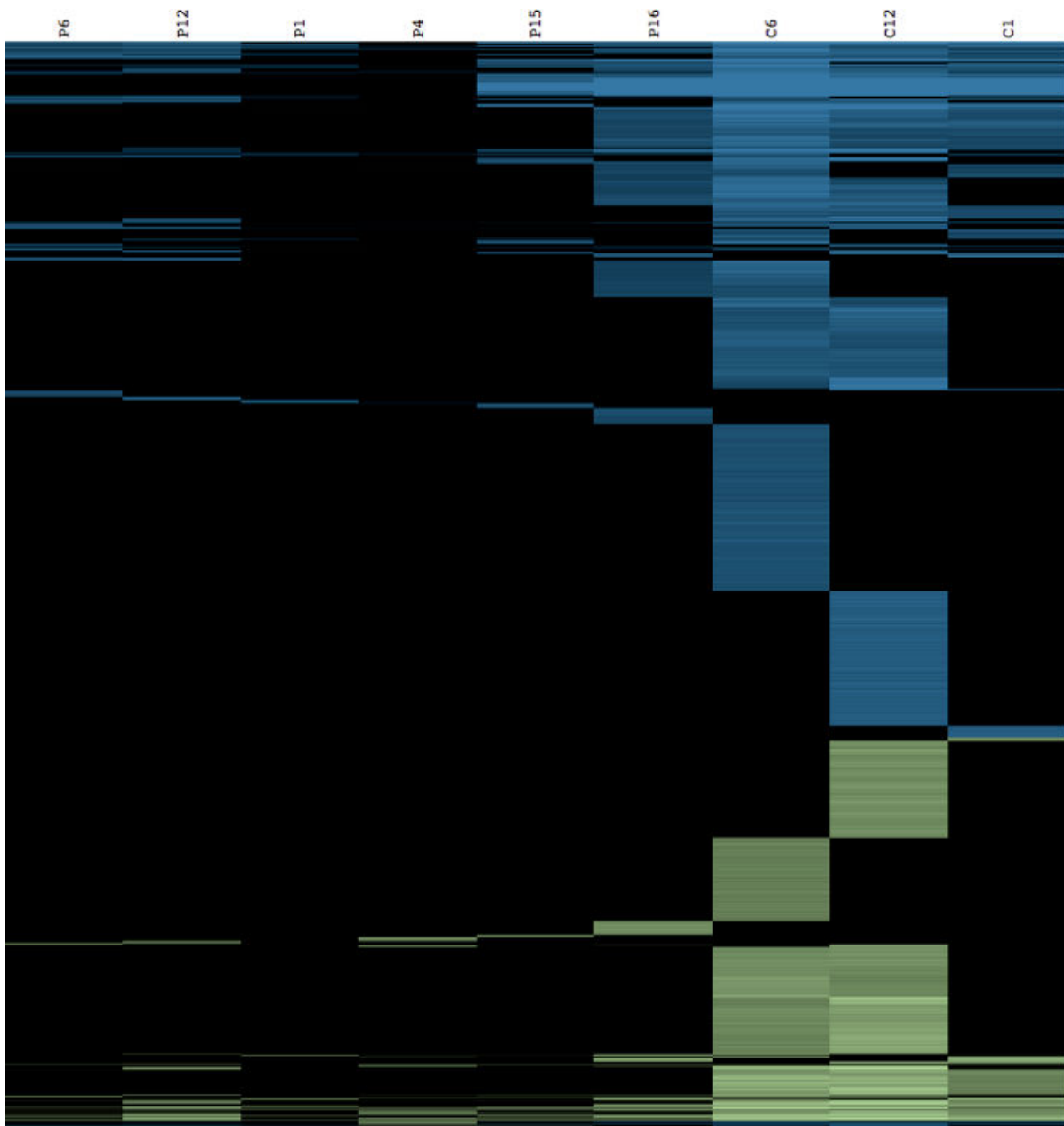


Figure 11. Heatmap de la représentation des transcrits de *Biomphalaria* au cours d'une cinétique d'infestation et de réinfestation homologue : (bleu signifie surreprésenté par rapport au contrôle et vert sous représenté). P6, P12, P1, P4, P15 et P16 pour 6h, 12h, 1

jour, 4 jours et 15 jours et 16j après première infestation ; C6, C12 et C1 pour 6h, 12h et 1 jour après challenge ; un triplicat d'hémocytes issus d'animaux naïfs a été utilisé comme contrôle.

Shift métabolique

L'expression du phénotype de l'immunité entraînée chez les vertébrés a été décrite comme étant dépendante d'une modification profonde du métabolisme, les cellules délaissant le métabolisme oxydatif habituel pour un métabolisme de glycolyse, excluant la chaîne respiratoire, cette source d'énergie est bien plus productive en ATP, mais bien plus toxique pour ces mêmes cellules (Arts, Joosten et al. 2016, Arts, Novakovic et al. 2016). Certaines molécules intermédiaires dans ce métabolisme ont donc été décrites comme proxy de conversion entre ces deux voies de production d'énergie, particulièrement le glutamate, l'aspartate et le fumarate (Arts, Joosten et al. 2016). Lorsqu'on s'intéresse aux transcrits nécessaires pour produire ces molécules allant des enzymes de synthèse jusqu'à des transporteurs spécifiques (Figure 12), nombreux sont ceux qui sont différenciellement sur-régulés lors de la deuxième rencontre avec le pathogène suggérant une potentielle conversion du métabolisme hémocytaire d'une manière similaire à celle observée pour les cellules de l'immunité entraînée des vertébrés (Arts, Joosten et al. 2016, Arts, Novakovic et al. 2016).

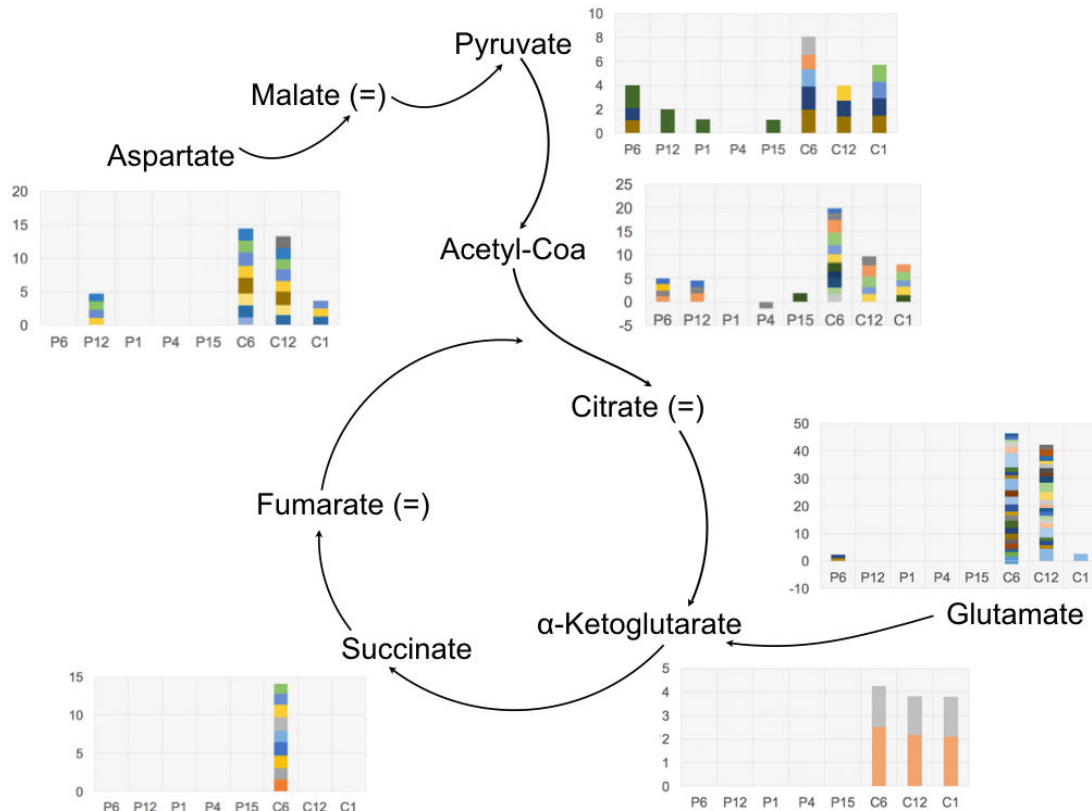


Figure 12. Expression différentielle de transcrits de *Biomphalaria* lors de l'acquisition de la résistance contre *Schistosoma* identifiés dans les voies métaboliques caractérisées comme

support de l'immunité entraînée des macrophages. (RNAseq sur hémocytes, $pvalue < 0.01$, $-1 < \log_2FC < 1$) (ordonnées : \log_2FC cumulé, chaque couleur correspond à un variant, la hauteur son \log_2FC ; abscisses : P6, P12, P1, P4 et P15 pour 6h, 12h, 1 jour, 4 jours et 15 jours après la première infestation ; C6, C12 et C1 pour 6h, 12h et 1 jour après le challenge).

Shift protéomique hémocytes/plasma

Des expériences de séquençage massif des protéomes (label-free) ont permis de confirmer la présence de biomphalysines qui sont fortement représentées dans le plasma d'animaux challengés par rapport au plasma d'animaux naïfs ou primés. De même de nombreux récepteurs potentiels de l'immunité comme les macrophages mannose-récepteurs, la famille des FREP ou les C1q ont été détectés dans le plasma d'animaux challengés. Le séquençage complémentaire de nouveaux répliquas devrait nous permettre d'analyser plus en profondeur la diversité et l'abondance des protéines lors de l'acquisition de la résistance chez *Biomphalaria glabrata* et ainsi confirmer ou infirmer les résultats obtenus en transcriptomique massive.

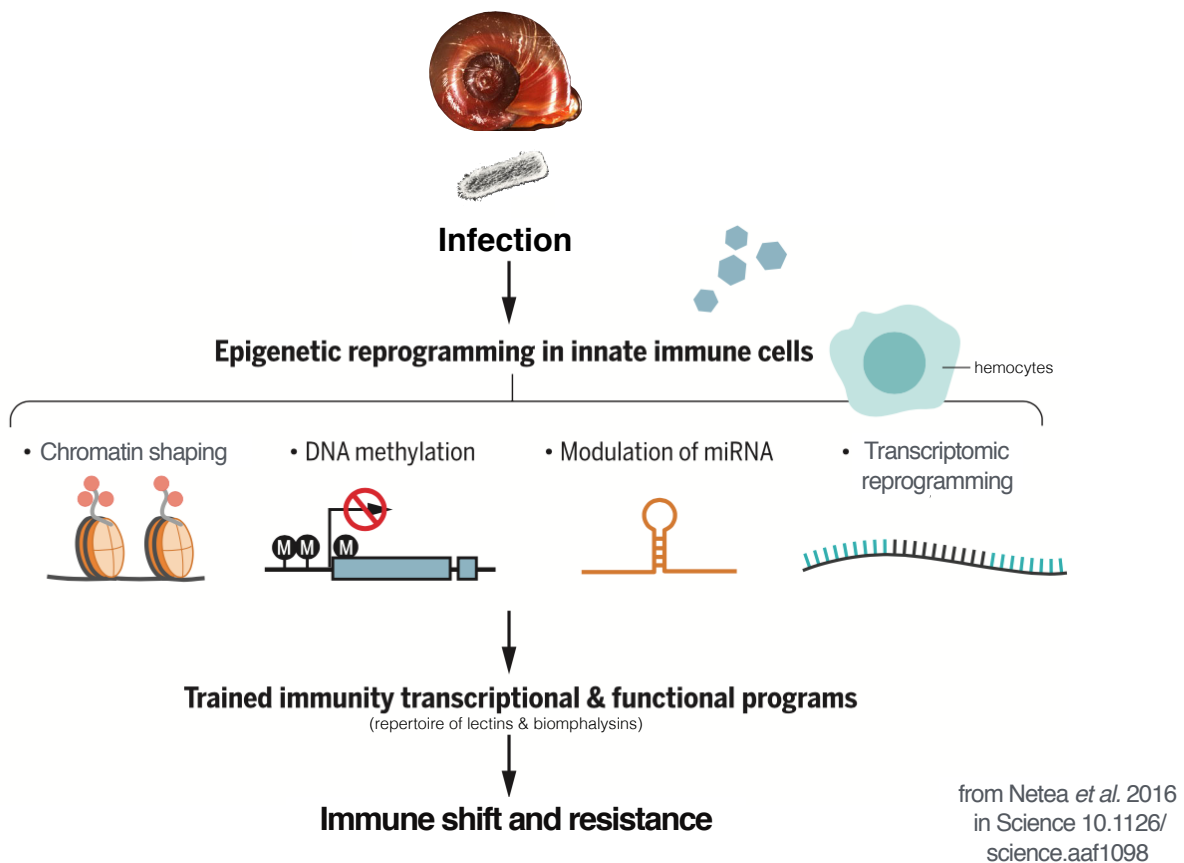


Figure 13. Support moléculaire de l'immunité entraînée chez *Biomphalaria glabrata* en réponse à une première infection par *Schistosoma mansoni* (d'après Netea *et al.* Science 2016 ; doi :10.1126/science.aaf1098)

Tous ces résultats convergent vers l'idée que c'est une réponse de type 'immunité-entraînée' qui supporte la résistance acquise chez *Biomphalaria glabrata*. À la suite d'une première rencontre avec le pathogène, les cellules immunitaires innées (Figure 13) détectent le parasite et subissent une profonde modification de leur structure chromatinienne, une déméthylation globale de leur génome, un changement de répertoire de miRNA pour *in fine* modifier leur métabolisme et acquérir de nouvelles capacités immunitaires correspondant au phénotype de résistance. Lors d'une seconde rencontre avec le parasite, ces cellules sont alors préparées à répondre plus efficacement et rapidement en produisant de nombreux effecteurs et récepteurs immunitaires capables d'éliminer rapidement les parasites, sans l'intervention d'une encapsulation hémocytaire.

Ce phénotype ressemble fortement à un phénotype typique de monocyte ou de NK de vertébrés en réponse à l'exposition au champignon *Candida albicans* par exemple dans le cadre d'une immunité mémoire innée dite entraînée. Ce mécanisme pourrait donc constituer un véritable trait d'union évolutif entre l'immunité des vertébrés et des invertébrés comme un support potentiel d'une immunité mémoire chez ces deux groupes phylogénétiquement distinct, voir chez l'ensemble des eucaryotes (Gourbal, Pinaud et al en préparation, voir Discussions et Réflexions)

Chapitre 3

Du Priming immunitaire à l'Immunité Entraînée

➔ **Trained Immune Cells Support Innate Immune Memory In Invertebrate.**

Pinaud S, Chaparro C, Lebron R, Cosseau C, Portet A, Grunau C, Netea M, Duval D & Gourbal B.

En preparation pour Cell Host & Microbe

Discussion et Réflexions

“Je sais que je ne sais rien” Socrate -400

Il n'est sans doute pas utile de passer en revue 2417 années d'histoire moderne depuis cette phrase de Socrate pour comprendre que c'est dans la soif de savoir que l'homme s'est rendu compte de son manque abyssal de connaissances. Il est bien évident que la nature s'est façonnée au travers d'innombrables générations d'êtres vivants, pour aujourd'hui donner la vie telle que nous la connaissons sur Terre, et telle qui nous est incapable de l'imaginer sous une autre forme ailleurs. Je crois pourtant qu'après cinq années de travail, ma problématique de travail qui est devenue ensuite ma thèse n'a pas progressé, elle a pourtant beaucoup évolué. À l'échelle évolutive, l'*homo sapiens*, notre espèce animale (Hublin, Ben-Ncer et al. 2017) ne représente que la dernière poignée de secondes sur l'horloge du vivant, nul doute qu'il nous est compliqué de comprendre l'intégralité de la vie qui nous entoure, mais tous les scientifiques essayent à leur tour d'apporter leur pierre à l'édifice de la connaissance.

Ma vie de jeune chercheur à Perpignan s'est intéressée à une interaction immunitaire entre un escargot tropical, *Biomphalaria glabrata* et un parasite trématode *Schistosoma mansoni*. Bien plus que le sentiment du devoir accompli, c'est le sentiment d'avoir su monter plus certains jours que d'autres, le rocher de la réflexion en attendant qu'un prochain Sisyphe se décide à le remonter à son tour. Même s'il est évident que mon travail n'est que la juste traduction de l'effort de recherche pratiqué dans l'équipe depuis plusieurs années, que la vision que je pourrais en avoir est largement influencée par les longues heures de réflexions partagées pour décortiquer ces différents résultats, la sensibilité de perception de tel ou tel résultat m'appartient, bien plus que les résultats eux-mêmes. Cette réflexion est exactement ce que je souhaite partager dans les pages de discussion générale qui vont suivre et clore ce travail de thèse.

Le couple *Biomphalaria glabrata* - *Schistosoma mansoni*, un système biologique modèle pour étudier l'immunité mémoire des invertébrés.

Une interaction durable, telle que qu'un système hôte-parasite et définie par Claude Combes (« L'art d'être parasite » Flammarion, 2001), est la pure résultante de millier d'années d'évolution voire peut-être de co-évolution. Dans un cycle complexe, le parasite nécessite de passer le plus inaperçu possible dans l'hôte afin d'y installer deux adultes, à sexes séparés, morphologiquement et fonctionnellement différents. Perpétuer le cycle de vie du parasite va ensuite dépendre d'une reproduction sexuée, de la libération d'œufs contrainte par la physiologie de l'hôte définitif et les conséquences mortelles que peuvent avoir ces œufs sur l'hôte définitif, puis de l'éclosion d'une larve dans le milieu extérieur qui devra rechercher un escargot d'eau douce pour l'infecter. Lors de ce contact, le parasite se transforme en trois nouveaux stades parasitaires dans un nouvel environnement qu'est l'hôte intermédiaire et lutter contre le système immunitaire de l'hôte pour assurer son développement parasitaire. N'oublions pas aussi le risque de mortalité associée à la virulence du parasite. Un deuxième type de larve nageante, la cercaire, aura la même mission de trouver l'hôte suivant et d'essayer de l'infester après avoir été libérée afin de boucler ce cycle hétéroxène. Ce n'est probablement pas l'interaction hôte/parasite la plus simple, mais c'est la manière efficiente qui a été sélectionnée par l'évolution et qui permet encore aujourd'hui à cet hôte et ce parasite de coexister depuis plusieurs milliers d'années.

Adapté de la roue pour mouvement perpétuel de Villard de Honnecourt (1200-1250)

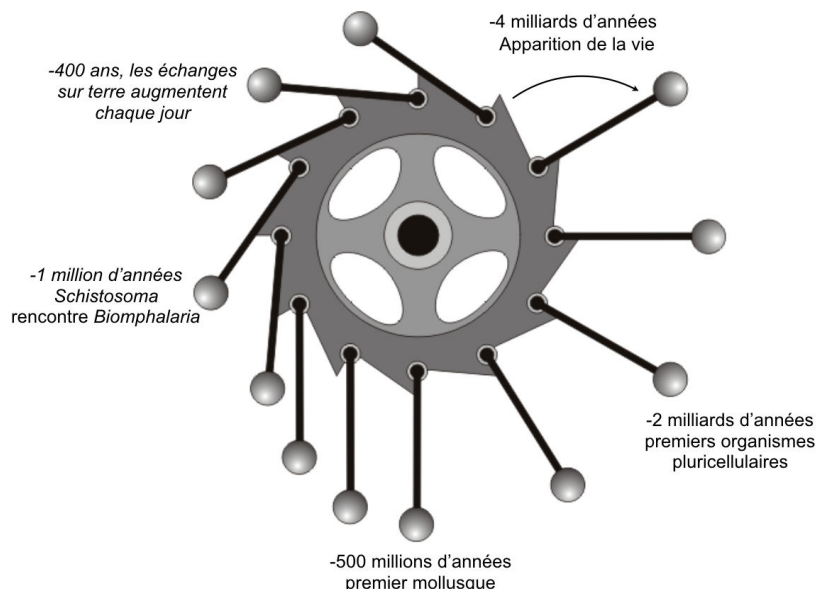


Figure 14. Schéma d'un mouvement perpétuel par Villard de Honnecourt

Lorsque le premier mouvement est effectué, la roue se lance jusqu'à consommer l'intégralité de l'énergie disponible. Chaque mouvement induit des modifications sur les prochains avec des répercussions à la fois inévitables et indispensables pour perpétuer le mouvement. Cette représentation peut être comparée à la fuite en avant de l'évolution de la vie sur terre en opposition

à la Reine rouge qui ne représente que l'action d'un belligérant dans son environnement, par exemple un parasite dans son hôte.

Cette sélection qui a donc forcé l'accumulation de caractères aux fils des générations ne nous renvoie sûrement pas aujourd'hui l'image consensuelle d'une interaction originelle, mais des milliers de pistes explorées par des populations mondiales de *Biomphalaria* et de *Schistosoma* pour tenter *in-fine* de dominer son opposant. Parmi ces pistes, certaines ne seront jamais sélectionnées, pas sélectionnables ou déjà contre sélectionnées quand d'autre attendront des milliers d'années pour trouver un sens évolutif. Difficile de faire le tri dans cette course en avant perpétuelle, souvent illustrée par l'image de la Reine Rouge de Lewis Carroll rendu célèbre par Leigh Van Valen en 1974, obligée de courir pour rester à sa place dans un même environnement changeant. L'hôte, le parasite, l'environnement, c'est en réalité un trio qui court chacun dans une direction qui lui semble pertinente, mais s'il est difficile de prévoir le point d'arrivée, il nous est possible d'en discuter le chemin. Telle la roue à mouvement perpétuel imaginée par Villard de Honnecourt (1200-1250) (Figure 14), l'évolution est une fuite en avant. À partir d'une énergie concentrée à un instant t (le big bang), l'évolution et les interactions physico-chimiques aléatoires la supportant, ont sélectionné pas à pas, la vie telle que nous la percevons aujourd'hui. Celle-ci est la résultante d'innombrables tractations et évènements expérimentés par ces différents acteurs.

Depuis le premier déséquilibre du système comme l'apparition de la vie sur Terre pouvant être comparée au premier mouvement sur la roue, le poids de chaque facteur (tel les bras portés par la roue) même s'ils sont tous très différents, vont donner une inertie à la roue entière et ainsi maintenir des interactions réciproques entre hôtes, parasites et environnements.

Il est impossible de résumer l'interaction *Biomphalaria/Schistosoma*, tout particulièrement le succès ou l'échec d'infestation dans un couple hôte/parasite donnée, à une poignée de responsables moléculaires. L'apport de l'environnement est primordial, et par environnement, on va bien évidemment parler de l'ensemble des autres facteurs biotiques et abiotiques qui vont entourer ces deux partenaires. L'interaction *Biomphalaria/Schistosoma* et principalement le succès ou l'échec d'infestation qui repose sur un système multifactoriel qui nécessite bons nombres d'acteurs peut-être encore ignorés aujourd'hui ; auquel il va dorénavant falloir ajouter la résistance acquise de l'hôte sur un parasite.

Un système multifactoriel

Dans son milieu naturel, le couple *Biomphalaria/Schistosoma* est soumis à davantage de variations de paramètres que dans nos laboratoires. Le premier est bien sûr le filtre de rencontre, base fondamentale pour initier un couple hôte/parasite. Afin de passer ce filtre, les Schistosomes sont soumis à une chronobiologie particulière fonction de leurs hôtes définitifs spécifiques et

particulièrement fonction de leurs activités et de l'heure préférentielle de leurs contacts à l'eau. Ainsi des Schistosomes guadeloupéens infestant des rats, auront tendance à être émis la nuit alors que leurs homologues infestant des humains, seront émis en journée (Theron 1981, N'Goran, Bremond et al. 1997, Theron 2015). Cette adaptation écologique permet aux parasites d'améliorer leurs capacités d'infestation. À l'inverse, les escargots présents à la fois la nuit et le jour peuvent être infestés par les deux types de parasites, (Theron 2015). Aussi, un *Biomphalaria* dans la nature n'est pas l'hôte exclusif des Schistosomes, mais peut être infecté par pas moins d'une trentaine d'autres parasites différents comme des Echinostomes et des Amphistomes (Nasir 1980, Loker, Moyo et al. 1981). Si l'on connaît la diversité de parasites capables d'infester les *Biomphalaria*, nous ne savons guère lesquels peuvent coexister chez *Biomphalaria*. Des expériences de co-infestations ont montré que les Echinostomes pouvaient physiquement consommer les Sp2 de Schistosomes et potentiellement être utilisés dans une lutte vectorielle (Barus, Moravec et al. 1974, Taha, Osama et al. 2004). Ce même phénotype a d'ailleurs été montré chez un hôte *Bulinus* (Mouahid and Mone 1990). Ces résultats ne montrent qu'un échantillon de la diversité de pathogènes à laquelle le système immunitaire de *Biomphalaria* est confronté, révélant ainsi la complexité des régulations immunitaires nécessaires à cet escargot pour survivre dans son environnement naturel.

Support moléculaire de ce système

À l'échelle moléculaire, l'interaction entre *Biomphalaria* et *Schistosoma* a été énormément étudiée. Il a ainsi été découvert des éléments codés, soit chez l'hôte, soit chez le parasite, et qui pourraient interagir lors des tout premiers moments de l'interaction et ainsi décider du succès ou de l'échec de l'infection. Les ROS (Reactive Oxygen Species) et RNS (Reactive Nitrogen Species) sont des composés cytotoxiques oxydants qui peuvent jouer un rôle dans les réponses immunitaires des vertébrés et des invertébrés (Nappi and Ottaviani 2000). Le rôle de ces molécules oxydantes est d'interagir avec les protéines (fonctions thiols, tyrosines), les acides nucléiques, et même des lipides et ainsi détériorer leurs fonctions. Les ROS/RNS agissent comme des effecteurs qui sont capables de limiter à la fois la fonction de certains composés endogènes de l'hôte (comme les protéines en fin de vie) et des composés exogènes (comme des facteurs de parasites) (Mone, Ribou et al. 2011). Pour lutter contre l'activité oxydante de ces molécules, les eucaryotes ont mis en place des contres mesures sous la forme de molécules de détoxification nommées Ros-Scavengers et chez lesquelles, on retrouve par exemple la superoxyde dismutase (SOD), les peroxydases, les glutathione S-transférases (GST) ou les peroxyrédoxines. De manière intéressante, une concordance phénotypique existe entre le niveau relatif de ROS ou de ROS-Scavenger et l'histoire évolutive de couples *Biomphalaria/Schistosoma* donnés (Roger, Mitta et al. 2008, Mone, Ribou et

al. 2011). Par exemple, dans une interaction sympatrique où l'hôte produit beaucoup de ROS, le parasite associé produira davantage de ROS-Scavenger pour se défendre (Mone, Ribou et al. 2011). Ce processus de sélection est issu d'un phénomène d'adaptation locale entre le parasite et son hôte.

À l'échelle protéique, des molécules hautement diversifiées et polymorphes de l'hôte (FREP) font face à des molécules hautement diversifiées et polymorphes du parasite (SmPoMuc). Bien que très différentes, l'interaction entre ces molécules pourrait activer chez l'hôte des voies de la réponse immunitaire à l'origine de la capacité de ce couple hôte/parasite à coexister ou non. Si un manque de validation fonctionnelle empêche aujourd'hui de conclure avec certitude sur leur rôle comme support du polymorphisme de compatibilité, des études *in-silico* apportent un faisceau de preuves concernant leurs éventuelles implications (Galinié, Roger et al. 2017).

Le projet d'éteindre l'expression de ce type de molécules hypervariables se heurte immédiatement à un phénomène d'épistasie, de compensation génique, où l'invalidation d'un variant est compensée par un autre. Cette possibilité peut d'ailleurs en partie expliquer pourquoi l'invalidation fonctionnelle des FREP ne réverse que faiblement la résistance acquise chez *Biomphalaria* (Pila, Tarrabain et al. 2016, Pinaud, Portela et al. 2016). Néanmoins, les résultats discutés dans le chapitre 1 suggèrent fortement que les FREP ne sont pas les seules molécules impliquées dans cette résistance acquise.

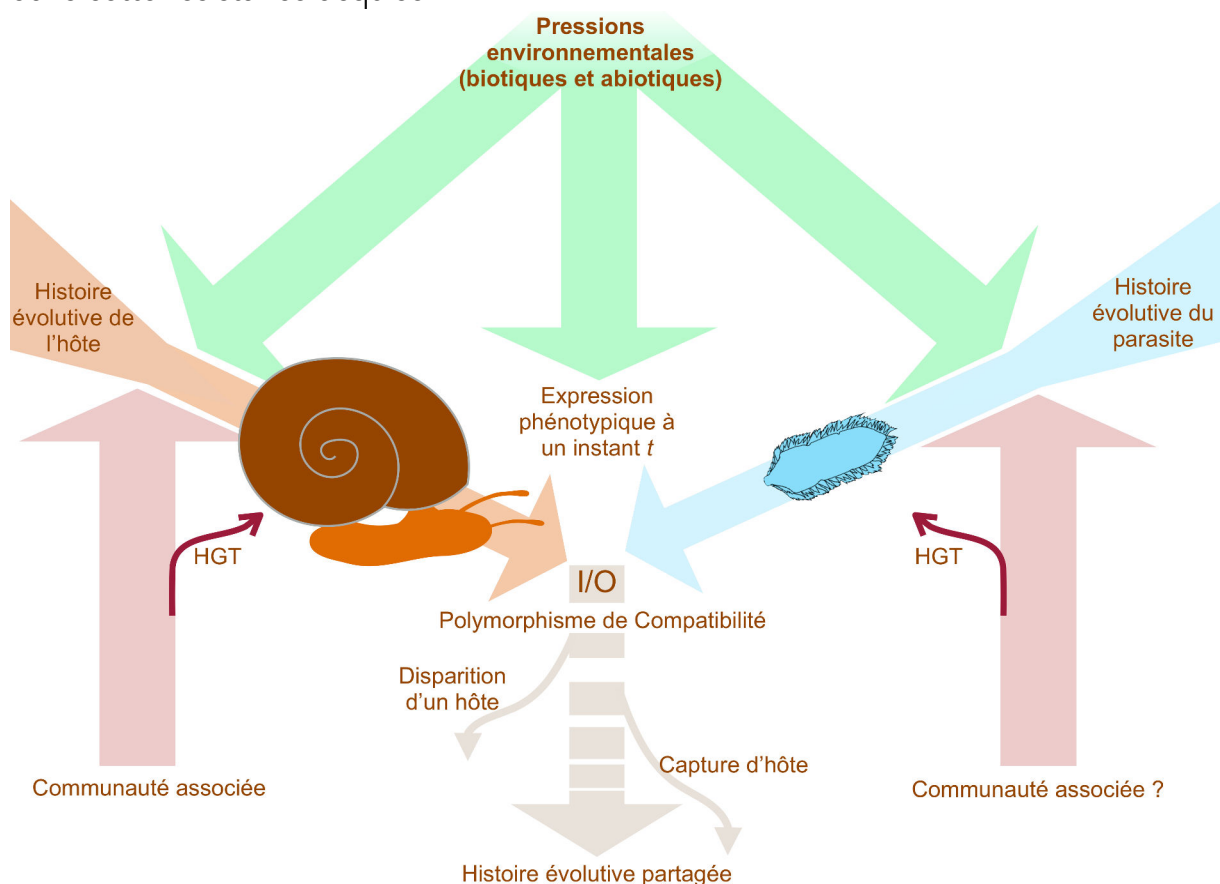


Figure 15. Pressions évolutives pesant sur le couple *Biomphalaria/Schistosoma*

HGT : transferts horizontaux de gènes

Cette interaction hôte/parasite repose à la fois sur l'histoire évolutive commune entre les deux organismes, mais également sur l'expression du phénotype de chaque belligérant dans son environnement au moment précis de leur rencontre.

Ainsi, les facteurs codés génétiquement peuvent largement être influencés par l'environnement affectant ainsi l'expression de ce polymorphisme de compatibilité. Il a été montré par exemple qu'une augmentation de température associée à une production plus importante de protéine chaperonne HSP70 pouvait modifier le succès de certaines interactions (Purnell 1966, Upatham 1973, Coelho and Bezerra 2006), voire même réduire le phénotype de résistance constitutive de la souche sélectionnée de *Biomphalaria* BS90 (Ittiprasert, Miller et al. 2010, Ittiprasert and Knight 2012). L'expression d'HSP70 est d'ailleurs dépendante d'une relocalisation nucléolaire et d'une baisse de la méthylation des sites CpG des régions intergéniques de ce gène (Arican-Goktas, Ittiprasert et al. 2014, Knight, Ittiprasert et al. 2016). La possibilité que la méthylation des génomes puisse être sous influence de l'environnement suggère que celui-ci pourrait directement impacter l'expression de gènes impliqués dans les résistances constitutives (Knight, Ittiprasert et al. 2016).

Puisque des facteurs environnementaux peuvent modifier des facteurs épigénétiques, pour valider la possibilité que des facteurs de l'environnement puissent modifier l'expression de gènes clés au centre du dialogue moléculaire de l'interaction, des modifications synthétiques de l'épigénome ont été proposées. L'utilisation d'une drogue capable d'inhiber les histones de-acétylases de type I et II (TSA, Trichostatin-A) d'escargot induit une augmentation de la transcription des SmPoMucs, ces molécules identifiées au cœur de l'interaction hôte/parasite (Perrin, Lepasant et al. 2013).

Cet environnement biotique ou abiotique peut également influencer sur les communautés d'organismes associées à l'hôte et au parasite dans une interaction qu'on pourrait qualifier de tripartite (hôte, parasite et microbiote) ou quadripartite (hôte, parasite, microbiote et environnement). La complexité de ces interactions entre chacun de ses éléments pourrait *in-fine* influencer l'expression du polymorphisme de compatibilité (Chiriboga, Ritchie et al. 1971, Ducklow, Boyle et al. 1979, Bereswill, Fischer et al. 2011, Silva, Melo et al. 2013, Gomez-Arreaza, Haenni et al. 2017, Mirzaei and Maurice 2017).

L'histoire évolutive du couple *Biomphalaria/Schistosoma* n'est alors que la résultante de chacun de ces mécanismes ou processus décrits ci-dessus qui s'adjoignent de générations en générations sous l'influence de la sélection et de l'évolution (Figure 15). Les communautés de micro ou macro-organismes associés à la fois à l'hôte et au parasite vont elles aussi être influencées par les pressions

environnementales et vont-elles à leur tour influencer le statut immunitaire de leur hôte respectif ? Ces communautés associées peuvent-elles être sources de nouveautés génétiques en fournissant de potentielles nouvelles créations moléculaires chez les eucaryotes via des transferts horizontaux de gènes ?

Biomphalaria et *Schistosoma* vont alors exprimer à un instant t un holophénotype qui va déterminer le succès ou non de l'infestation et ainsi le polymorphisme de compatibilité. À travers les générations, une course à l'armement se met en place entre les répertoires de l'hôte et du parasite qui pourra traduire aujourd'hui leur histoire évolutive.

Le succès ou l'échec d'infestation lors d'une interaction *Biomphalaria/Schistosoma* donnée, serait donc la résultante d'un système multifactoriel très complexe qui va dépendre entre autres de facteurs codés génétiquement, de l'environnement et sa répercussion sur les facteurs épigénétiques, les communautés micro et macrobiotique associées, etc. Le résultat de l'addition de ces dizaines de facteurs expliquera que le parasite se développe ou que l'hôte élimine le parasite.

Si cette armada de facteurs peuvent expliquer le résultat d'une rencontre entre un hôte et un parasite, il n'est pas évident que ces déterminants aient les mêmes rôles lorsque des hôtes sont exposés une seconde fois à des parasites. Le priming, cette résistance acquise à la suite d'une première infestation, semble reposer sur un grand nombre de facteurs également impliqués dans le polymorphisme de compatibilité. Si le polymorphisme de compatibilité est le résultat d'une première rencontre, il pourrait être comparé au résultat de la réponse immunitaire innée de l'hôte. À la suite d'une première infestation, *Biomphalaria* est capable de générer une réponse anticipatoire pour se préparer à une potentielle future exposition. Lors de cette seconde rencontre, il sera capable de générer une réponse adaptative comparable à l'immunité mémoire innée des vertébrés.

Balayer la barrière des phénomènes propre à chaque espèce

« L'imbrication de la réponse innée dans la réponse adaptative montre la force de l'évolution sur un système intégratif comme l'immunité » (Hoffmann, Kafatos et al, Science 1999).

Il semblerait qu'il soit vraisemblablement imprécis de vouloir opposer l'immunité innée et l'immunité adaptative. En réalité, il apparaît surtout que deux volets autonomes de l'immunité ont été sélectionnés par l'évolution et que les deux sont intimement imbriqués.

Il est parfois considéré que les invertébrés se contentent de l'immunité innée pendant que les vertébrés ont pu sélectionner une immunité adaptative. Pourtant avec des pressions de sélections causées par des agents pathogènes identiques, la nécessité d'un système adaptatif se pose à la fois chez les uns et chez les autres.

Cela aurait potentiellement conduit tous les organismes vivants, invertébrés compris, à posséder une immunité adaptable et mémorielle malgré des mécanismes sous-jacents profondément différents au sein des différents clades (Milutinovic and Kurtz 2016).

L'utilisation des invertébrés comme modèle de l'immunité innée permet de s'affranchir des mécanismes de l'immunité adaptative et ainsi mieux appréhender la complexité du premier volet immunitaire essentiel qu'est l'immunité innée. C'est d'ailleurs l'utilisation de la thyméctomie pratiquée chez des souris (ablation du thymus, organe de maturation des lymphocytes T), qui a révélé les premiers indices d'une immunité adaptative, porté pourtant par les acteurs de l'immunité innée (Bistoni, Vecchiarelli et al. 1986).

Comment améliorer la compréhension de l'immunité mémoire chez les invertébrés ? *Vers la fin de la simplification des systèmes biologiques ?*

À l'heure actuelle, la confirmation de l'existence d'une immunité mémoire chez tous les invertébrés reste difficile à atteindre. En effet, la méthodologie appliquée pour montrer parfois de la résistance acquise, est majoritairement dépendante du système biologique étudié. Ainsi, les conclusions de ces études sont incomparables quand un système étudie la réponse antivirale d'un insecte social et un autre la réponse immunitaire antibactérienne d'un bivalve solitaire. Pour essayer d'approcher la diversité potentielle de mécanismes sélectionnés dans les différents clades des invertébrés, il pourrait être proposé d'unifier les méthodes de travail (Figure 16).

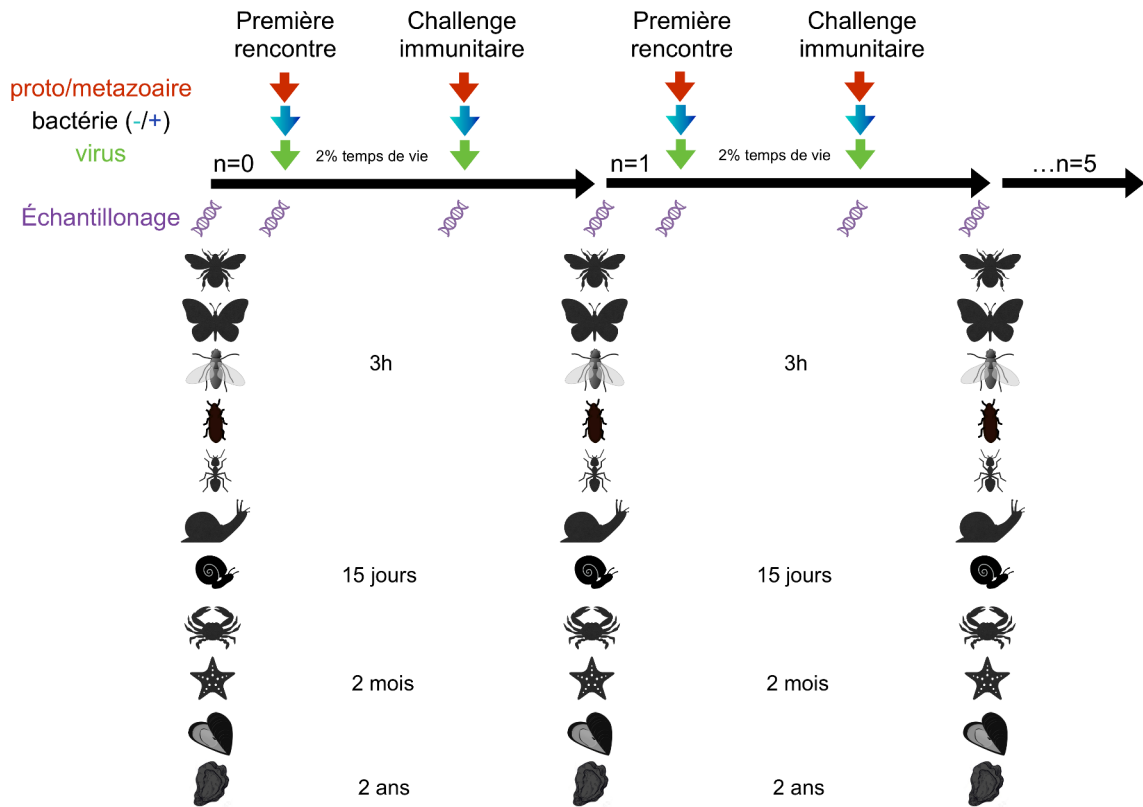


Figure 16. Proposition d'un plan expérimental pour une étude comparée de l'immunité chez les Invertébrés

Un plan expérimental pourrait permettre de comparer pour la première fois la réponse de différents hôtes invertébrés au regard de quatre types de pathogènes très différents : virus, bactérie gram+, bactérie gram-, et un eucaryote unicellulaire comme des protozoaires ou des pluricellulaires métazoaires avec les mêmes procédures expérimentales.

Une expérience simple consisterait à confronter le sujet à de nombreux pathogènes de manière standardisée pour identifier les différents changements phénotypiques et moléculaires subis à la suite d'une première rencontre. Ensuite une seconde rencontre serait réalisée de manière à respecter un pas de temps pondéré entre les modèles étudiés. Ce temps serait basé sur une valeur proportionnelle à la durée de vie ou de la fin de l'optimum de reproduction des individus. Par exemple 2 % du temps de vie de l'animal pourrait séparer le priming et le challenge immunitaire, ce qui ouvrirait un espace entre quelques heures pour les insectes et plusieurs jours, voire mois pour les modèles mollusques bivalves. Cette expérience serait maintenue sur plusieurs générations (ex, n=5) pour voir si une transmission à la descendance est imaginable en terme de phénotype ou de facteurs moléculaires. Dans un premier temps l'échantillonnage se basera sur une extraction de l'animal entier d'une part, et sur ses hémocytes d'autre part. Les

extraits d'animaux seront combinés (pool) pour compenser la variance individuelle. Différents supports moléculaires pourraient être explorés sur plusieurs échelles pour regarder la diversité et l'abondance des molécules utilisées chez les différents systèmes pour devenir résistant : (i) génétique (gDNA), (ii) épigénétique (ATACseq, spectrométrie de masse des histones), (iii) transcriptomique (mRNA, sRNA), (iv) protéomique (label-free) et (v) métabolomique (spectrométrie de masse).

Une expérimentation de la sorte nécessiterait des années de travail et le traitement des données qui en résulterait tout autant. De nombreux projets à dimension internationale émergent en biologie pour accélérer les connaissances sur des sujets très sensibles comme le réchauffement climatique (Union of Concerned Scientists, ucsusa.org), la cartographie du monde planctonique (Tara Ocean) ou les changements globaux et leurs impacts sur les récifs coralliens (Tara Pacific), preuve que pour certaines thématiques, des projets scientifiques à l'échelle planétaire peuvent émerger. Une tentative pour sensibiliser les chercheurs étudiants les vecteurs de maladies infectieuses mollusques avait d'ailleurs fait grand bruit en 2012 avec trop peu de retombées aujourd'hui (Adema, Bayne et al. 2012). Un plan expérimental comme celui-ci ne pourrait exister qu'au travers d'une coopération entre équipes qui se composeraient de différents spécialistes qui maîtrisent individuellement mieux leur modèle. Une expérience de la sorte permettrait de générer la première base de données comparatives génétiques, transcriptomiques, épigénétiques d'invertébrés en réponse à des stress immunitaires et pourrait faire progresser les connaissances biologiques très rapidement pour un clade qui regroupe des problèmes socio-économiques majeurs : des pestes agricoles (insectes), vecteurs (insectes, mollusques), économie de la mer (crustacés, mollusques).

Acquisition de l'immunité mémoire : une stratégie des pathogènes ?

Comment les pathogènes auraient pu être les artisans évolutifs majeurs de l'acquisition de l'immunité adaptative au sein du monde vivant ?

En biologie, les systèmes hôtes-pathogènes permettent d'accéder aux réponses respectives d'un organisme sur un autre et montrent comment cette relation réussit à se perpétuer dans le temps. Malheureusement, il est parfois compliqué, lorsqu'on regarde un phénotype à un instant t , d'attribuer le poids relatif des différents acteurs sur ce phénotype.

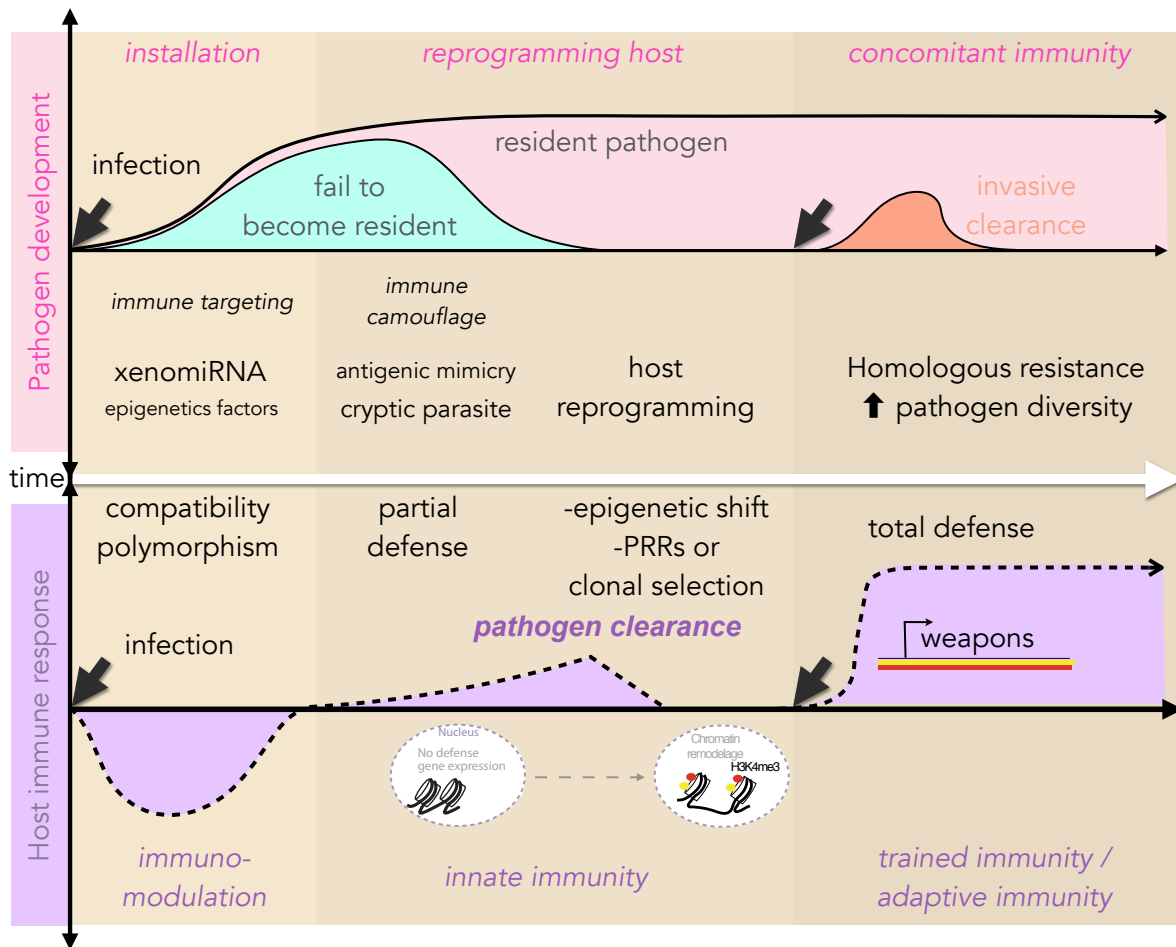


Figure 17. Réponse immunitaire d'un invertébré dans le cadre de la théorie de la discontinuité antigénique

Il est par exemple intéressant de comparer la diversité des pathogènes qui font face à des hôtes et les mécanismes de l'immunité adaptative d'autre part. La diversité des pathogènes potentiels est tellement grande, que les organismes hôtes semblent avoir sélectionné une immunité très spécifique pour y faire face. Mais est-ce que la réponse immunitaire adaptative n'est pas à l'inverse responsable de l'augmentation de la diversité génétique des pathogènes ?

La théorie de la discontinuité antigénique (Pradeu, Jaeger et al. 2013, Pradeu and Vivier 2016) suggère que la concentration d'un antigène lors d'une première rencontre qui va guider la réponse immunitaire. Ainsi, lors d'une première rencontre avec un pathogène, si cette concentration est suffisamment élevée, la réponse associée sera très intense et conduira à l'élimination du pathogène (Figure 17). Par contre, à cette concentration élevée, si l'hôte est incapable d'éliminer cet organisme et que cette association ne conduit pas à la mort de l'hôte, l'organisme étranger pourra devenir résident, voire même être inclus plus tard dans le microbiote. La réponse immunitaire va donc rapidement s'arrêter contre cet organisme, limitant ainsi les coûts énergétiques associés (Figure 17). Lorsqu'un pathogène se développe

très lentement dans un hôte, sa diversité antigénique exposée à l'hôte est très diluée. Malgré la présence de ce pathogène chez l'hôte, celui-ci n'a pas engendré de conséquences suffisamment délétères pour exiger son élimination. Cette interaction devient donc durable et devrait probablement conduire à une interaction moins unilatérale et passer d'un statut de pathogène/parasite vers un statut de symbionte/microbionte ou de mutualiste.

Quel lien entre la théorie de la discontinuité et la réponse immunitaire mémoire innée d'un invertébré : la connexion par l'immunité concomitante.

Lorsqu'un agent pathogène infeste un hôte, soit il est éliminé par le système immunitaire de l'hôte, soit il résiste à l'hôte et pourrait devenir à long terme un résident s'il diminue suffisamment voire annihile ses potentiels effets délétères. Un grand nombre de résultats actuels montre que la quasi-totalité des pathogènes sont capables de modifier, détourner à leur profit, ou « hacker » la réponse immunitaire de l'hôte pour assurer leur développement. On trouve ainsi différentes stratégies : (i) Des bactéries *Salmonelle* sont capables de modifier l'état inflammatoire de l'hôte pour permettre leur propre développement (Hobbie, Chen et al. 1997) ; (ii) des bacilles de la lèpre sont capables de forcer la différenciation cellulaire indispensable à la dissémination bactérienne (Masaki, Qu et al. 2013) ; (iii) les légionnelles peuvent sécréter des histones méthyltransférase-like pour modifier spécifiquement l'expression des gènes immunitaire de l'hôte (Cazalet, Gomez-Valero et al. 2010, Rolando, Rusniok et al. 2013, Rolando, Escoll et al. 2016) ; ou encore (iv) des *Listeria* sécrètent des protéines capables de modifier les patrons de méthylation génomique de l'hôte. Cette immunomodulation par le pathogène existe aussi chez les apicomplexes comme *Toxoplasma* qui sont capables, pour se développer, de modifier l'expression du répertoire de microRNA de son hôte via la libération de kinases spécifiques (Cai and Shen 2017, Hakimi, Olias et al. 2017) ou pour maintenir une prolifération cellulaire à sa convenance (Chaussepied, Janski et al. 2010, Marsolier, Pineau et al. 2013, Marsolier, Perichon et al. 2015) mais aussi potentiellement chez les parasites métazoaires (Buck, Coakley et al. 2014, Fromm, Trelis et al. 2015, Cai and Shen 2017).

Cette liste non exhaustive pourrait être complétée par le modèle *Biomphalaria/Schistosoma* où des résultats récents montrent que *Schistosoma* est capable d'interférer avec la réponse immunitaire via des miRNA, soit chez l'hôte définitif (Zhu, He et al. 2014, Nowacki, Swain et al. 2015, Zhao, Luo et al. 2015, Zhu, Dao et al. 2015, Hong, Fu et al. 2017) soit chez l'hôte intermédiaire (Portet, Pinaud et al, soumis dans MBE ; Pinaud, Lassalle et al, en préparation). D'autres molécules peuvent être le support de cette modulation de l'immunité du mollusque par le parasite comme le venom allergen-like 9 (SmVAL9) altérant la matrice extra-cellulaire et l'expression de gènes de l'immunité de l'hôte (Yoshino, Brown et al. 2014).

Cette capacité intrinsèque de tous les pathogènes de contourner la réponse

immunitaire de l'hôte pour se développer suggère une potentielle course à l'armement au cœur de cette interaction moléculaire. Ce qui signifie que l'avenir idéal d'un pathogène n'est certes pas la mort de son hôte (Combes 1997, Combes 1997, Combes 2000), mais de faire perdurer cette interaction le plus longtemps possible. Dans le cas d'un système hôte/parasite, le développement du pathogène va conduire à la mort inéluctable de l'hôte, mais plus celle-ci interviendra tard et plus l'hôte et le parasite pourront produire un maximum de descendance. La toxicité du parasite doit alors être suffisamment forte pour qu'il puisse se développer, mais pas trop pour ne pas tuer rapidement son hôte trop rapidement et ainsi avoir suffisamment de temps pour se reproduire et produire une descendance.

Un organisme, qu'il soit microbiontique ou parasite résident, n'est plus un pathogène à éliminer en priorité, car ses effets néfastes interviendront tardivement temps pour laisser à l'hôte l'opportunité de se reproduire. Dans le cas de l'interaction entre *Biomphalaria* et *Schistosoma*, la réponse immunitaire de l'hôte semble se concentrer sur les tout premiers stades de développement du parasite. Comme indiqué par les expériences de vaccination, seul le stade précoce de Sp1 est vaccinant à l'inverse des stades Sp2 ou cercaires. Une fois le stade Sp1 établi dans l'hôte, le mollusque ne semble plus investir dans l'immunité contre ce parasite en essayant par exemple de cibler les Sp2 ou les cercaires. Dans le cas d'une infestation par *Schistosoma* où le parasite est capable de s'implanter dans les tissus du mollusque, le parasite sous ses deux dernières formes larvaires pourrait être considéré comme un pathogène résident.

Chez les hôtes capables de générer une réponse mémorielle très spécifique contre les futurs pathogènes qui se présenteraient à eux, c'est-à-dire non résidents, ils vont forcer la sélection de parasites qui seront différents du premier rencontré ? Le seul moyen d'infester à nouveau ces hôtes primo-infectés qui ont acquis une mémoire immunitaire est alors, pour la communauté de pathogènes, de sélectionner de la diversification afin de ne plus ressembler au parasite utilisé lors de la primo-infection (Alizon 2013, Fraser, Lythgoe et al. 2014). Ce scénario se produit de la même façon dans l'univers viral, où un individu peut devenir très résistant à une souche donnée de virus, mais sera toujours très sensible à une autre dissimilaire forçant ainsi de-facto la diversification des pathogènes (Fraser, Lythgoe et al. 2014).

La réponse immunitaire adaptative semble posséder une limite fondamentale, dans la mesure où elle force irrémédiablement la diversification des pathogènes en sélectionnant toujours les plus dissimilaires qui peuvent alors passer inaperçus vis-à-vis de ce système immunitaire.

Des expériences d'évolution expérimentale pourraient être utilisées pour étudier le poids relatif de la spécificité de la réponse immunitaire dans l'acquisition de processus de diversification « antigénique » chez les pathogènes. Est-ce que la pression évolutive qui pèse sur l'immunité adaptative est perdue lorsqu'on force la rencontre avec des agents pathogènes de moins en moins diversifiés ? Est-ce que l'augmentation de la diversité de pathogène force l'acquisition d'armes

immunitaires de plus en plus complexes ?

Dans cette course à l'armement, qui opposerait la virulence des pathogènes à l'immunité mémoire des hôtes, la création d'un système immunitaire adaptatif chez les hôtes potentiels pourrait conduire d'une part, à l'augmentation de sa spécificité de reconnaissance des hôtes et d'autres parts, à la diminution de la résistance contre des pathogènes de plus en plus dissimilaires.

Ce mécanisme n'est pas sans rappeler l'immunité concomitante décrite chez les Vertébrés et plus particulièrement dans les interactions mammifères/trématodes (Clegg, Smithers et al. 1971, Jordan, Cook et al. 1974, Wilson, Coulson et al. 1983, Brown and Grenfell 2001, He, Yi et al. 2004, Salim and Al-Humiany 2013). Suite à une infection par le Schistosome, l'individu chez qui le parasite se développe met en place une réponse immunitaire innée puis mémoire qui est efficace contre les nouvelles infections et d'autant plus efficace que la dissimilarité génétique est faible entre le parasite de la primo-infection et les parasites issus des réinfections. Cette réponse immunitaire efficace contre les infections parasitaires successives, reste toutefois sans effet sur le parasite de la primo-infection, qui est à l'origine de la mise en place de cette réponse immunitaire. C'est ce processus tout à fait étonnant que l'on appelle l'immunité concomitante. Cette stratégie peut être interprétée comme une stratégie parasitaire visant à favoriser un brassage génétique puisque seuls les parasites les plus dissimilaires réussissent à infecter l'hôte, mais également cela peut protéger leur niche écologique de nouvelles arrivées de parasites (compétition parasitaire par utilisation du système immunitaire de l'hôte).

Dans le cas de l'interaction entre *Biomphalaria* et *Schistosoma*, la résistance s'acquiert grâce à une première infection où le parasite se développe sans être affecté d'aucune manière par le système immunitaire ou par la réponse innée mémoire qui se met en place. Ainsi, deux hypothèses peuvent alors être avancées, la mémoire immunitaire innée est la résultante de : (i) une réponse immunitaire de l'hôte afin de se défendre de nouvelles infections ; (ii) d'une compétition directe entre parasites. Néanmoins, une troisième possibilité existe : le parasite pourrait détourner l'immunité de l'hôte en le rendant ainsi résistant à une nouvelle infection pour protéger sa niche écologique. Cette dernière hypothèse s'intègre parfaitement dans la définition de l'immunité concomitante, le premier parasite arrive à s'implanter, puis l'hôte est capable d'éliminer tous les prochains parasites similaires sans effet sur le premier entrant.

Sur la base de nos résultats, il est compliqué de définitivement conclure sur la présence d'immunité concomitante chez *Biomphalaria*. Il semblerait que le genre des Schistosomes ait développé des stratégies très particulières pour se soustraire au système immunitaire de l'hôte, par du mimétisme moléculaire (Yoshino, Wu et al. 2012), l'utilisation de molécules immuno-suppressives (Yoshino, Brown et al. 2014) jusqu'à la libération d'exosomes contenant des acides nucléiques capables

d'interférer avec des gènes de la réponse immunitaire des vertébrés (Nowacki, Swain et al. 2015). Pour autant, si des expériences ont pu montrer l'existence de cette immunité concomitante dans l'interaction entre un Schistosome et son hôte définitif (He, Yi et al. 2004, Salim and Al-Humiany 2013), l'absence d'immunité mémoire caractérisée chez l'hôte invertébré a retardé ce questionnement chez le mollusque vecteur. Aujourd'hui, nos résultats décrivent les bases moléculaires de l'immunité mémoire innée chez *Biomphalaria*, ce qui amène automatiquement à rappeler que cette forme d'immunité induite par les trématodes doit être explorée plus intensément chez *Biomphalaria*. Il serait très intéressant de comparer aujourd'hui la réponse immunitaire de l'ensemble des vecteurs mollusques contre les *Schistosoma* et observer ainsi, si cette stratégie parasitaire pourrait être retrouvée contre l'ensemble des hôtes. Que la résistance observée chez *Biomphalaria* puisse être induite activement par un Schistosome ne remettra jamais en cause que les molécules impliquées dans l'élimination du parasite lors de la seconde rencontre sont produites exclusivement par le *Biomphalaria*. Le vecteur de la bilharziose possède donc l'ensemble des armes nécessaires pour générer une résistance contre des parasites Schistosomes.

Un cas de résistance acquise atypique

L'interaction entre les *Biomphalaria* et les *Schistosoma* a été façonnée par des milliers d'années d'évolution. La diversification de molécules du parasite potentiellement ciblées par l'hôte d'une part, et la complexification du système immunitaire de l'hôte d'autre part, indique une course à l'armement ou une adaptation entre ces deux protagonistes. Cette interaction nous indique probablement pour la première fois le chemin qu'aurait pu emprunter l'évolution pour cibler, se souvenir, et éliminer un pathogène dans un système immunitaire d'invertébrés. Il serait dans un premier temps très pertinent de rechercher chez un panel d'invertébrés (voir figure 16) la présence de phénomènes proches du shift identifié chez *Biomphalaria*. Une grande divergence de mécanismes ne serait pas particulièrement étonnante lorsqu'on observe par exemple le poids important des transferts horizontaux chez les invertébrés pour acquérir spécifiquement de nouvelles fonctions comme précédemment montrées pour les aérolysines par exemple (Moran, Fredman et al. 2012, Galinier, Portela et al. 2013).

Scénario de l'acquisition de la résistance ?

L'immunité de *Biomphalaria* repose à la fois sur un panel de répertoire de récepteurs immunitaires, capables (peut-être en étant combinés) de différencier un grand nombre de pathogènes (Deleury, Dubreuil et al. 2012) et (Pinaud, Portet et al. soumis), et d'effecteurs toxiques capables de les éliminer (Baron, van West et al. 2013, Galinier, Portela et al. 2013, Adema, Hillier et al. 2017).

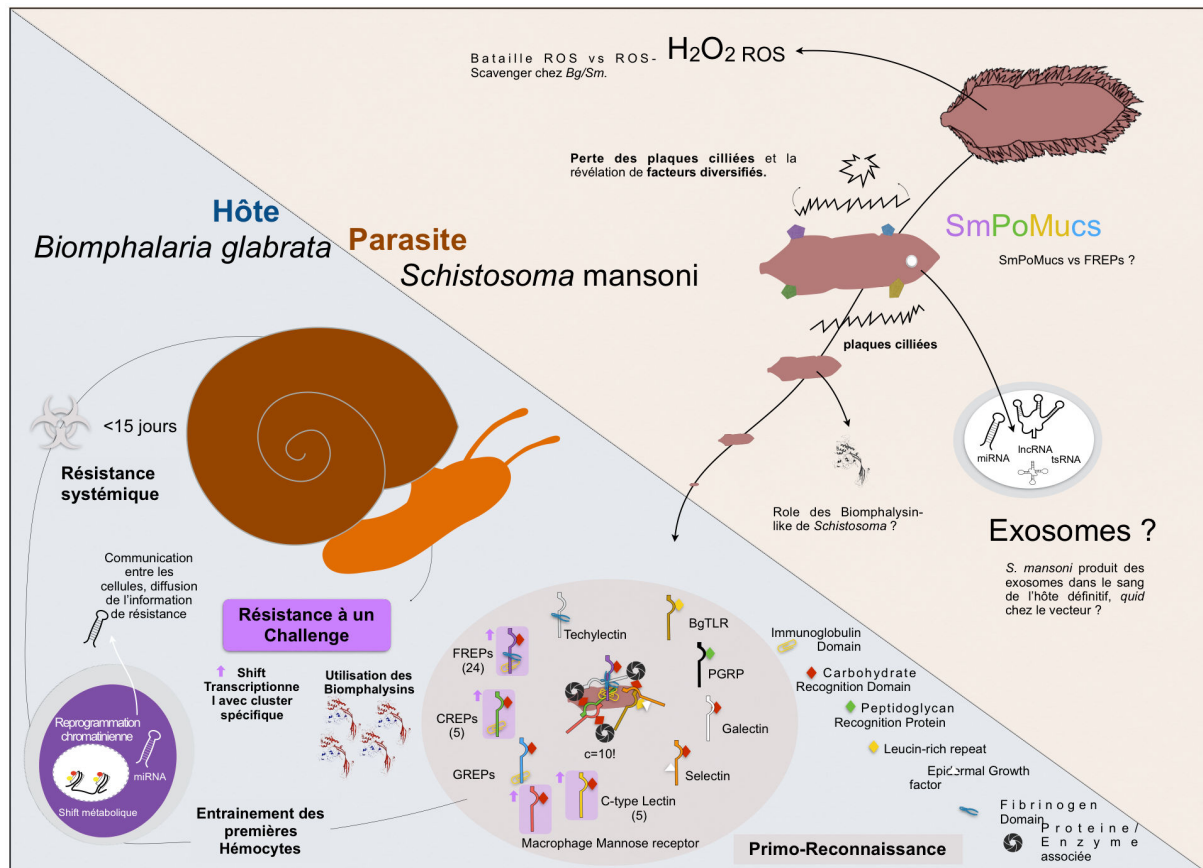


Figure 18. Acquisition de la résistance chez *Biomphalaria glabrata* en réponse à *Schistosoma mansoni*. Un miracidium nage vers un hôte *Biomphalaria* et va lors de l'infestation perdre ses plaques ciliées. Cette libération pourrait être associée à des exosomes contenant des acides nucléiques pouvant jouer un rôle dans l'immunosuppression induite par le parasite. Ensuite, le neo-Sp1 en contact avec les tissus de l'hôte ainsi que ses hémoctes s'expose à une reconnaissance par des molécules de types lectines (FREPs, CREPs, GREPs, C-Type, Selectine, Galectine), Toll (BgTLR), PGRP voire des molécules de reconnaissance non-canoniques (actine, tubuline, hemoglobine) ainsi que des enzymes (GAPDH, alpha-amylase). Cette reconnaissance conduit au déclenchement de l'acquisition de la résistance de priming. Après une quinzaine de jours, l'animal est résistant, et, ses hémoctes ont subi une reprogrammation épigénétique profonde (changement de structure chromatinienne, déméthylation globale, changement de répertoires de miRNA) permettant d'exprimer plus fortement des récepteurs et des toxines (biomphalysines) lors d'une future rencontre avec un parasite homologue.

Ces différents récepteurs et effecteurs immunitaires révèlent une stratégie très complexe pour devenir résistant qui pourrait être synthétisée dans un modèle de l'acquisition de la résistance chez *Biomphalaria* en réponse à *Schistosoma* (Figure 18) :

- un hôte BgBRE est exposé à 10 miracidia de *Schistosoma mansoni* SmbRE
- des miracidia s'introduisent activement dans les tissus du mollusque
- la transformation des miracidia en Sp1 entraîne la libération de nombreux composés (dont des acides nucléiques sous la forme de miRNA) capables d'interférer avec la réponse immunitaire de l'hôte
- des hémocytes circulants se déplacent pour entourer ± 7 parasites en développement, une capsule peut être observée après une douzaine d'heures et disparaît moins de 48h après
- ± 3 parasites arrivent à démarrer leur développement en dépit de la réponse immunitaire de l'hôte, ces derniers ne sont pas encapsulés
- au niveau transcriptomique, la représentation de différentes lectines est fortement perturbée
- au niveau protéomique, de nombreuses lectines sont identifiées conduisant potentiellement à la formation d'un complexe immun, différentes biomphalysines sont également retrouvées
- des molécules non canoniques pour leurs rôles dans l'immunité permettent de structurer ou maturer ce complexe immun
- des effecteurs ainsi que des enzymes protéolytiques doivent être utilisées pour détruire les parasites au cœur des capsules
- des miRNAs de parasites sont retrouvés dans le plasma de l'hôte
- certains Sp1 ont réussi à se développer pour donner au bout d'une dizaine de jours des Sp2, sans la formation d'aucun complexe hémocytaire.
- en dix à quinze jours, les cellules de l'hôte ont subi une restructuration chromatinienne, des changements de méthylation de leur génome et un changement d'expression de miARN endogènes
- au bout d'une quinzaine de jours, l'hôte est totalement résistant à une nouvelle infestation par un nouveau parasite homologue
- lors d'une deuxième rencontre, de 15j à la mort de l'hôte (>140j), l'hôte et ses cellules immunitaires produisent un panel très complexe de récepteurs immunitaires qui doivent lui permettre de détecter ces nouveaux parasites plus efficacement et réussir à l'éliminer sans structuration d'un complexe immunitaire d'encapsulation, par le biais d'une réponse immunitaire humorale.
- des biomphalysines sont détectées en protéomique massive (label-free) en grande quantité dans le plasma et pourraient expliquer ce phénotype humoral

Le rôle des biomphalysines dans cette résistance mémorielle est très intéressant. En effet un faisceau de preuves indique que les biomphalysines pourraient être les

effecteurs terminaux de la résistance acquise chez *Biomphalaria* contre *Schistosoma*.

Enfin des aérolysines similaires à celles identifiées chez *Biomphalaria* ont été très récemment identifiées chez *Schistosoma*. Si leur caractérisation est pour le moment très préliminaire, il est possible d'envisager qu'elles puissent constituer une arme supplémentaire utilisée par le parasite pour se soustraire de l'immunité de son hôte.

Quel rôle pour les biomphalysines lors d'une première rencontre avec un pathogène ?

La liaison spécifique des biomphalysines avec différents pathogènes et leur expression constitutive suggèrent une action sentinelle d'élimination de pathogènes dans le plasma du mollusque. Lors de la première rencontre, les biomphalysines expriment des capacités à se lier spécifiquement à différents pathogènes, mais n'entraînent lors de nos tests in-vitro aucune mortalité de ces pathogènes. Ceci suggère que lors d'une première rencontre avec un pathogène, le rôle des biomphalysines n'est peut-être pas d'être toxiques pour le pathogènes, mais pourrait être un acteur de la reconnaissance. Leur activité lytique pourrait être liée à l'absence de synthèse d'un cofacteur nécessaire à leurs activations. Lors d'une première rencontre les biomphalysines pourraient arborer différentes fonctions :

- reconnaissance spécifique portée par les récepteurs portés par le petit lobe
- structuration et maturation du complexe immun
- sélection des populations activées d'hémocytes dans l'activation de l'acquisition de la résistance et de la mémoire immunitaire.

À l'inverse, lors d'une seconde rencontre, la production d'une quantité beaucoup plus importante de biomphalysines dans le plasma, indique qu'elles sont impliquées dans l'élimination du parasite par leur action lytique. Leur activité cytotoxique, peut-être plus que leur concentration, pourrait se trouver dans l'expression d'un cofacteur plasmatique qui va permettre leur activation, par clivage ou par libération d'une chaperonne nécessaire à la conversion du pré-pore en pore fonctionnel. Ce rôle d'activation d'une molécule toxique par liaison avec un cofacteur spécifique rappelle fortement l'activité des facteurs du complément.

Rôle dual dans une réponse innée

La présence de molécules capables d'exercer plusieurs fonctions immunitaires « dual-role » comme la reconnaissance et l'élimination d'un pathogène, ont été plusieurs fois décrites chez les invertébrés (Cambi and Figdor 2003, Galinier, Portela et al. 2013). Les processus évolutifs pesant sur ces familles auraient-ils pu entraîner la production de famille à double rôle fonctionnel, récepteurs/effecteurs expliquée par la présence de domaine protéiques supplémentaires utiles à l'élimination directe d'un pathogène (Cambi and Figdor

2003, Sarray, Berthet et al. 2004, van Vliet, Gringhuis et al. 2006, Galinier, Portela et al. 2013, Vazquez-Mendoza, Carrero et al. 2013, Dambuza and Brown 2015) ou d'une autre fonction autre que l'immunité comme ce qui est connu pour les TEPs (Pompon and Levashina 2015). Ce dual-role suggère donc : (i) l'activation de la réponse immunitaire des invertébrés pourrait ne pas dépendre toujours d'une structuration d'un complexe immun conduisant à l'élimination de la cible, mais de l'utilisation d'effecteurs doués de reconnaissance ; (ii) que la deuxième fonction portée par ces molécules amène une nouvelle source de pression évolutive pour cette molécule pour conserver non pas la fonction de cette molécule, mais les deux fonctions de celle-ci.

Parmi ces protéines à fonction duales, les BgTEP, thioester containing protein, possèdent à la fois un rôle d'inhibiteur de protéase (A2M) et un rôle d'opsonine. Les c-type lectines et les biomphalysines possèdent toutes des domaines de reconnaissance spécifiques ou des domaines impliqués dans la cytolysse. Ces différentes fonctions doivent-elles être obligatoirement liées à une seule et unique fonction ici immunitaire ? Ainsi la AgTEP est également impliquée dans l'élimination des cellules lors de la production des spermatozoïdes chez le moustique (Pompon and Levashina 2015) tout en étant capable de participer à l'élimination des sporozoïtes de *Plasmodium* (Levashina, Moita et al. 2001).

Ces molécules immunitaires diversifiées/polymorphes, malgré un rôle potentiellement autre que la reconnaissance, pourraient donc tout de même être retrouvées dans des complexes immunitaires dus à leur seconde fonction. Des études d'interactomes chez *Biomphalaria* suggèrent ainsi l'implication de nombreuses molécules dans un rôle de reconnaissance, au sens reconnaissance d'épitopes spécifiques, (Wu, Dinguirard et al. 2017). Pourtant l'activité de reconnaissance de ces molécules reste difficile à décrire sans expériences complémentaires telles que proposées par nos expériences d'interactome plus similaire à la réalité biologique. Il serait intéressant d'utiliser des anticorps dirigés contre la fonction de reconnaissance de ces molécules dual-role, et voir si pendant une infection elles seraient toujours retrouvées dans une capsule immunitaire. Leurs présences pourraient signifier que c'est pour leur deuxième fonction qu'elles sont utiles à ce complexe immun.

Le cas des infestations mono-miracidiales

Dès les premiers résultats de résistance acquise (Sire, Rognon et al. 1998), les primo-infestations réalisées avec un seul miracidium ont révélé chez le couple BgBRE/SmBRE que les hôtes infestés deviennent totalement résistants à une seconde infestation homologue. Dans ce contexte, il n'y a alors aucun parasite éliminé par une encapsulation hémocytaire. Ceci suggère que l'acquisition de la résistance, ne dépend pas du contact des hémocytes avec le parasite lors de la formation du complexe immun conduisant à l'encapsulation. Ce résultat pourrait

suggérer deux choses : la résistance acquise s'induit à partir d'un seul parasite infestant donc une compétition larvaire pourrait encore expliquer cette résistance acquise ; l'activation de l'acquisition de la réponse immunitaire de priming peut être déclenchée même lorsqu'un qu'aucun parasite n'est éliminé. Cette dernière proposition, suggère donc qu'une reconnaissance pourrait être efficace bien que le parasite réussisse à se développer. Si l'étape de reconnaissance ne semble pas évitable par le parasite, il semblerait qu'il soit capable de stopper ensuite la réponse immunitaire initiée par l'hôte.

Cette acquisition de la résistance pourrait être portée par la détection de déterminants de surfaces lors de la rencontre avec le tout premier parasite. Différentes combinaisons de récepteurs immuns (Figure 18) pourraient être alors sélectionnées malgré l'absence de capsule. La bonne combinaison pourrait être ré-exprimée lors de la seconde rencontre et ainsi expliquer le phénotype mémoriel. Or, les résultats de transcriptomique révèlent que le panel de récepteurs potentiels est plus complexe lors de la seconde rencontre, suggérant la nécessité d'augmenter la machinerie de reconnaissance.

L'immunité entraînée comme immunité anticipatoire innée

L'immunité entraînée, un succès évolutif partagé chez tous les eucaryotes ?

Une version de la réponse immunitaire mémorielle semble avoir eu un succès évolutif largement plus important qu'imaginé jusqu'à lors, c'est l'immunité entraînée ou « trained immunity » (Netea, Quintin et al. 2011). Portée par une modification massive de la biologie nucléaire des cellules de l'immunité innée, une reprogrammation de leur métabolisme qui permettrait de se préparer à répondre à un prochain stimulus. Les plantes, les invertébrés et les vertébrés seraient tous capables de subir ce type de modifications pour se préparer à mieux répondre contre un pathogène précédemment rencontré.

L'immunité entraînée, comme discutée précédemment, est une immunité mémoire qui repose sur une résistance acquise d'une première rencontre avec un pathogène et qui n'est pas assurée par des cellules du système immunitaire adaptatif, ni par une sélection clonale d'anticorps.

Cette immunité est maintenant bien caractérisée et repose entre autres sur une reprogrammation épigénétique de cellules de l'immunité innée, qui sont alors capables de produire davantage de récepteurs, d'effecteurs immunitaires et de molécules de communication (Interleukine, Cytokine) pour potentiellement recruter et stimuler de nouvelles cellules circulantes et ainsi, mieux se préparer à répondre à une nouvelle infection (Netea, Quintin et al. 2011, Bekkering, Joosten et al. 2014, Cheng, Quintin et al. 2014, Ifrim, Quintin et al. 2014, Kleinnijenhuis, Quintin et al.

2014, Blok, Arts et al. 2015, Ifrim, Quintin et al. 2015, Arts, Carvalho et al. 2016, Arts, Joosten et al. 2016, Arts, Novakovic et al. 2016, Bekkering, Blok et al. 2016, Netea, Joosten et al. 2016, Rizzetto, Ifrim et al. 2016, Netea and van der Meer 2017, Smith, Kleinnijenhuis et al. 2017).

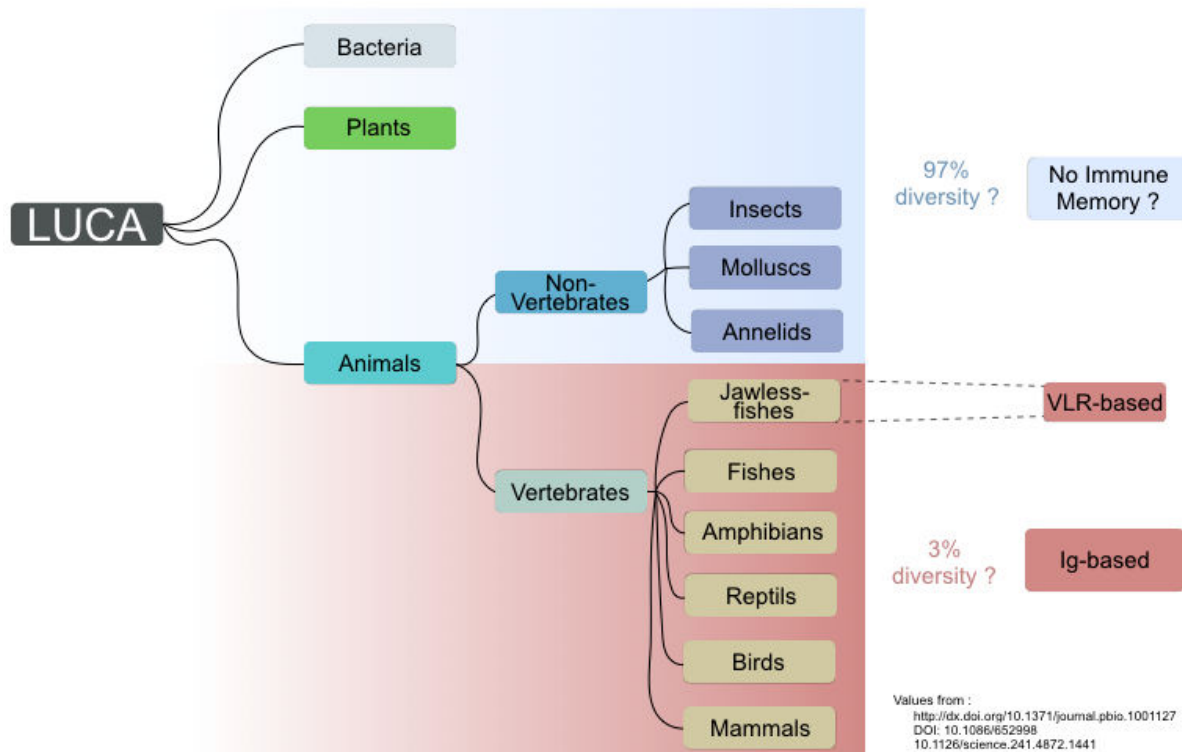


Figure 19. Phylogénie simplifiée du vivant pour comparer le succès évolutif des différents système immunitaires mémoriels. Depuis LUCA (last universal common ancestor), les organismes ont évolué pour donner de manière caricaturale, bactéries, plantes, invertébrés et vertébrés. Il apparaît que les bactéries, plantes et invertébrés représentent 97% de la diversité spécifique sur terre. Pourtant ces organismes ne possèdent pas d’immunité adaptative. Cela sous-entend qu’il y a un succès évolutif à ne pas avoir d’immunité adaptative basée sur les Ig.

Si on regarde à l’échelle du vivant (Figure 19) avec un cloisonnement simple, eucaryotes, plantes, animaux ; puis vertébrés et non-vertébrés, il apparaît que seul 3 % des espèces sur terre ont acquis une immunité adaptative mémorielle dépendante des immunoglobulines (Ig). 97 % des autres organismes sur Terre prospéreraient donc sans faire appel à un système immunitaire mémoire dépendant des Ig. Cela signifie-t-il pour autant qu’ils ne possèdent pas d’immunité mémoire, rien n’est moins sûr. En réalité de nombreux organismes parmi ces 97%, comme les ecdysozoaires et les mollusques, ont montré des capacités à acquérir une résistance à la suite d’une première rencontre avec un pathogène soutenues par des mécanismes sous-jacents très différents (Jorge Contreras-Garduno, Humberto Lanz-Mendoza et al. 2016, Milutinovic and Kurtz 2016). De même chez les plantes, des stratégies de résistance supportées par des modifications épigénétiques et l’expression de récepteurs homologues du système immunitaire innée, suggèrent une forte conservation de ce type d’immunité chez les plantes (Conrath, Beckers et

al. 2015).

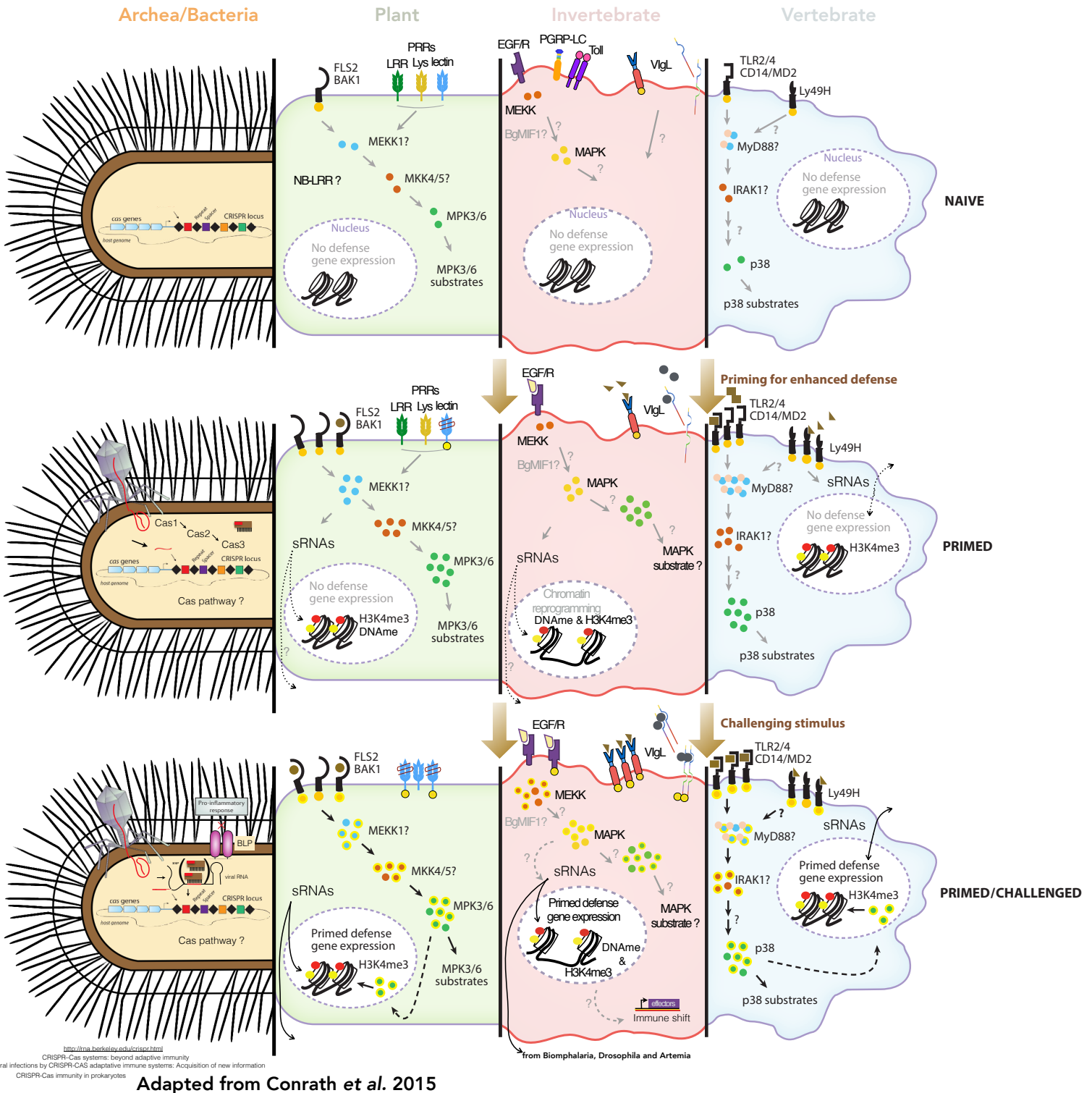


Figure 20. Support moléculaire de la résistance acquise chez les procaryotes, plantes et invertébrés et vertébrés

Adapté à partir de Conrath et al. 2015 dans Annual Reviews of Phytopathology

Ainsi, un faisceau d'évidences tend à montrer que cette immunité mémoire innée aurait pu être acquise précocement dans l'arbre du vivant et être partagée par l'ensemble des eucaryotes dont les principales caractéristiques sont les suivantes (Figure 20) :

- un phénotype de résistance acquise à un pathogène
- des cellules portant ou sécrétant des récepteurs immunitaires
- des voies de transduction de signaux incluant des Kinases
- un support épigénétique (au sens des modifications affectant la régulation des gènes sur une ou plusieurs générations) tel que la méthylation du génome, la régulation de l'état chromatinien (marques d'histones), la production d'ncARN (lncARN, miARN, etc)
- des effecteurs immunitaires capables d'éliminer totalement le pathogène.

En partageant ces caractéristiques, ces organismes ouvrent la voie vers une généralisation de l'immunité entraînée comme phénomène ancestral conservé au cours de l'évolution chez un très grand nombre d'espèces, mais ayant pu être, jusqu'à présent, masquée chez les vertébrés par la réponse adaptative dépendante des Ig.

Cette observation pourrait même être poussée à l'extrême en incluant les procaryotes. En effet, de plus en plus de travaux proposent de comparer le système CRISPR-Cas à un système immunitaire adaptatif chez les Procaryotes (Koonin and Makarova 2009, Marraffini and Sontheimer 2010, Terns and Terns 2011). Si cette réponse venait à être supportée par des modifications épigénétiques, elle pourrait en ce sens être assimilée à une réponse entraînée.

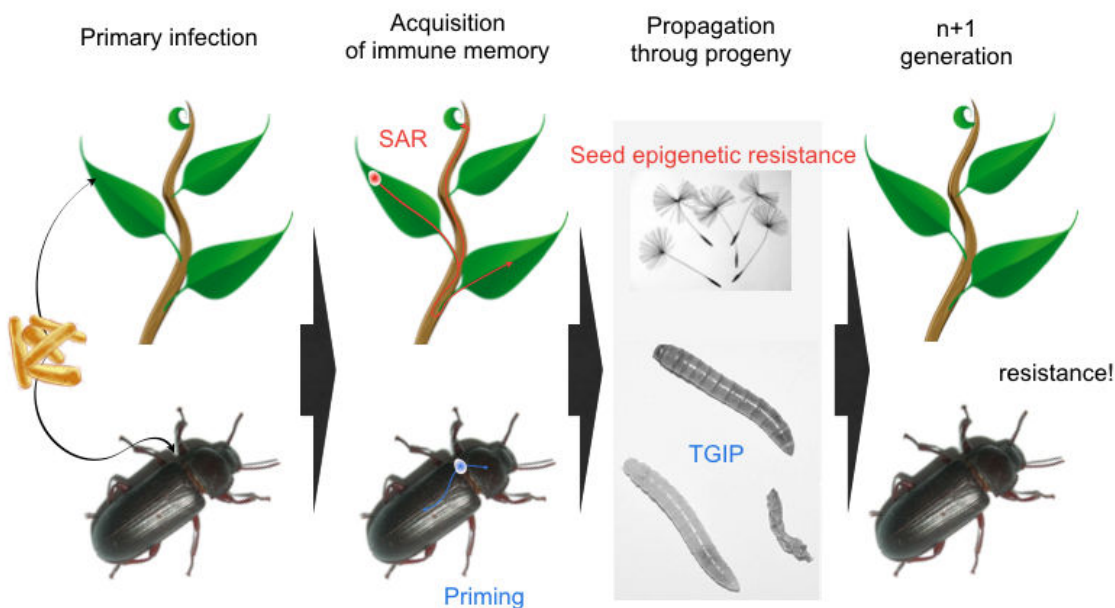


Figure 21. Modèle de transmission de résistance acquise chez plantes et invertébrés. L'immunité entraînée comme base du SAR (systemic acquired resistance) et TGIP (priming trans-générationnel) ?

L'immunité entraînée suggère également qu'un mécanisme somatique porté par des processus épigénétiques améliore la réponse immunitaire d'un individu. Ces processus pourraient affecter les cellules germinales et ainsi participer à la transmission de la protection à la descendance. En effet, des phénotypes de transmission de caractères basés sur des modifications épigénétiques ont été observés (Hanson and Skinner 2016, Huypens, Sass et al. 2016, Whipple and Holeski 2016, Houri-Zeevi and Rechavi 2017), tout comme une transmission de facteurs de résistance de parents vers la descendance (Figure 21) (Moret and Siva-Jothy 2003, Grindstaff, Hasselquist et al. 2006, Moret 2006, Jokela 2010, Tidbury, Pedersen et al. 2011, Zanchi, Troussard et al. 2011, Dubuffet, Zanchi et al. 2015, Knorr, Schmidtberg et al. 2015, Trauer-Kizilelma and Hilker 2015, Rizzetto, Ifrim et al. 2016, Dhinaut, Chogne et al. 2017).

Darwin (1809-1882, Angleterre) a expliqué dans de nombreux ouvrages sa théorie de la sélection naturelle. Elle explique que les êtres vivants vont accumuler des innovations « technologiques » qui pourront en fonction des conditions environnementales être sélectionnées ou non pour améliorer leurs conditions de vie. Ces êtres vivants ne sélectionnent pas la meilleure combinaison de phénotypes, mais s'ils survivent, les facteurs de résistances nécessaires à cette survie seront irrémédiablement transmis. Lamarck au contraire (1744-1829, France), pensait que les êtres vivants, forgés par les pressions de sélection, allaient s'adapter graduellement à leur environnement en se complexifiant jour après jour. S'il a ainsi décrit la première théorie de l'évolution des espèces, il a négligé les sources potentielles d'innovations et donc d'adaptations : le hasard et la contingence (Stephen Jay Gould, 1941-2002, USA). Bien que ces deux théories de l'évolution ne s'opposent pas vraiment, la temporalité entre l'acquisition d'un caractère et son effet adaptatif étant difficilement observable, il reste un affrontement théorique entre ce qui pourrait être issu de la pensée de Darwin ou celle de Lamarck.

À l'heure actuelle, l'acquisition de caractère de résistance, permettant un gain de fitness chez un individu et pouvant être transmis à sa descendance par des processus épigénétiques, dispose de toutes les caractéristiques d'un phénomène Néo-Lamarckien.

Cette réflexion pourrait ouvrir la voie à considérer qu'un statut immunitaire pourrait être transmis à la descendance via l'expression somatique de l'immunité entraînée qui, par l'expression de processus épigénétiques pourrait modifier certains caractères de la lignée germinale. De ce fait, l'immunité entraînée pourrait donc véritablement être une immunité adaptative et mémorielle. Du fait qu'elle soit adaptative et potentiellement transmissible à la descendance, elle pourrait être décrite comme un processus lamarckien. Les mécanismes pour supporter cette immunité ont néanmoins été sélectionnés par des processus décrits par Darwin. L'immunité entraînée pourrait être une évolution biologique majeure chez l'ensemble des eucaryotes en proposant une immunité adaptative, mémorielle et

transmissible.

En conclusion : Observations, Principe, Théorie, Hypothèses, Dogme, Lois ?

La résistance observée chez *Biomphalaria glabrata* à la suite d'une infection par le vers trématode *Schistosoma mansoni* reste singulière. Si les multiples explorations des bases moléculaires ne font que prouver tant et plus la complexité de l'immunité de cet hôte et l'activation de nombreux facteurs endogènes pour mieux répondre à une future infection, il reste toujours difficile de confirmer l'origine de cette résistance. Il est nécessaire aujourd'hui de rechercher dans différentes interactions entre des schistosomes et des vecteurs mollusques la présence ou l'absence de ce phénomène de résistance acquise (*heamatobium/Bulinus, rodhaini/pfeifferi*, par exemple).

Il est important de rappeler, qu'une résistance induite par le parasite est toujours envisageable, mais elle aurait le mérite de démontrer que cet escargot possède des armes assez puissantes pour éliminer ce parasite. Ce phénotype de résistance acquise observé dans le couple BgBRE/SmBRE, pourrait apporter les premiers éléments moléculaires pour sélectionner à l'avenir des souches résistantes à l'infestation et donc à la transmission du parasite sur le terrain. Ces résultats montrent également que la tolérance à un parasite pourrait être tout aussi importante que de lui résister dans le cas où l'hôte aurait suffisamment de temps pour se reproduire. Il est d'ailleurs intéressant de constater qu'une augmentation de l'activité de reproduction est observable chez *Biomphalaria* à la suite d'une infestation par *Schistosoma* (Crews and Yoshino 1989). Dans les stades plus avancés de parasitose par contre cette augmentation est suivie d'une castration totale liée entre autres à la localisation du parasite dans la glande digestive et la gonade qui perturbe profondément le processus de maturation des gamètes.

Cette notion de tolérance pourrait donc être une stratégie pour investir dans la reproduction rapidement après l'infestation. La résistance acquise pourrait permettre d'accroître cette période de reproduction, et sans elle une seconde infestation pourrait être potentiellement fatale à l'individu.

Le but de ce travail était d'explorer les mécanismes moléculaires supportant la résistance acquise de *Biomphalaria* à la suite d'une infestation par *Schistosoma*. Dans ce sens, de nombreuses expériences et techniques ont été utilisées pour enrichir les connaissances de cette immunité mémoire innée chez *Biomphalaria glabrata* et donc chez les invertébrés.

Ainsi, nous avons pu montrer que le priming immunitaire, ou immunité innée mémoire, ou résistance acquise, ou immunité entraînée était dépendante d'un « shift » qui permettait aux cellules spécialisées de l'immunité de l'hôte de se préparer à mieux répondre lors d'une deuxième rencontre avec le pathogène. Cette amélioration de la réponse est d'ailleurs caractérisée par l'arrêt de la réponse immunitaire d'encapsulation typique de la première rencontre avec un parasite, vers

une réponse humorale plus efficace et spécifique. Ce shift possède d'ailleurs une composante épigénétique qui le rapproche fortement de l'immunité entraînée des vertébrés suggérant sa potentielle conservation évolutive chez l'ensemble des animaux voire des eucaryotes.

Nous avons ainsi pu montrer que cette résistance spécifique du génotype, est dépendante de l'expression de différents répertoires de récepteurs et d'effecteurs immunitaires. Cette spécificité de reconnaissance est portée par une combinatoire de nombreux récepteurs immunitaires tels que les FREP, GREP, CREP, les G/C/H-type lectines, les macrophage-mannose récepteurs, les selectines, PGRP et peut être d'autres encore jusqu'ici peu décrites dans l'immunité (récepteurs non-canoniques). En effet de nouvelles expériences d'interactome nous ont d'ailleurs permis de caractériser également une spécificité de reconnaissance portée par des molécules à double rôle telle que les TEP, certaines C-type lectin ou encore les biomphalysines. Ces biomphalysines peut-être héritées d'un ou plusieurs transferts horizontaux, ont été sélectionnées, domestiquées puis diversifiées afin d'être aujourd'hui une arme au cœur de l'immunité innée mémoire de *Biomphalaria*.

Ces différents résultats amènent maintenant autant de théories et de perspectives que de conclusions. Les bases moléculaires apportées par ce travail constituent une avancée majeure pour la compréhension de l'immunité innée mémoire chez un invertébré. Les résultats de cette thèse pourraient même tracer les premières lignes d'une conservation de ce phénomène immunitaire pour l'ensemble des invertébrés, alors que ce n'était jusqu'à lorsqu'une caractéristique singulière observée uniquement chez les vertébrés.

Projets secondaires

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

Portet A, **Pinaud S**, Chaparro C, Charriere G, Allienne JF, Duval D, Galinier R, Gourbal B.
Soumis dans Molecular Biology and Evolution, Juin 2017

➤ A Multistrain Approach To Studying The Mechanisms Underlying Compatibility In The Interaction Between *Biomphalaria Glabrata* And *Schistosoma Mansoni*.

Galinier R, Roger E, Moné Y, Duval D, Portet A, **Pinaud S**, Chaparro C, Grunau C, Genthon C, Dubois E, Rognon A, Arancibia N, Dejean B, Théron A, Gourbal B, Mitta G.
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Soumis dans Frontiers Immunology, Septembre 2017

🍏 premiers auteurs ➤ article publié ↻ article soumis

Projets secondaires

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

Portet A, Pinaud S, Chaparro C, Charriere G, Allienne JF, Duval D, Galinier R, Gourbal B.
Soumis dans Molecular Biology and Evolution, Juin 2017

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L'adaptation locale pour des interactions hôtes/pathogènes données est souvent observée à un niveau phénotypique. Ici nous proposons qu'à l'échelle moléculaire, un phénotype de résistance ou virulence puisse masquer une adaptation locale. Ici, j'ai particulièrement participé à la caractérisation de l'immunosuppression de l'immunité de l'hôte par le parasite, via l'utilisation de données in-silico de miARN. On y découvre que les parasites sympatriques, après une longue co-évolution produisent plus de miARN que leurs homologues allopatriques, et que ces miARN peuvent cibler potentiellement plus de gènes de l'immunité que les allopatriques, suggérant ainsi une adaptation locale dans cette discussion moléculaire, reposant sur l'utilisation d'acides nucléiques.

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

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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.
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30 **Key Words:** local adaptation, host-parasite interaction, transcriptomic, immunity
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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.
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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;
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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.
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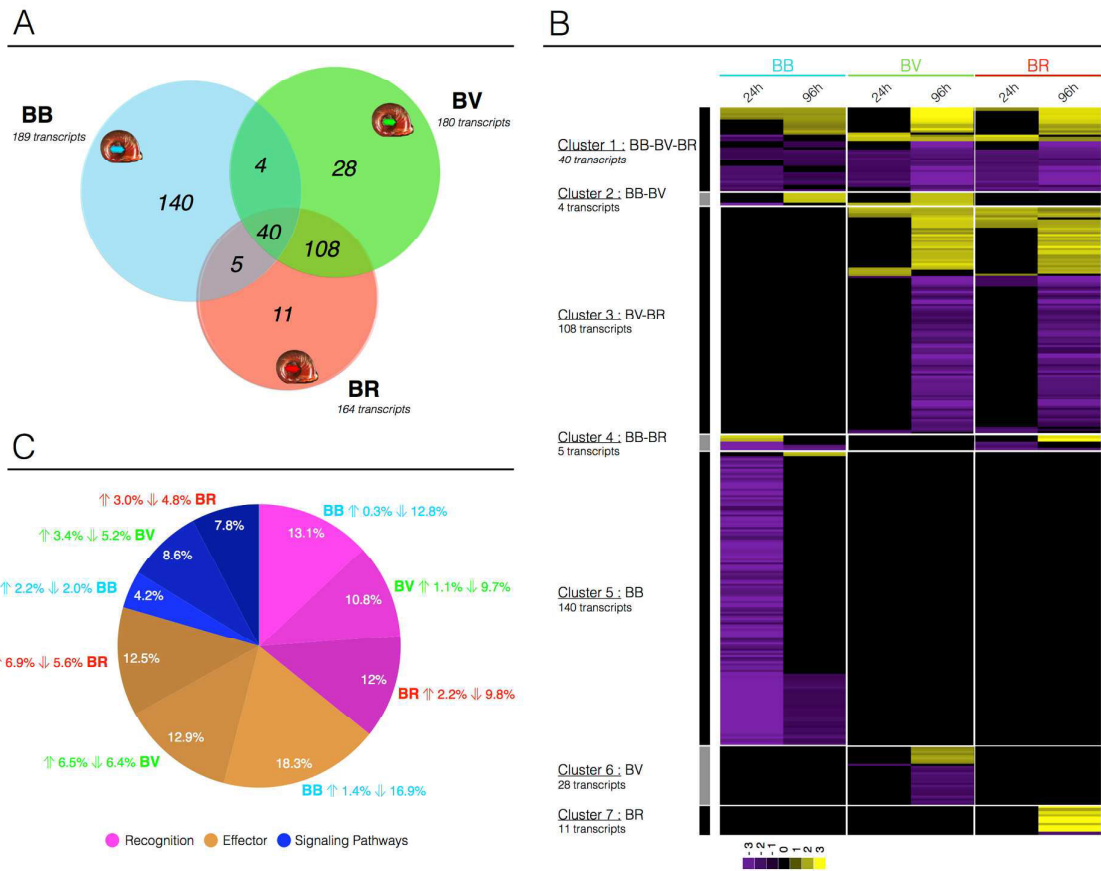
29 128 **Results**

30 129 **A whole-snail transcriptomic approach for investigating the molecular basis of the innate** 31 130 **immune response in the sympatric and allopatric contexts**

32 131 To the best of our knowledge very few studies have investigated the molecular basis of local
33 132 adaptation in host/parasite interactions (Robertson, et al. 2017). Moreover, the previous studies were
34 133 conducted on a limited set of candidate genes using targeted approaches (i.e. quantitative PCR). Here,
35 134 we used a powerful global approach to identify the differentially regulated transcripts in snails of a
36 135 single *B. glabrata* strain under infection by three sympatric or allopatric compatible strains or species
37 136 of the parasite, *Schistosoma* sp. The global transcript representation was analyzed using the previously
38 137 described RNAseq pipeline developed in our laboratory (Dheilly, et al. 2015; Pinaud, et al. 2016;
39 138 Galinier, et al. 2017). The *BgBRE* snail host had the same prevalence and intensity phenotypes when
40 139 exposed to the *SmBRE*, *SmVEN* or *Srod* parasites (see Materials and Methods section). To compare
41 140 the host responses during the sympatric or allopatric interactions, we used the DEseq2 software to
42 141 analyse the response of duplicate *BgBRE* control strains (uninfected *BgBRE1* and *BgBRE2*) to the
43 142 sympatric and allopatric conditions. Of the 159,711 transcripts of the *BgBRE* transcriptome, 1,895
44 143 (1.2%) were differentially represented in all sympatric and allopatric conditions compare to naive
45 144 snails (table 1, supplementary fig. S1). We performed automatic Blast2GO annotation and discard the
46 145 non-annotated transcripts, and retained 1,017 annotated transcripts (53.7% of the differentially
47 146 represented transcripts). Finally, as we were interested in the innate immune response of the snail to
48 147 the sympatric and allopatric parasites, we focused on transcripts known to have immune-related
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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.

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

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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).

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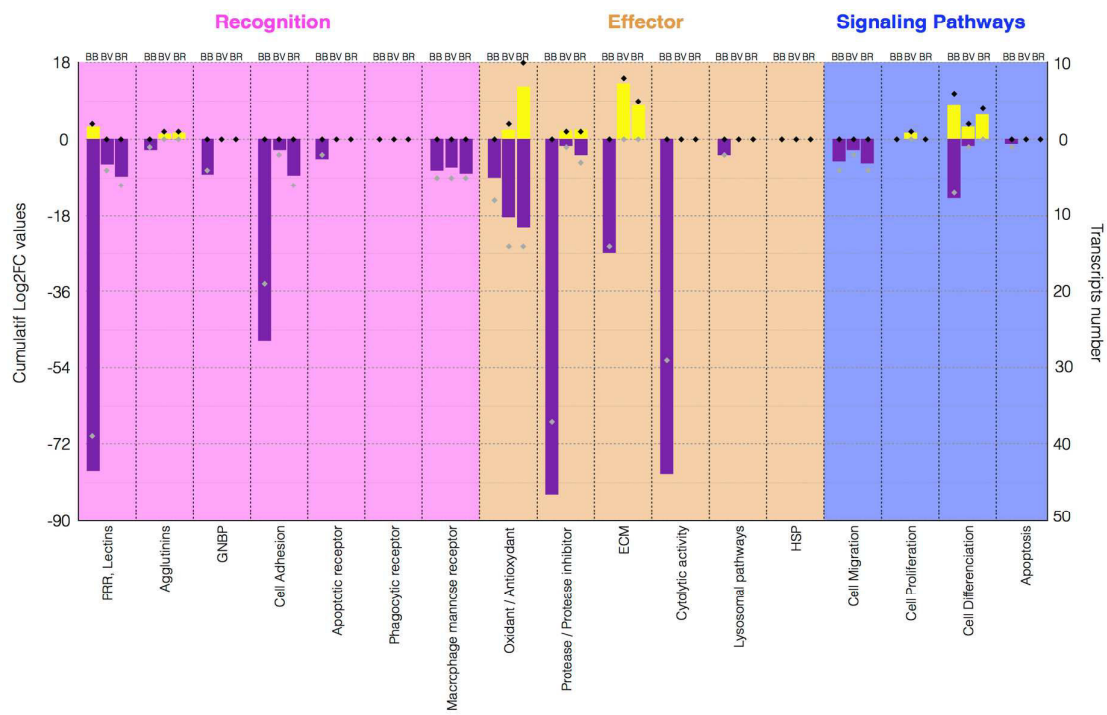
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3 166 Very similar transcript expression patterns were observed for the two allopatric interactions (BV and
4 167 BR). Clusters 6 and 7, comprising only 28 and 11 transcripts, were exclusive to BV and BR,
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6 168 respectively. In BV interaction (fig. 1B, cluster 6), at 96 h after infection 96.5% of the transcripts were
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8 169 expressed: 22% over-represented. In BR interaction (fig. 1B, cluster 7), 100% of the transcripts were
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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
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18 174 between the sympatric and allopatric interactions. While most of the selected transcripts were
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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
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26 178 5), whereas allopatry yielded an activation of the immune response (fig. 1B, cluster 3).
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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
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34 182 differentially expressed immune transcripts into the groups of immune recognition molecules, immune
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36 183 effectors, and immune signaling molecules (fig. 1C), and then subdivided these groups into functional
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38 184 categories (fig. 2). In the BB sympatric interaction, immune recognition molecules, immune effectors
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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
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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
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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
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54 192 interaction (fig. 1C; fig. 2). In fact, different immune transcripts (grey and black diamond in fig. 2)
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56 193 belonging to the same immune functions showed similar differentially regulations following sympatric
57
58 194 and allopatric infections (fig. 2).
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A



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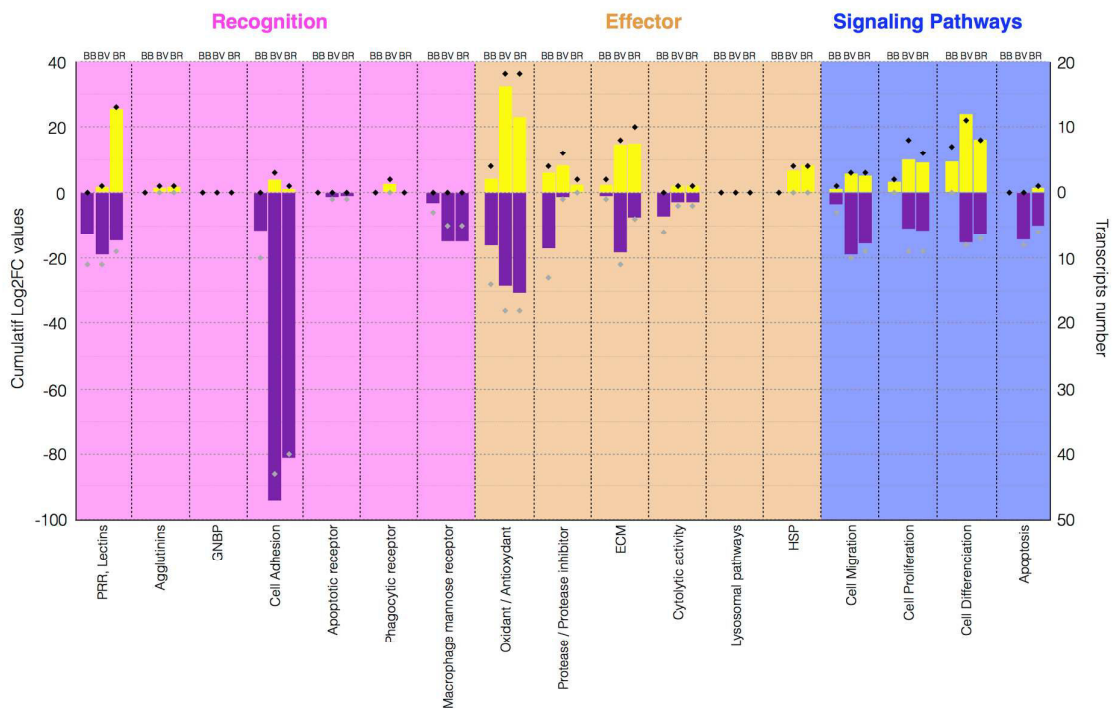


Fig. 2: Differentially represented immune-related transcripts in sympatric and allopatric interactions.

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

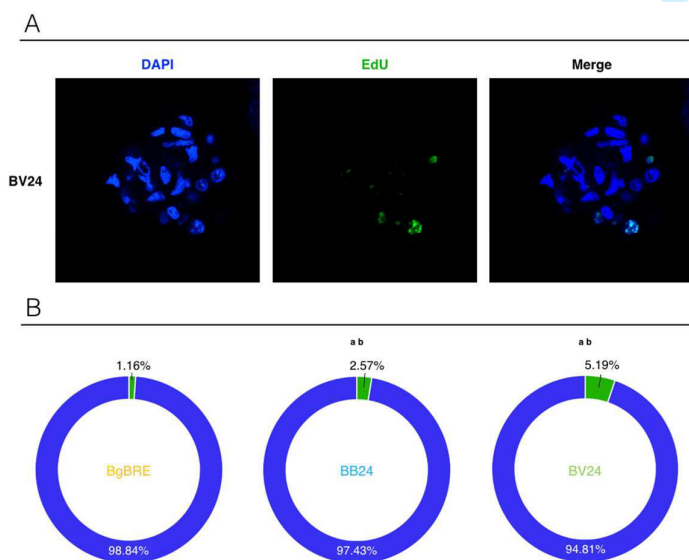
202 migration, cell differentiation and cell proliferation. All of these functions were under-represented in
 203 sympatric interaction (76%), whereas many were over-represented in allopatric ones (39%) (fig. 2).

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.

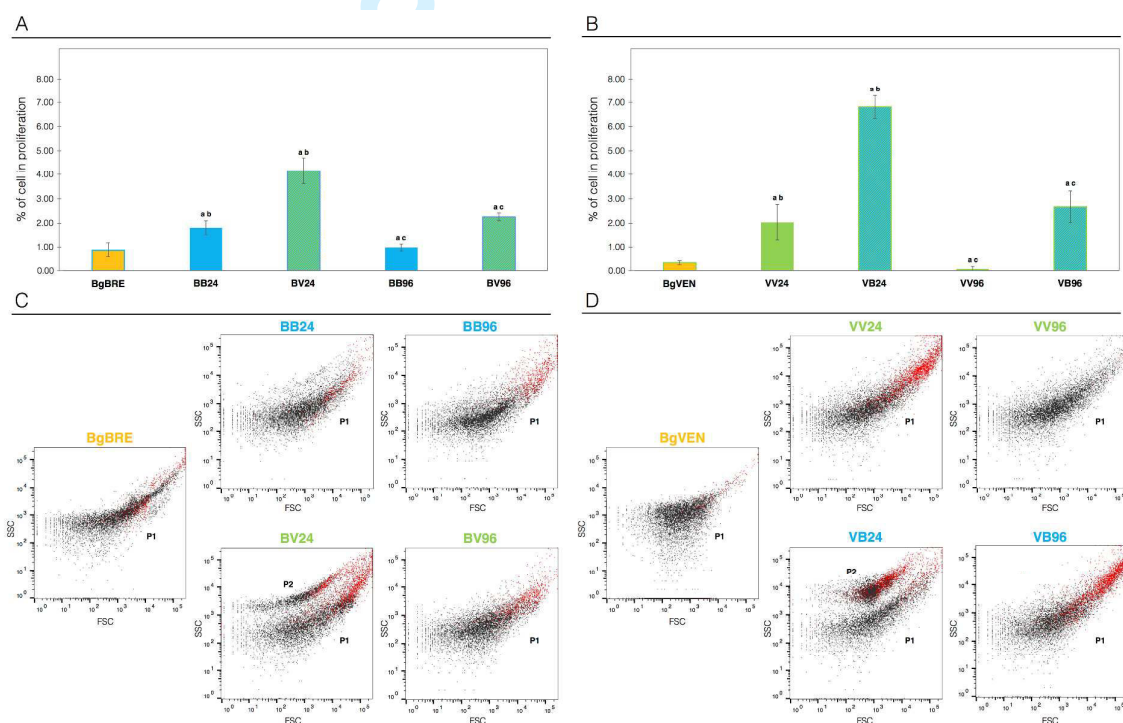


224 Fig. 3: Microscopic investigation of snail hemocyte proliferations

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).

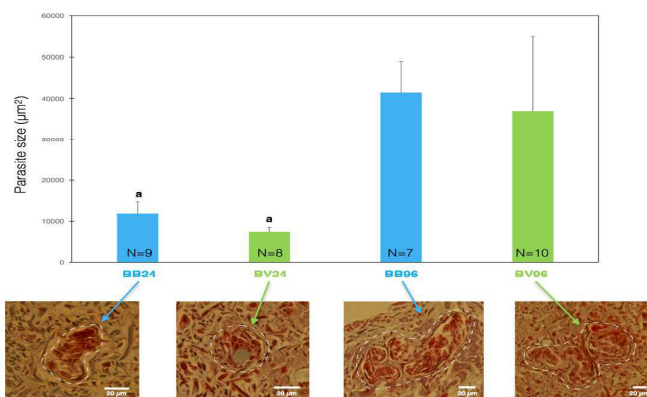
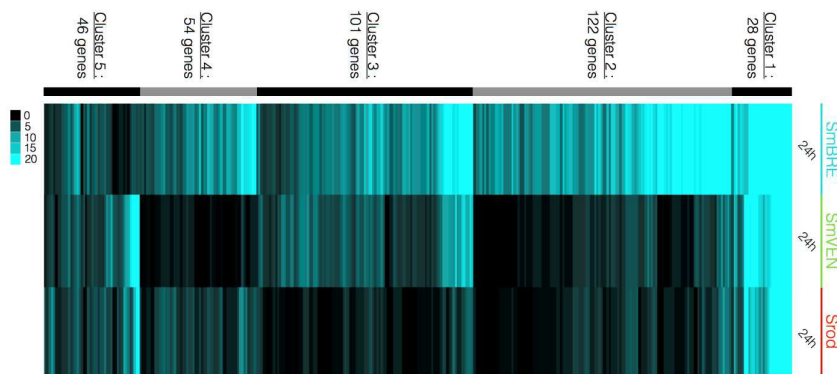


Fig. 5: Development of parasites into snail tissues

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.

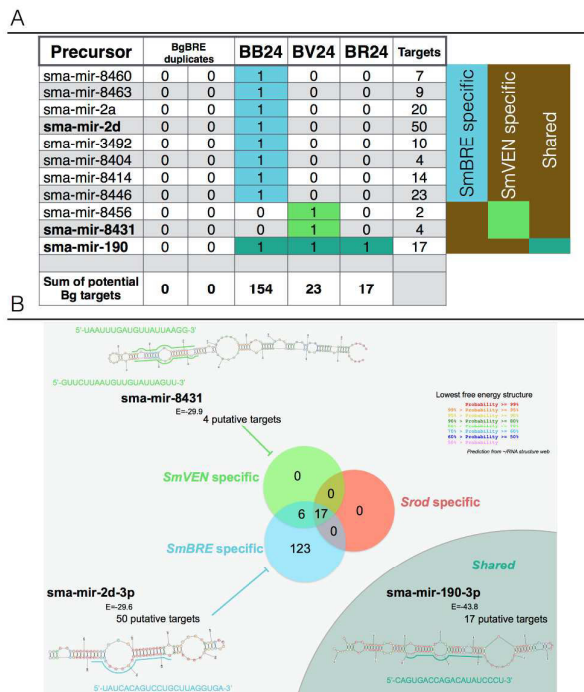
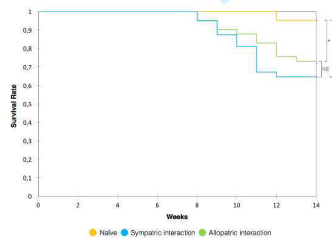


Fig. 7: In-silico identification of parasite miRNAs

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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).

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

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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
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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.

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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.

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

31 435 We next examined the generated dual-RNAseq libraries in an effort to identify whether sympatric
32 436 and/or allopatric Schistosomes could hijack the host immune system. We confronted the dual-RNAseq
33 437 data to the *Schistosoma mansoni* subset of miRBase to identify the presence of microRNAs (miRNAs)
34 438 in our datasets. At 24 h after infection, several parasite miRNAs were identified and differentially
35 439 expressed. As sRNAs and miRNAs are known to regulate numerous biological processes, including
36 440 key immune response genes (Li, et al. 2017; Zawada, et al. 2017), we investigated the role played by
37 441 these miRNAs on host immunobiological response to intruders. We identified some predicted targets
38 442 of such schistosome miRNAs in the *Biomphalaria* immune reference transcriptome. We found that
39 443 schistosomes miRNAs identified in the snails 24 h after sympatric infection may target 43.5% of the
40 444 differentially regulated immune transcripts (fig. 7). In contrast, far fewer correspondences were
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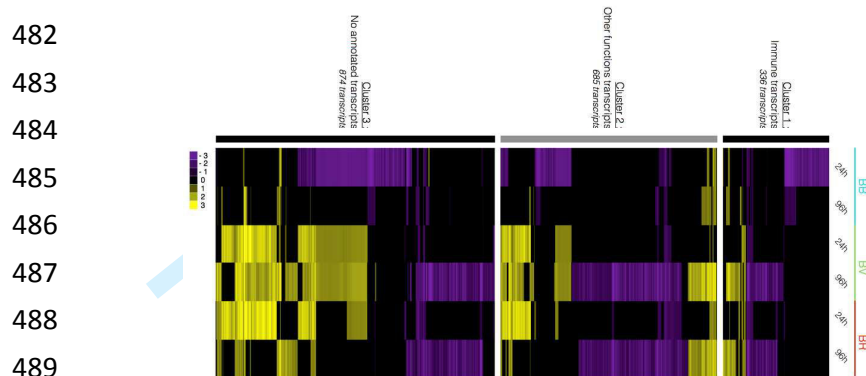
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3 445 identified for the allopatric interaction (fig. 7). Recent work has demonstrated that circulating small
4 446 non-coding RNAs from parasites have hijack roles against host metabolism, especially in the
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6 447 interaction of schistosomes with their vertebrate hosts (Nowacki, et al. 2015; Meninger, et al. 2017;
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8 448 Walzer and Chi 2017). Such non-coding RNAs could act as exogenous miRNAs to interfere with or
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10 449 circumvent the host immune system. These results and our findings show that parasite miRNAs might
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12 450 help subvert the snail's immune defenses by targeting immune weapons or directly targeting genes
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14 451 that regulate innate cellular response.. Furthermore, our observation that more host immune genes
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16 452 appear to be more targeted in the sympatric combination (fig. 7) demonstrates that miRNAs may act as
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18 453 potential support of local adaptation in the transcriptional regulation of a sympatric interaction
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20 454 between host and parasite. This hypothesis deserves further investigation; small RNA (<50pb)
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22 455 sequencing approach is actually on going to confirm the role of non-coding RNAs in host-parasite
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24 456 local adaptation processes.

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

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34 461 Based on our present observations, we propose that sympatric and allopatric interactions trigger totally
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36 462 different responses. In the sympatric interaction, the parasite is able to induce a host
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38 463 immunosuppression within the first day of infection enabling it to quickly infect the host and readily
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40 464 begins to develop. This very efficient strategy reflects a close match between the host and parasite,
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42 465 originating from fine and complex local adaptation processes. In the allopatric interaction, the host's
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44 466 immune system immediately recognizes the parasite and mounts a potent immune response. Although
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46 467 the need to resist the immune system seems to disrupt the activation of the parasite's developmental
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48 468 program during the first day of infection, however the allopatric parasite seems able to circumvent the
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50 469 host immune response and develops in the snail tissues, such that no significant between-interaction
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52 470 difference is seen at 96 h post-infection. Indeed, one major objective of the parasite is to block and/or
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54 471 resist to the innate immune response of the host organism, however, this is not exclusive. Parasites
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56 472 may also hijack more globally the host to their own benefit. Herein we demonstrate that the global
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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.

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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

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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

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3 556 S1). Prevalence of SmBRE and SmVEN for the African vector snail *Biomphalaria pfeifferi* from
4 557 Senegal (BpSEN), and prevalence of the corresponding parasite SmSEN on South American snails
5 558 were also tested (supplementary table S1).
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8 560 ***RNAseq experimental protocol***

9 561 One hundred and twenty BgBRE snails were infected with SmBRE, SmVEN or Srod. Each snail was
10 562 individually exposed for 12 h to 10 miracidia in 5mL of pond water. For each experimental infection,
11 563 20 snails were recovered at 24 and 96 h after infection. The samples were named as follows: BB24,
12 564 BB96 for BgBRE infected with SmBRE; BV24, BV96 for BgBRE infected with SmVEN; and BR24,
13 565 BR96 for BgBRE infected with Srod. Two pools of 30 uninfected BgBRE snails (Bre1 and Bre2) were
14 566 used as controls.

15 567 - *Whole-snail RNA extraction and sequencing*

16 568 Total RNA was extracted using TRIZOL[®] (Sigma Life Science, USA) according to the
17 569 manufacturer's instructions. Sequencing was realized in paired-end 72-bp read lengths, using an
18 570 Illumina Genome Analyzer II (MGX-Montpellier GenomiX, Montpellier, France).

19 571 - *De novo transcriptome assembly*

20 572 *De novo* transcriptome assembly, using all time points, was performed using an in-house pipeline
21 573 created with the Velvet (1.2.02), Oases (v0.2.04), and CD-HIT-EST (v4.5.4) programs. The assembly
22 574 of consensus reference transcriptome was optimized using various parameters, including k-mer length,
23 575 insert length and expected coverage, as previously described (Dheilly, et al. 2015; Pinaud, et al. 2016).
24 576 A *de novo* transcriptome was created and contained 159,711 transcripts.

25 577 - *Differential expression analysis*

26 578 High-quality reads (Phred score >29) were aligned to the *de novo* transcriptome using Bowtie2
27 579 (v2.0.2), which was run locally on a Galaxy server. The DESeq2 (v2.12) software was used to identify
28 580 differential transcript representation between uninfected (Bre1 and Bre2) and infected conditions (p
29 581 value < 0.1) (Pinaud, et al. 2016). A Venn diagram was generated using the Venny 2.1 software to
30 582 highlight which differentially expressed transcripts were specific to a certain interaction versus shared
31 583 between them. A heatmap was then created to analyze transcript expression patterns, using log₂ Fold
32 584 Change with Hierarchical Ascending Clustering (HAC) and Pearson correlation (uncentered) as
33 585 applied by the Cluster (v3.0) and Java TreeView (v1.1.6r4) softwares packages. The differentially
34 586 represented transcripts were functionally classified using a BlastX analysis with the cutoff set to e-
35 587 value < 1e⁻³ (NCBI dataset; thanks to the Roscoff Datacenter Cluster, UPMC) and gene ontology was
36 588 assigned using Blast2GO (v3.0.8) (supplementary table S2).

37 589 ***Innate immune cellular response analysis: microscopy and flow cytometry***

38 590 A total of 105 BgBRE and 90 BgVEN were used. Snails were infected as described above, using
39 591 either SmBRE or SmVEN parasites. For each experimental infection, snails were recovered at 24 and
40 592 96 h after infection, and designated as follows: BB24 and BB96 for BgBRE infected with SmBRE;

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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 naïve 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.

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38 628 ***Histological procedures***

39 629 BgBRE snails (n=8 per group) were infected as described above with either SmBRE or SmVEN
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630 parasites. For each experimental infection time point, at 24 and 96 h after infection, two snails were
631 fixed in Halmi's fixative (4.5% mercuric chloride, 0.5% sodium chloride, 2% trichloroacetic acid,
632 20% formol, 4% acetic acid and 10% picric acid-saturated aqueous solution). Embedding in paraffin
633 and transverse histological sections (3- μ m) were performed at the RHEM platform (Montpellier,
634 France). The slides were stained using Heidenhain's azan trichromatic staining solution as follows: (i)
635 serial re-hydration was performed in toluene followed by 95%, 70%, and 30% ethanol and then
636 distilled water; (ii) coloration was performed using azocarmine G (70% ethanol, 1% aniline, 1% acetic
637 alcohol, distilled water, 5% phosphotungstic acid, distilled water, Heidenhain's azan) and (iii) serial
638 dehydration was performed using 95% ethanol, absolute ethanol, and toluene. The preparations were
639 then mounted with Entellan (Sigma Life Science, St. Louis Missouri, USA) and subjected to
640 microscopic examination. The parasite sizes were measured with the ImageJ software (v2.0.0), and
641 between-group size differences were tested using the Mann-Whitney U-test with statistical
642 significance accepted at a p value < 0.05 based on the mean of the three largest serial cuts of each
643 sporocyst in sympatric or allopatric conditions.

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

646 - *Schistosoma* read subtractions

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

654 - *Gene analysis*

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

663

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

665 *S. mansoni* miRNAs were analyzed *in-silico* by comparing the relevant miRNA database (miRBase) to
666 our RNAseq libraries generated at the 24 h time point. *S. mansoni* precursor sequences were

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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*
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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.
12 674 Putative miRNA targets were predicted from among the differentially represented immune-related
13 675 transcripts (figure 1) using Miranda tools (`miranda input_miR input_Transcriptome -out results.txt -`
14 676 `quiet -sc 140 -en -15`) (Enright, et al. 2003). Because mature miRNAs may exist in two forms
15 677 depending on which strand (5'-3') of the precursor stem-loop is matured the predicted interactions
16 678 could involve the 5' and/or 3' forms, as noted. The results were extracted in line code using the awk
17 679 tool, compiled in supplementary files (supplementary table S4), and used to generate a Venn diagram.
18 680 To confirm the ability of a selected pre-miRNA to produce the stem-loop necessary to produce the
19 681 mature form, the secondary structures of precursor were predicted using RNAstructureWeb tool
20 682 (<http://rna.urmc.rochester.edu/RNAstructureWeb>, 03/09/2017) under default parameters.
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29 684 *Snail survival analysis*

30 685 One hundred and sixty BgBRE snails were infected as described above with SmBRE or SmVEN
31 686 strains (n=50), and 60 non-infected BgBRE snails were retained as controls. The numbers of dead
32 687 snails were compiled weekly for 14 weeks. A Kaplan-Meier estimator was used to estimate the
33 688 survival function from lifetime data. Survival curves were generated using the xlstatsMac software and
34 689 the log-rank test was applied with significance accepted at $p < 0.05$.
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42
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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.

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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.

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

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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.

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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.

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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.

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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

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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	

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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.

Projets secondaires

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

Portet A, Pinaud S, Chaparro C, Charriere G, Allienne JF, Duval D, Galinier R, Gourbal B.
Soumis dans Molecular Biology and Evolution, Juin 2017

➤ **A Multistrain Approach To Studying The Mechanisms Underlying Compatibility In The Interaction Between *Biomphalaria Glabrata* And *Schistosoma Mansoni*.**

Galinier R, Roger E, Moné Y, Duval D, Portet A, Pinaud S, Chaparro C, Grunau C, Genthon C, Dubois E, Rognon A, Arancibia N, Dejean B, Théron A, Gourbal B, Mitta G.
PLoS Neglected Tropical Disease, 2017 doi: 10.1371/journal.pntd.0005398.

➤ **First Characterization Of Viruses From Freshwater Snails Of The Genus *Biomphalaria*, The Intermediate Host Of The Parasite *Schistosoma Mansoni*.**

Galinier R, Tetreau G, Portet A, Pinaud S, Duval D, Gourbal B.
Acta Tropica, 2017 doi: 10.1016/j.actatropica.2016.12.021

Alcool absorption in human must increase *Schistosoma* transmission in snail.

Aliaga B 🍏, Pinaud S 🍏, Portet A 🍏, Rognon A, Duval D.
En preparation, Automne 2017

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

Portet, A., Galinier, R., Pinaud, S., Portela, J., Nowacki, F. Gourbal, B. and Duval, D.
Soumis dans Frontiers Immunology, Septembre 2017

🍏 premiers auteurs ➤ article publié ↳ article soumis

Un polymorphisme de compatibilité est décrit dans l'interaction *Biomphalaria* et *Schistosoma* en fonction des combinaisons de souches et d'espèces utilisées. Les FREP et les SmPoMucs ont été souvent utilisés comme proxy de la co-évolution entre ces deux protagonistes. Une étude intégrative a permis de décrire l'évolution génomique de ces deux potentiels supports du polymorphisme de compatibilité. J'ai particulièrement participé à l'exploration de l'expression transcriptomiques des FREP chez les différentes souches.

RESEARCH ARTICLE

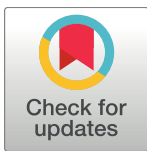
A multistrain approach to studying the mechanisms underlying compatibility in the interaction between *Biomphalaria glabrata* and *Schistosoma mansoni*

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)



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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.

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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

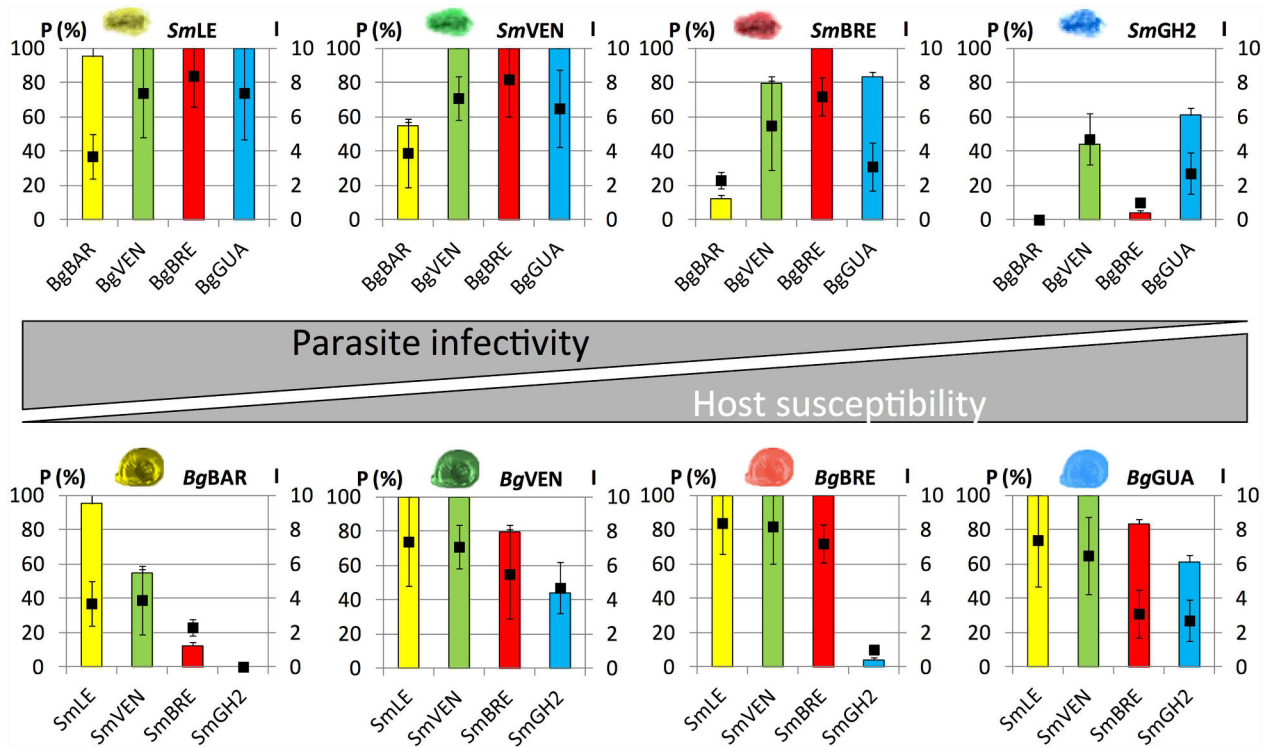


Fig 1. Compatibility trials between different strains of parasites and snails. Each pairwise combination of the studied strains of schistosomes (*SmLE*, *SmVEN*, *SmBRE*, and *SmGH2*) and snails (*BgBAR*, *BgVEN*, *BgBRE*, and *BgGUA*) were tested for compatibility. The upper graphs present the prevalence (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 exceed 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

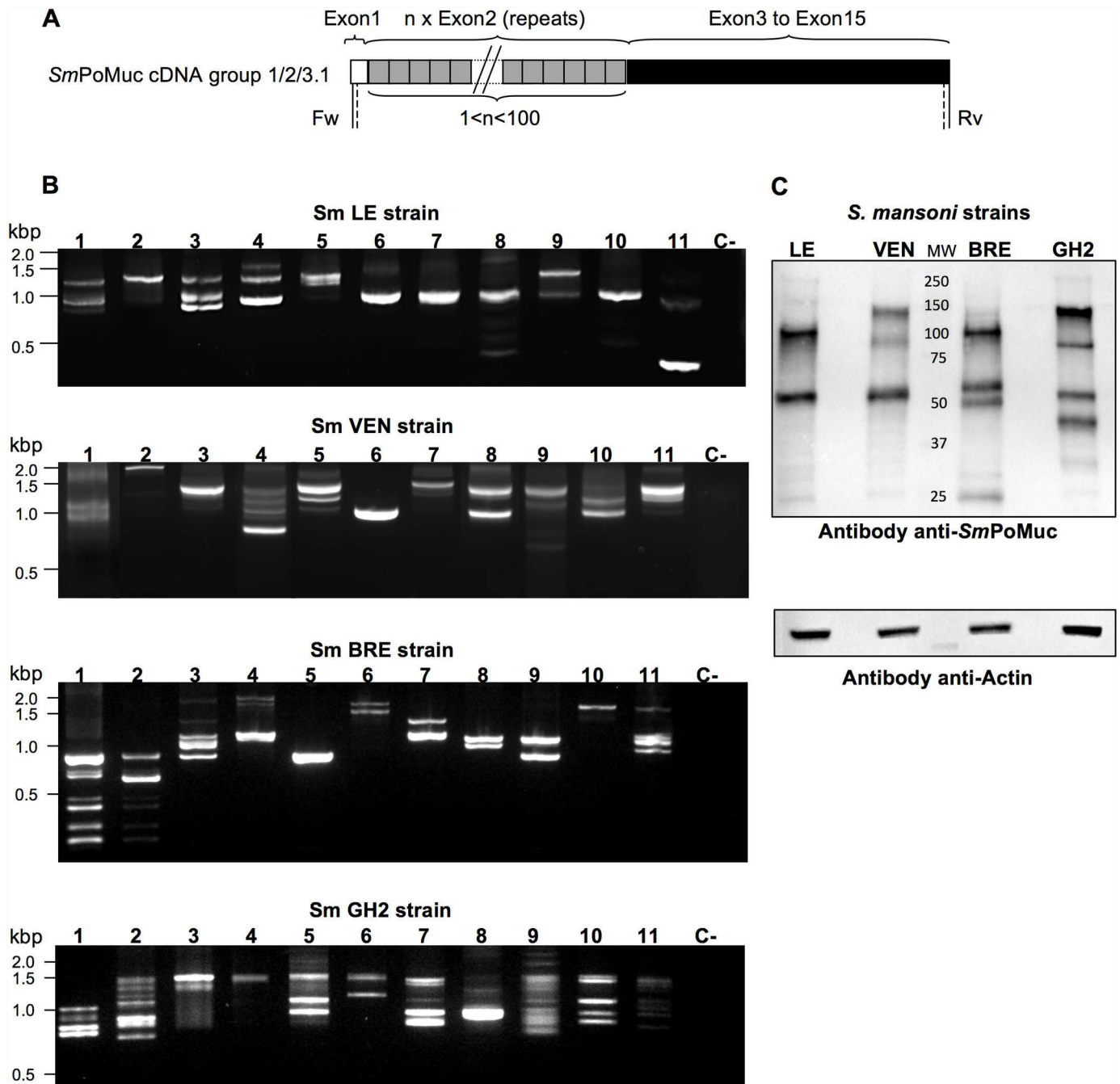


Fig 2. Diverse *SmPoMucs* are expressed by the different strains of *S. mansoni*. (A) *SmPoMuc* cDNA structure. The 5' region is characterized by a variable number of tandem repeats. Two different types of repeats (r1 and r2) were mainly identified in previous studies [19,20]. *SmPoMuc* cDNA structure 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.

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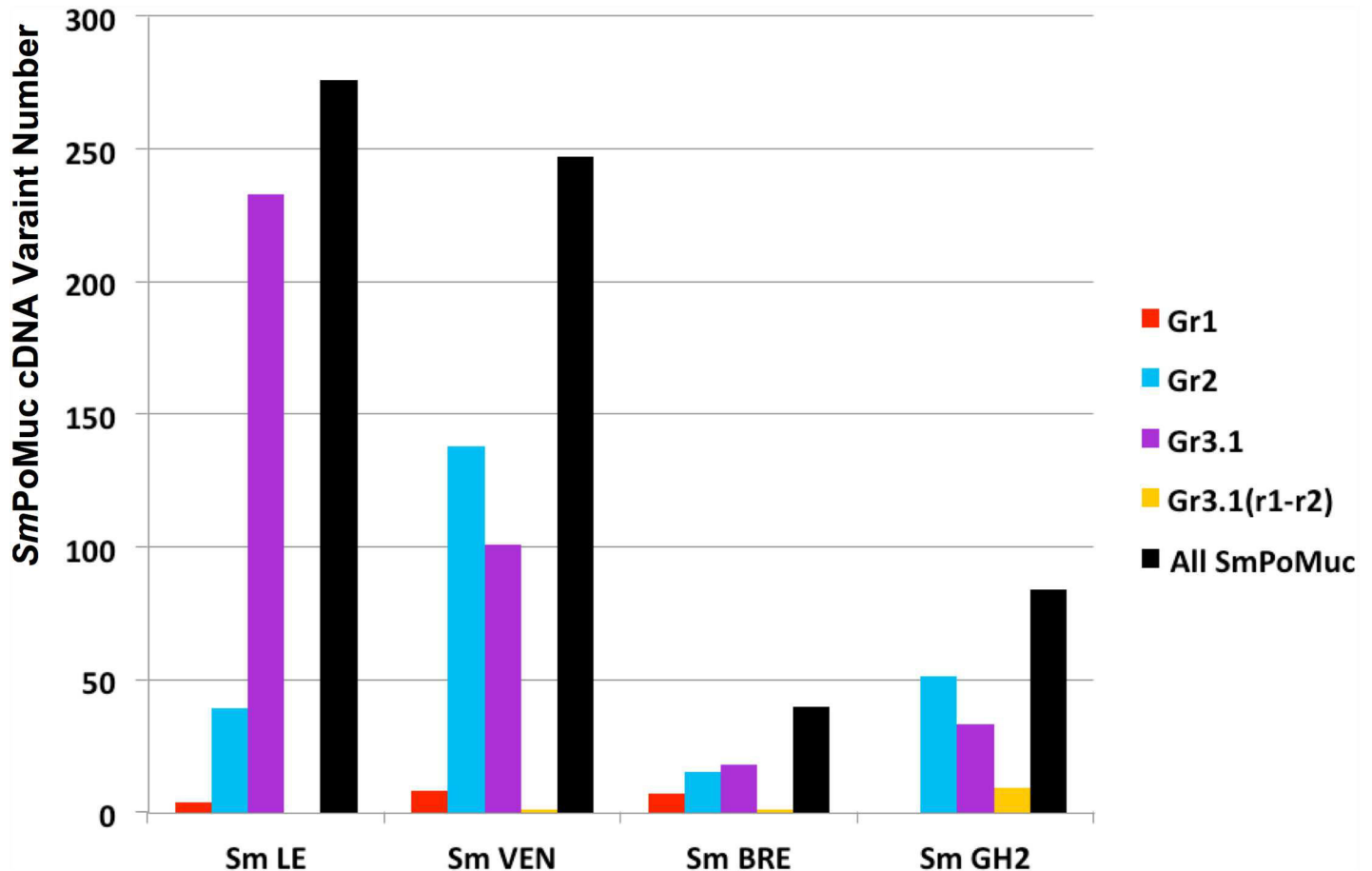


Fig 3. *SmPoMuc* variant numbers in the different *S. mansoni* strains. Nested RT-PCR for *SmPoMuc*s was performed on 11 individual sporocysts for each *S. mansoni* strain (*SmLE*, *SmVEN*, *SmBRE*, and *SmGH2*), and the obtained fragments were cloned and analyzed by Sanger sequencing (see S1 Table). 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

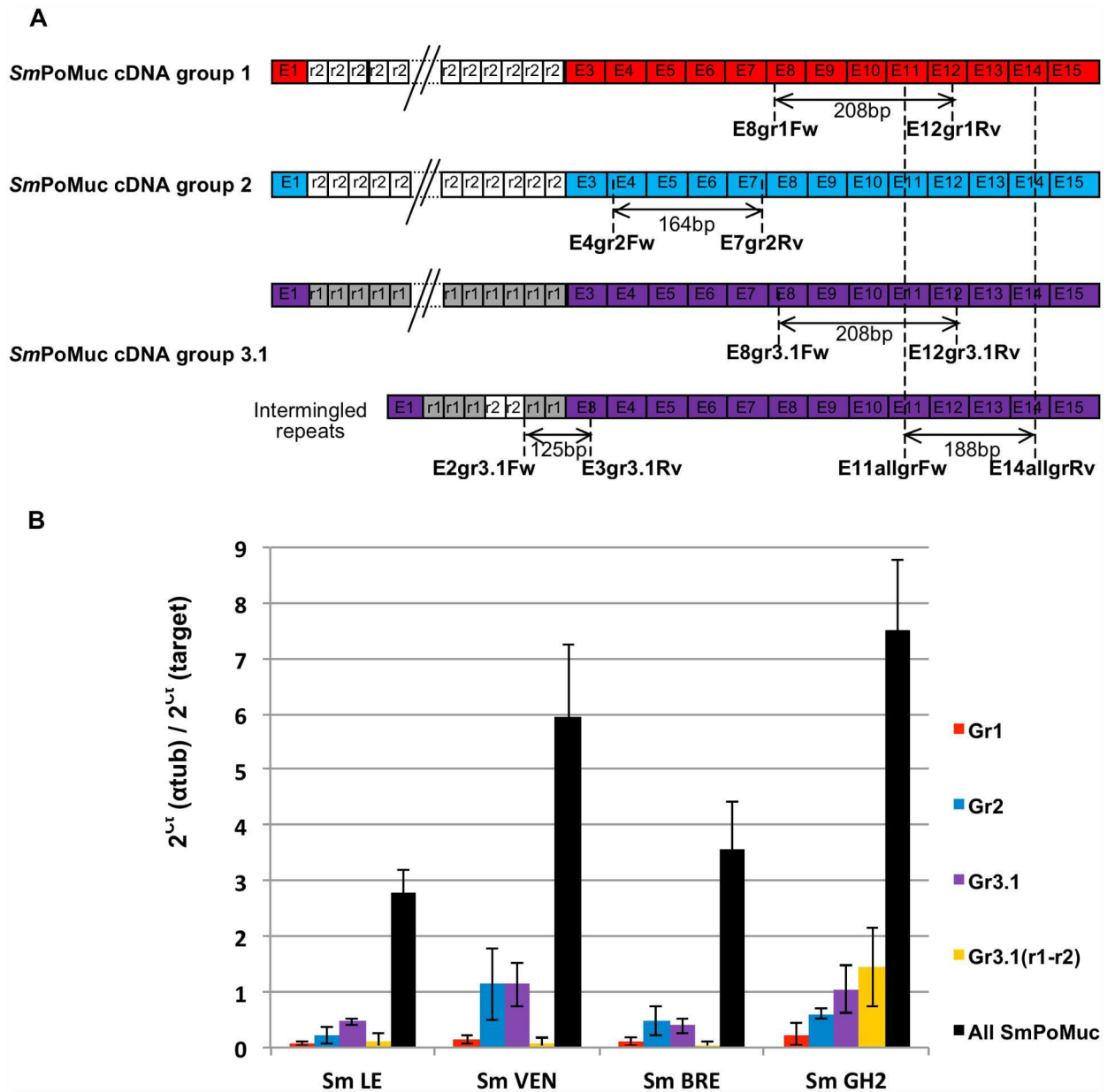


Fig 4. *SmPoMuc* expression between *S. mansoni* strains. (A) Quantitative PCR was performed with primers that targeted all *SmPoMuc* transcripts or discriminated among groups 1, 2, 3.1, and 3.1(r1-r2). The primer positions and amplicon sizes are indicated. (B) RNA was extracted from pooled sporocysts 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.

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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

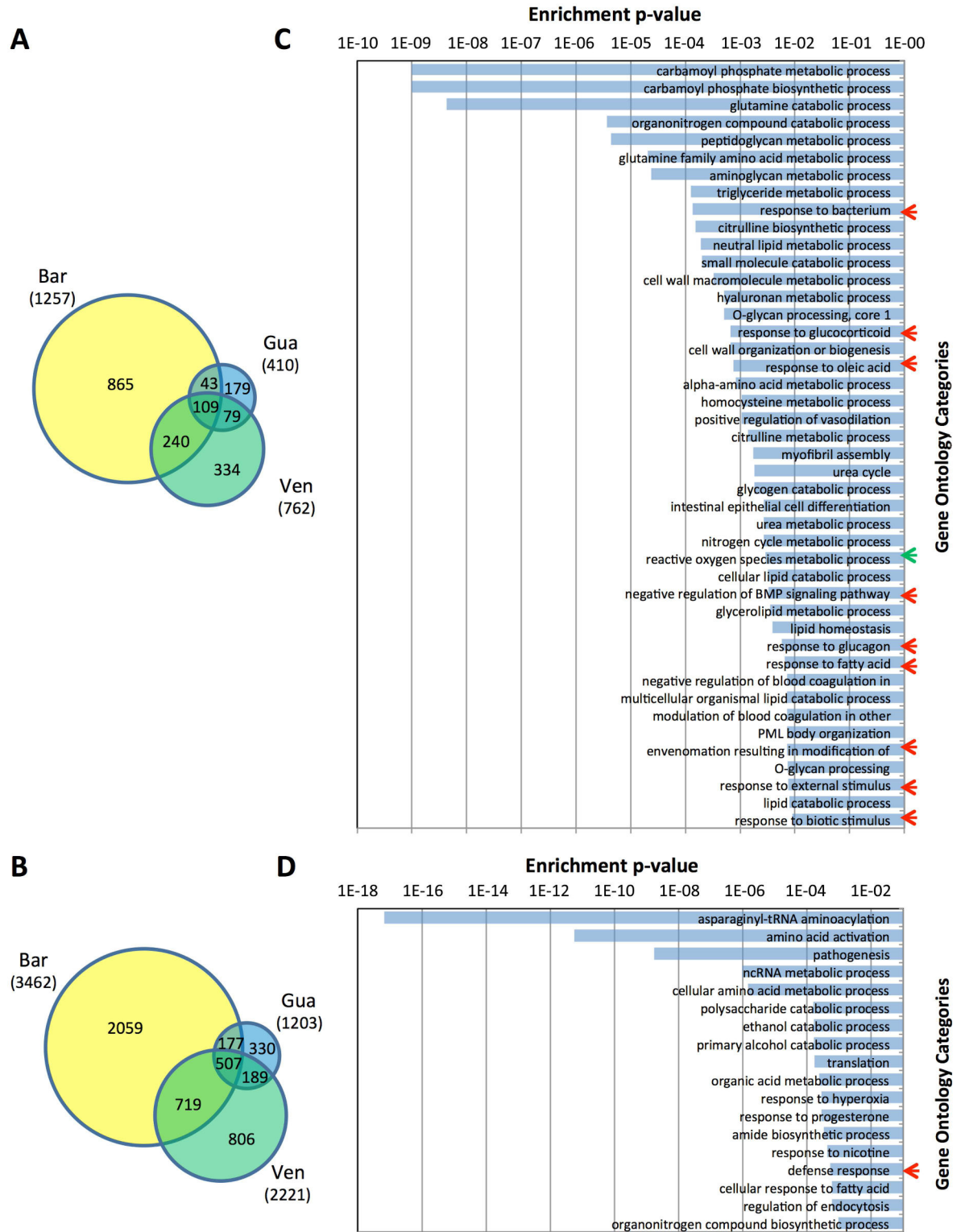


Fig 5. RNA-seq of the different *B. glabrata* strains, and enrichment analysis. Venn diagram presenting the transcripts found to be over-represented (A) and under-represented (B) in the transcriptomes of *BgBAR*, *BgVEN*, and *BgGUA* versus *BgBRE*. Gene Ontology (GO) enrichment analysis of genes that 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.

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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

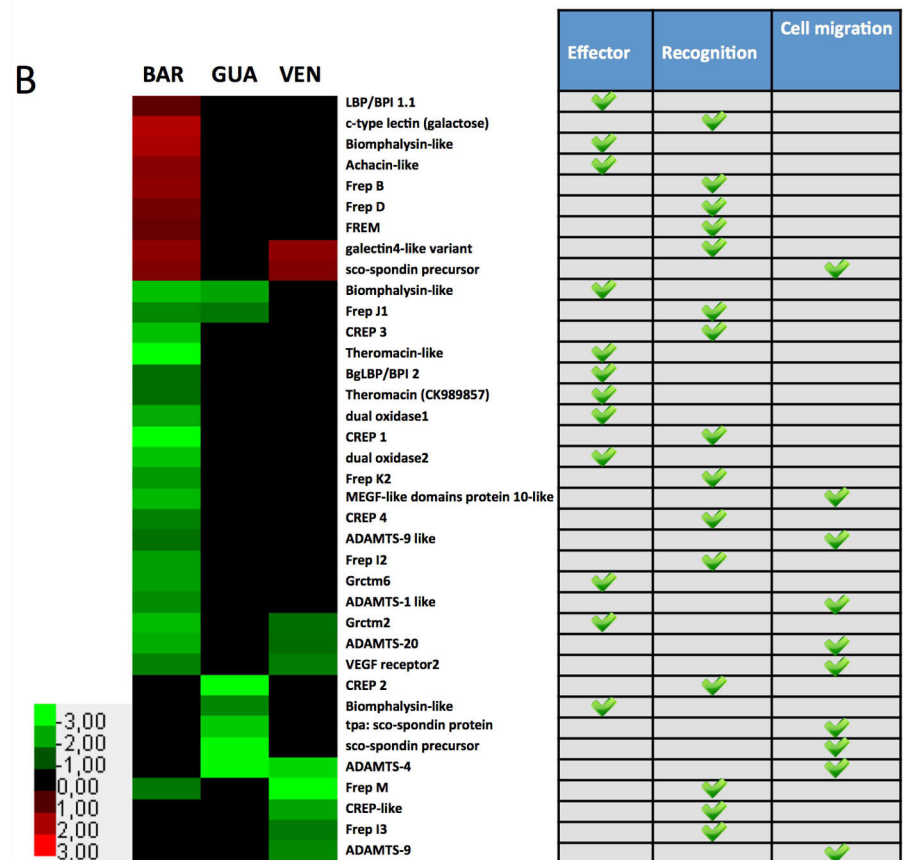
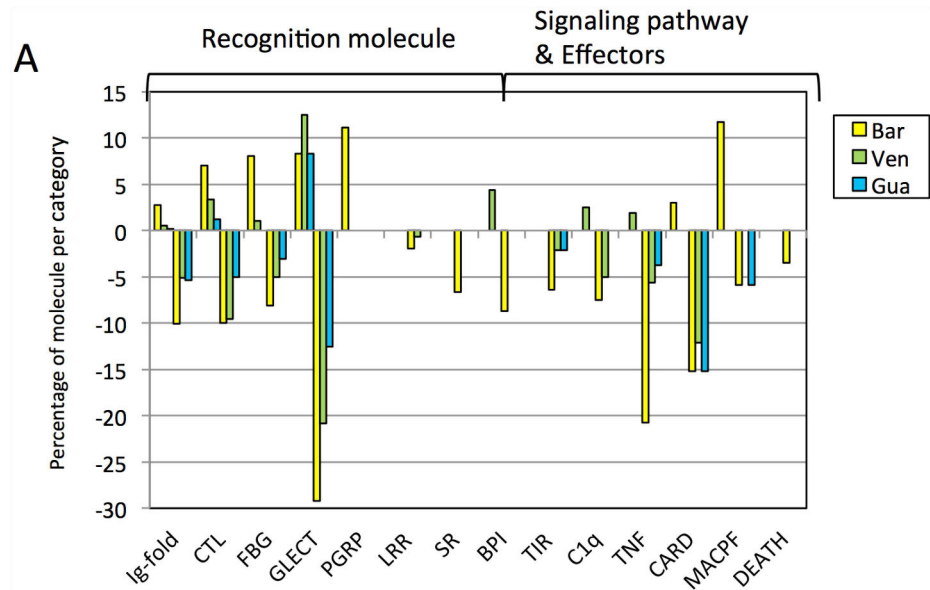


Fig 6. Analysis of immune-relevant genes differentially represented between *BgBAR*, *BgVEN*, *BgGUA* and *BgBRE* reference transcriptome. (A) Histograms showing the proportion of genes bearing immune-relevant protein domains that were differentially represented (DESeq 2 analysis) in *BgBAR* (yellow), *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.

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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

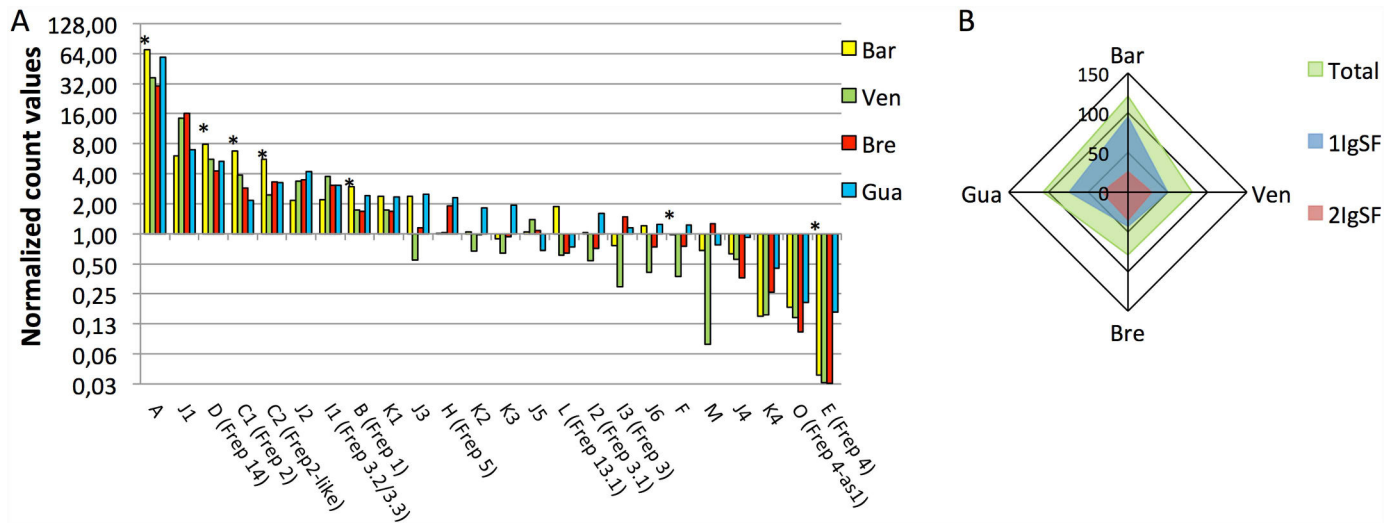


Fig 7. Abundances of transcripts for the different FREP classes in the four *B. glabrata* strains. (A) The histograms represent normalized count values (upper quartile method) obtained when Bowtie 2 was used to map the *BgBAR*, *BgVEN*, *BgBRE*, and *BgGUA* reads with respect to the sequences of the 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.

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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

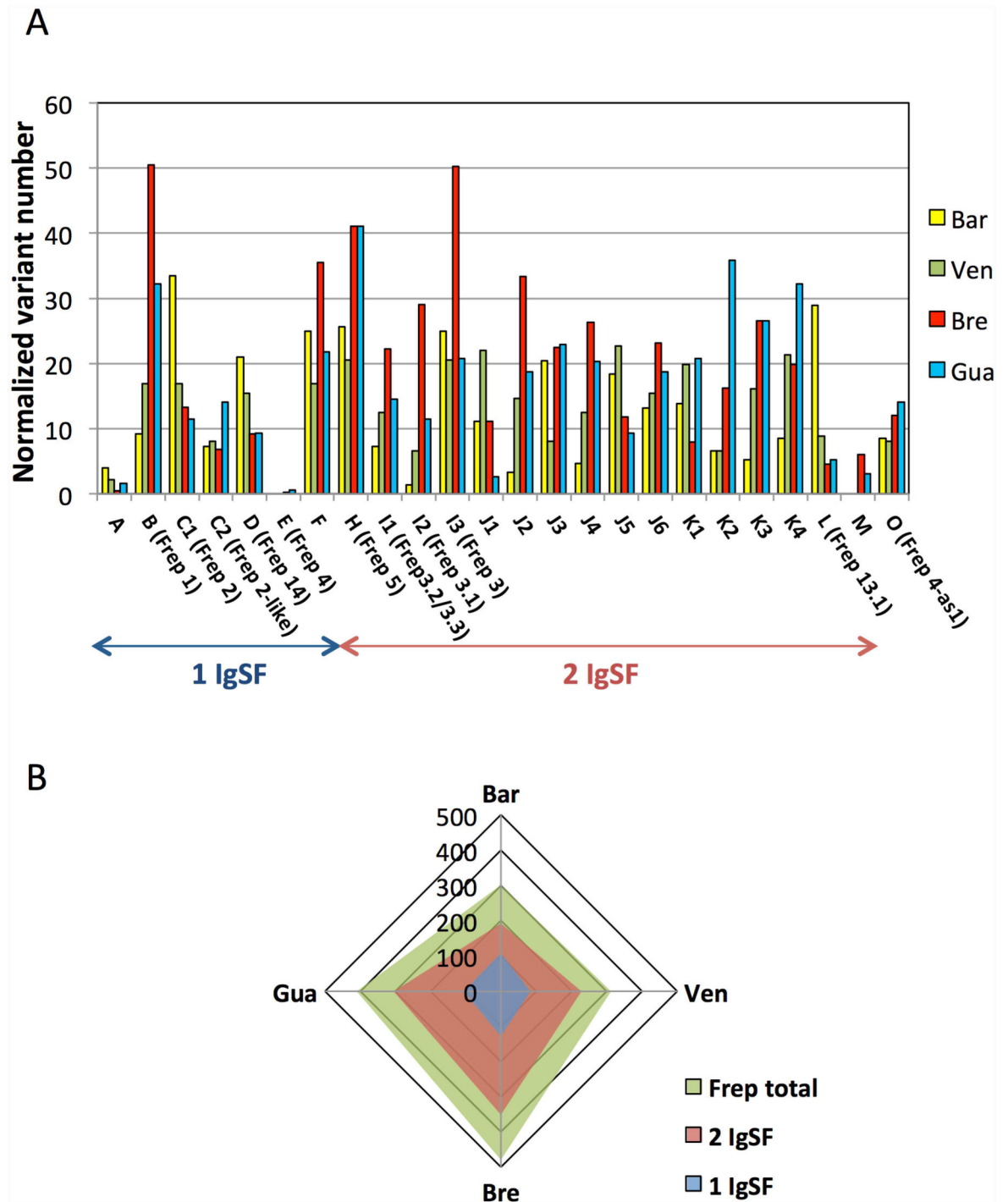


Fig 8. FREP variant polymorphism analysis. Diversity was assessed by aligning the longest transcript from each FREP class to the transcriptomes of *BgBAR*, *BgVEN*, *BgBRE*, and *BgGUA* using Blastn (similarity, at least 95%). (A) Histograms showing the number of variants normalized by the total number 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.

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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)

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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.

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Projets secondaires

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

Portet A, Pinaud S, Chaparro C, Charriere G, Allienne JF, Duval D, Galinier R, Gourbal B.
Soumis dans Molecular Biology and Evolution, Juin 2017

➤ A Multistrain Approach To Studying The Mechanisms Underlying Compatibility In The Interaction Between *Biomphalaria Glabrata* And *Schistosoma Mansoni*.

Galinier R, Roger E, Moné Y, Duval D, Portet A, Pinaud S, Chaparro C, Grunau C, Genthon C, Dubois E, Rognon A, Arancibia N, Dejean B, Théron A, Gourbal B, Mitta G.
PLoS Neglected Tropical Disease, 2017 doi: 10.1371/journal.pntd.0005398.

➤ First Characterization Of Viruses From Freshwater Snails Of The Genus *Biomphalaria*, The Intermediate Host Of The Parasite *Schistosoma Mansoni*.

Galinier R, Tetreau G, Portet A, Pinaud S, Duval D, Gourbal B.
Acta Tropica, 2017 doi: 10.1016/j.actatropica.2016.12.021

Alcool absorption in human must increase *Schistosoma* transmission in snail.

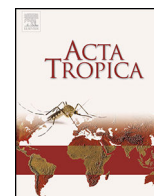
Aliaga B 🍏, Pinaud S 🍏, Portet A 🍏, Rognon A, Duval D.
En preparation, Automne 2017

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

Portet, A., Galinier, R., Pinaud, S., Portela, J., Nowacki, F. Gourbal, B. and Duval, D.
Soumis dans Frontiers Immunology, Septembre 2017

🍏 premiers auteurs ➤ article publié ↳ article soumis

Aucun virus à ARN n'avait été décrit jusqu'à présent chez *Biomphalaria*. Chose faite avec cette première description d'une partie négligée des communautés associées au mollusque (microbiote). Mon expérience dans l'analyse transcriptomique de gènes d'intérêts m'a permis d'aider Richard Galinier pour explorer l'abondance des différents virus chez différentes souches grâce à l'étude de transcriptomes disponibles au laboratoire.



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

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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.

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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

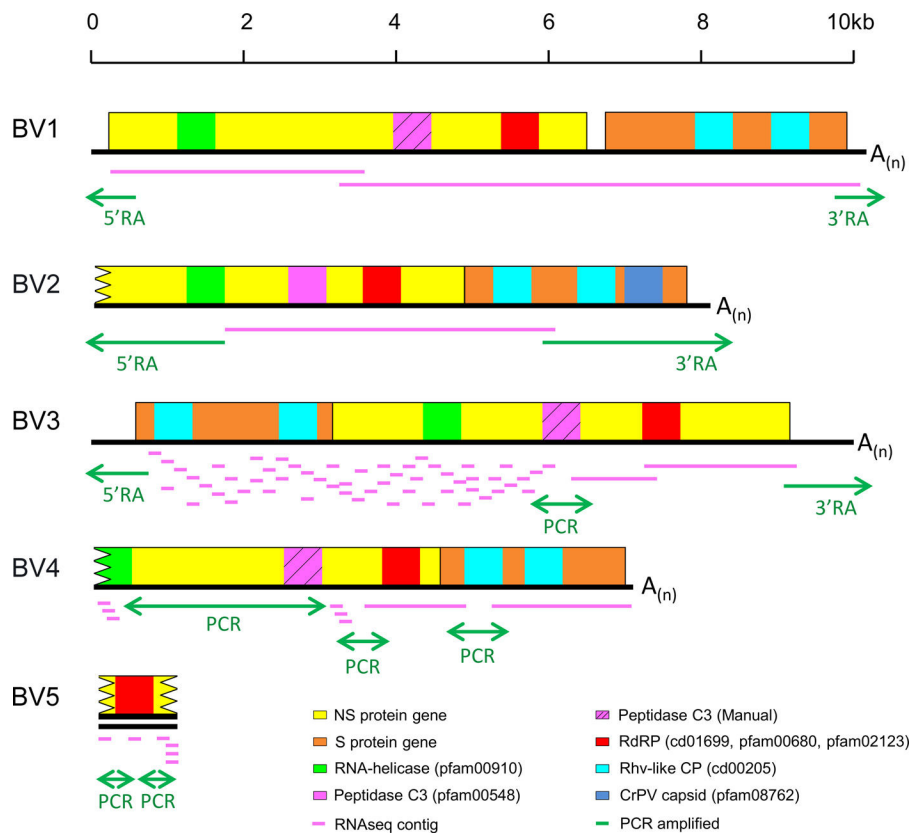


Fig. 1. Genome structures of *Biomphalaria* RNA-viruses. Black lanes represent the RNA strands (one for BV1, BV2, BV3 and BV4 or two for BV5). Yellow and orange boxes correspond to the region coding for non-structural proteins and structural proteins, respectively. Colored squares in these boxes relate 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. Rearing 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-

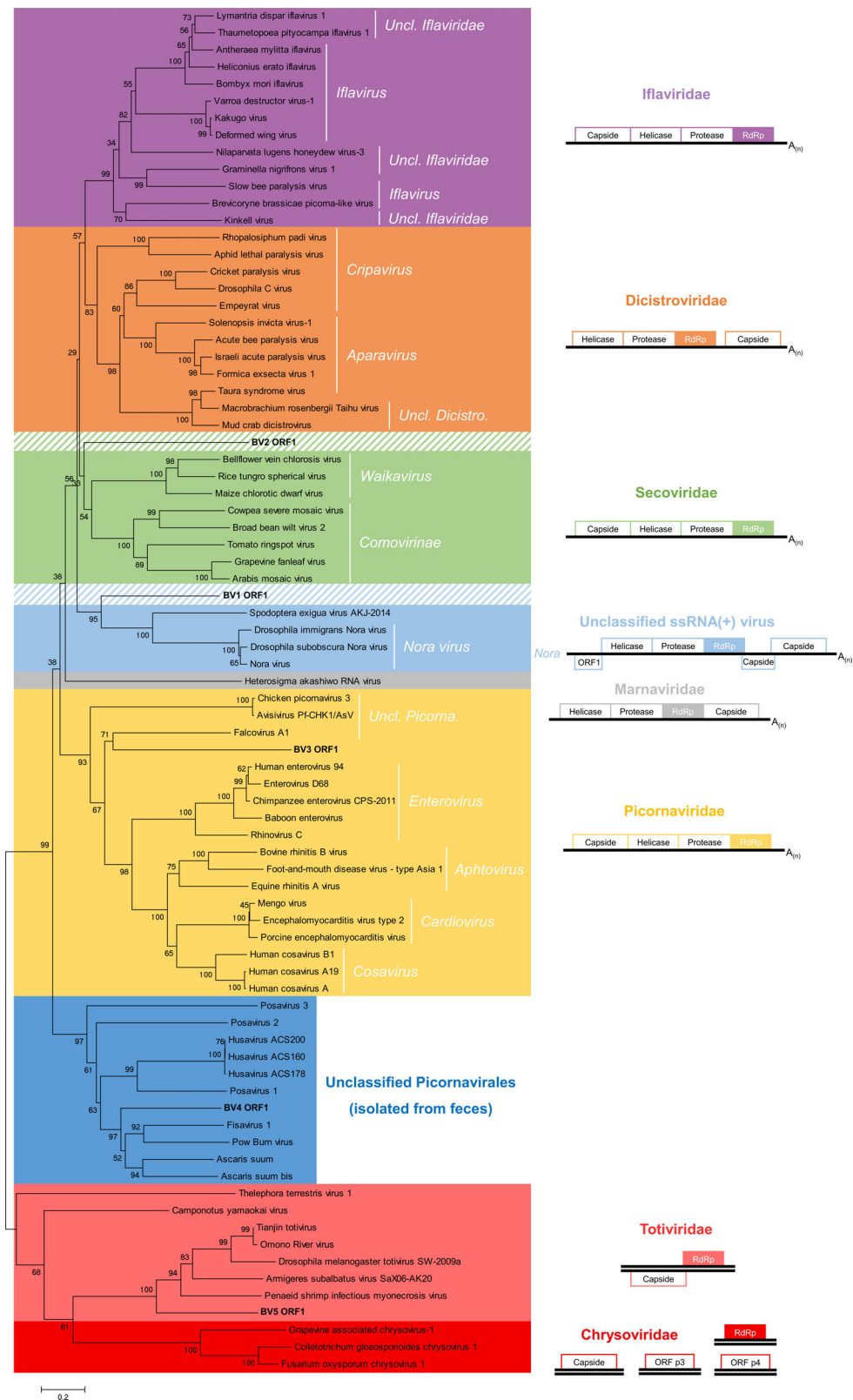


Fig. 2. Phylogenetic analysis of the RdRp protein sequence domains from 81 different viruses belonging to the *Iflaviridae* (13; purple), *Dicistroviridae* (12; orange), *Secoviridae* (8; green), Unclassified ssRNA(+) virus (4; light blue), *Marnaviridae* (1; grey), *Picornaviridae* (17; yellow), Unclassified *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.

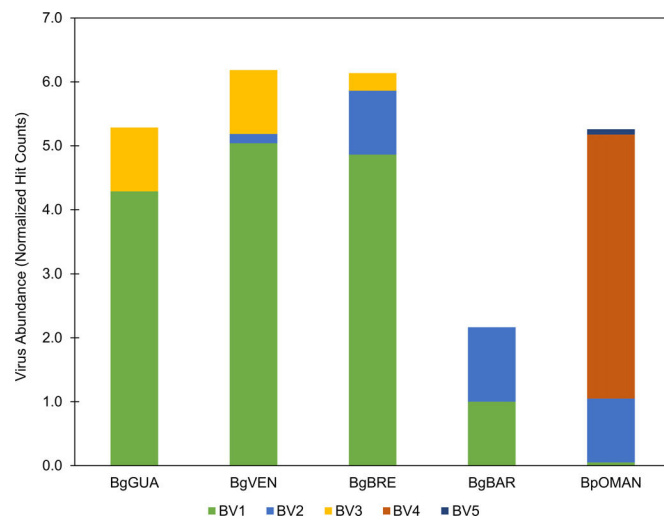


Fig. 3. Relative abundance of virus into *Biomphalaria* strains. Cumulative expression (Hit count normalized by the upper-quartile) of the *Biomphalaria* virus transcripts, in the different *Biomphalaria* strains, determined by Bowtie2 analysis. Each histogram corresponds to a *Biomphalaria* strain (BgGUA, 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.

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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>.

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Projets secondaires

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

Portet A, [Pinaud S](#), Chaparro C, Charriere G, Allienne JF, Duval D, Galinier R, Gourbal B.
Soumis dans Molecular Biology and Evolution, Juin 2017

➤ A Multistrain Approach To Studying The Mechanisms Underlying Compatibility In The Interaction Between *Biomphalaria Glabrata* And *Schistosoma Mansoni*.

Galinier R, Roger E, Moné Y, Duval D, Portet A, [Pinaud S](#), Chaparro C, Grunau C, Genthon C, Dubois E, Rognon A, Arancibia N, Dejean B, Théron A, Gourbal B, Mitta G.
PLoS Neglected Tropical Disease, 2017 doi: 10.1371/journal.pntd.0005398.

➤ First Characterization Of Viruses From Freshwater Snails Of The Genus *Biomphalaria*, The Intermediate Host Of The Parasite *Schistosoma Mansoni*.

Galinier R, Tetreau G, Portet A, [Pinaud S](#), Duval D, Gourbal B.
Acta Tropica, 2017 doi: 10.1016/j.actatropica.2016.12.021

Alcool absorption in human must increase *Schistosoma* transmission in snail.

Aliaga B , Pinaud S , Portet A , Rognon A, Duval D.
En preparation, Automne 2017

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

Portet, A., Galinier, R., Pinaud, S., Portela, J., Nowacki, F. Gourbal, B. and Duval, D.
Soumis dans Frontiers Immunology, Septembre 2017

 premiers auteurs ➤  article publié ↳  article soumis

La thioester-containing protein est souvent décrite comme une opsonine chez de nombreux organisme. Leurs rôles dans l'immunité des invertébrés sont souvent très différents en fonctions des espèces. Ici nous proposons la première caractérisation chez *Biomphalaria* : BgTEP. C'est majoritairement pour mon expérience dans l'infestation de mollusques *Biomphalaria* que j'ai participé à ce projet, dans le but d'explorer la réponse spécifique de cette BgTEP en fonction de différents pathogènes : gram +, gram -, levure ou schistosomes. Les animaux exposés ont vu leurs ARN extraits et reverse-transcripts avant une analyse en q-rtPCR.

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

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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 (FREP) 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.

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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

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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.**

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

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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

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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
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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

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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 A₂M
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 10^8 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 μ L volume comprising 2 μ L of cDNA diluted to 1:200 with ultrapure-water, 5 μ L of No
317 Rox SYBR Master Mix blue dTTP (Takyon), 1 μ L of ultrapure-water and 10 μ M 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 Δ Ct 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- μ m) 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

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705

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932
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 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 Presence of BgTEP in *B. glabrata* plasma was assessed by western blot using the monoclonal
969 anti-BgTEP-PEP antibody. Eight microliters of *B. glabrata* ultracentrifugated plasma were
970 run on each two wells of a 12 % SDS-PAGE gel before protein transfer onto a PVDF
971 membrane. Membrane was then divided into two parts for subsequent revelation steps.
972 Western blot using the monoclonal anti-BgTEP antibody as primary antibody (Ab) revealed
973 the presence different forms of BgTEP in the plasma, while western blot on the second part of
974 the membrane using the anti-BgTEP antibody pre-incubated with BgTEP peptide (Ab+pep)
975 showed the Ab specificity since no band was detected. Arrows correspond to putative full-
976 length and two processed forms of the protein present in naive hemolymph.

977

978 Figure 5: Immune-Inducible Transcription of BgTEP

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

985

986 Figure 6: Immunoblotting analysis of BgTEP

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

997 *profile. In this figure, the different images come from two different original gels; naïve*
998 *hemolymph and E.coli interactions come from a same first gel, whereas images of*
999 *interactions with M. luteus and S. cerevisiae come from a same second gel. Brightness and*
1000 *contrast balance was applied equally for each pixel of the entire image. B. Comparison of*
1001 *BgTEP binding and time-dependent processing after 30 minutes and 3 hours of interaction*
1002 *between snail cell-free hemolymph and S. mansoni at two development-stages (miracidia and*
1003 *primary sporocyst).*

1004

1005 Figure 7: Cellular Expression of BgTEP

1006 *A. Immunolocalization of BgTEP in hemocyte populations by confocal microscopy. Three cell*
1007 *types are represented in Biomphalaria glabrata hemolymph; the hyalinocytes with cytoplasmic*
1008 *projections, the granulocytes with granules into the cytoplasm and the blast-like cells, the*
1009 *smallest cells determined with May-Grünwald Giemsa coloration (Supp. Figure 2), with a*
1010 *proportion expressing the BgTEP. The green labelling corresponds to the actin, the blue is*
1011 *cell nucleus and the red is the BgTEP. B. Flow cytometry profile of BgTEP-positive*
1012 *hemocytes in hemolymph. A total number of hemocytes of n=13,785 were recovered.*
1013 *Comparison of hemocytes labelled with anti-BgTEP antibody and hemocytes labelled only*
1014 *with secondary anti-body (fluorescent antibody) as negative control. The total hemocytes are*
1015 *shown according to FSC (forward-scattered light, representing cell size) and SSC (side-*
1016 *scattered light, representing cell granularity). The red dots correspond to BgTEP-positive*
1017 *hemocytes. C. Flow cytometry quantification of BgTEP-positive hemocytes in hemolymph.*
1018 *Comparison of the hemocyte fluorescence between the hemocytes labelled with anti-BgTEP*
1019 *antibody (red) and hemocytes labelled only with secondary anti-body (fluorescent antibody)*
1020 *as negative control (bleu). A fluorescent cut-off was determined and 20.7% of hemocytes are*
1021 *BgTEP-positive.*

1022

1023 Figure 8: Opsonin Role of BgTEP

1024 *A. Co-immunolocalisation of phagocytic and BgTEP-positive hemocytes by confocal*
1025 *microscopy. Labelling has been performed on a hemolymph fraction. On picture 1, green*
1026 *fluorescent-yeast phagocytosis by hemocytes is shown, for that the hemocyte nucleus are*
1027 *labelled in blue (dapi) and the actin is labelled in red. On picture 2, green fluorescent-yeasts*
1028 *are phagocytosed, hemocyte nucleus are labelled in blue and the BgTEP is labelled in red*
1029 *with an anti-BgTEP antibody coupled with red fluorescent-secondary-antibody. B. Histologic*
1030 *slide of a S. mansoni parasite encapsulation by hemocytes 24h post-infection. White arrows*
1031 *show the BgTEP-positive cells around and in the capsule and also in the head-foot. The*
1032 *BgTEP is labelled by anti-BgTEP antibody coupled with red fluorescent-secondary-antibody,*
1033 *on histological slide.*

1034

1035 *Supplementary Figure 1:*

1036 *Web-logo representation of the consensus sequence of intron-exon junction in BgTEP gene.*

1037

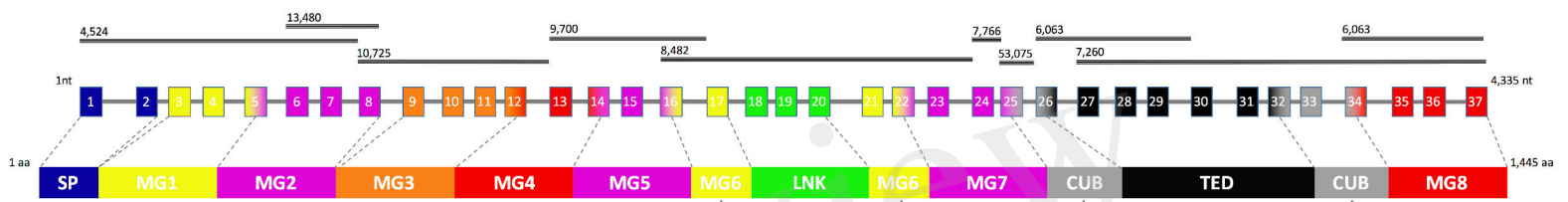
1038 *Supplementary Figure 2:*

1039 *May-Grünwald Giemsa staining of B. glabrata hemocytes revealing the three hemocyte sub-*
1040 *types; hyalinocytes, granulocytes and blast-like cells.*

1041

Figure 1.TIFF

A



B

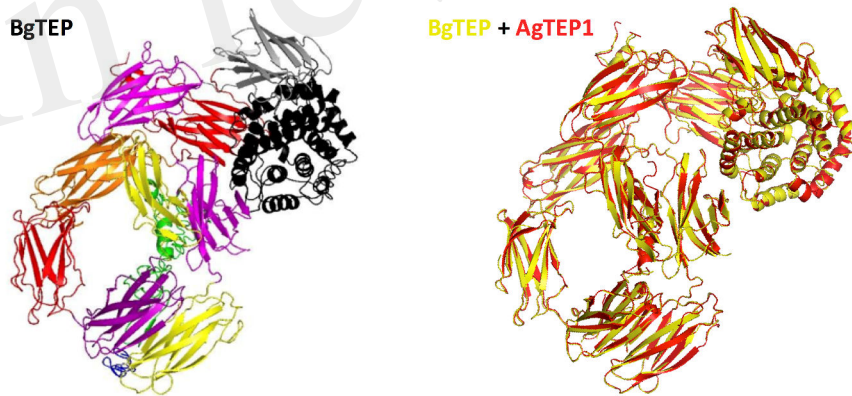


Figure 2.TIFF

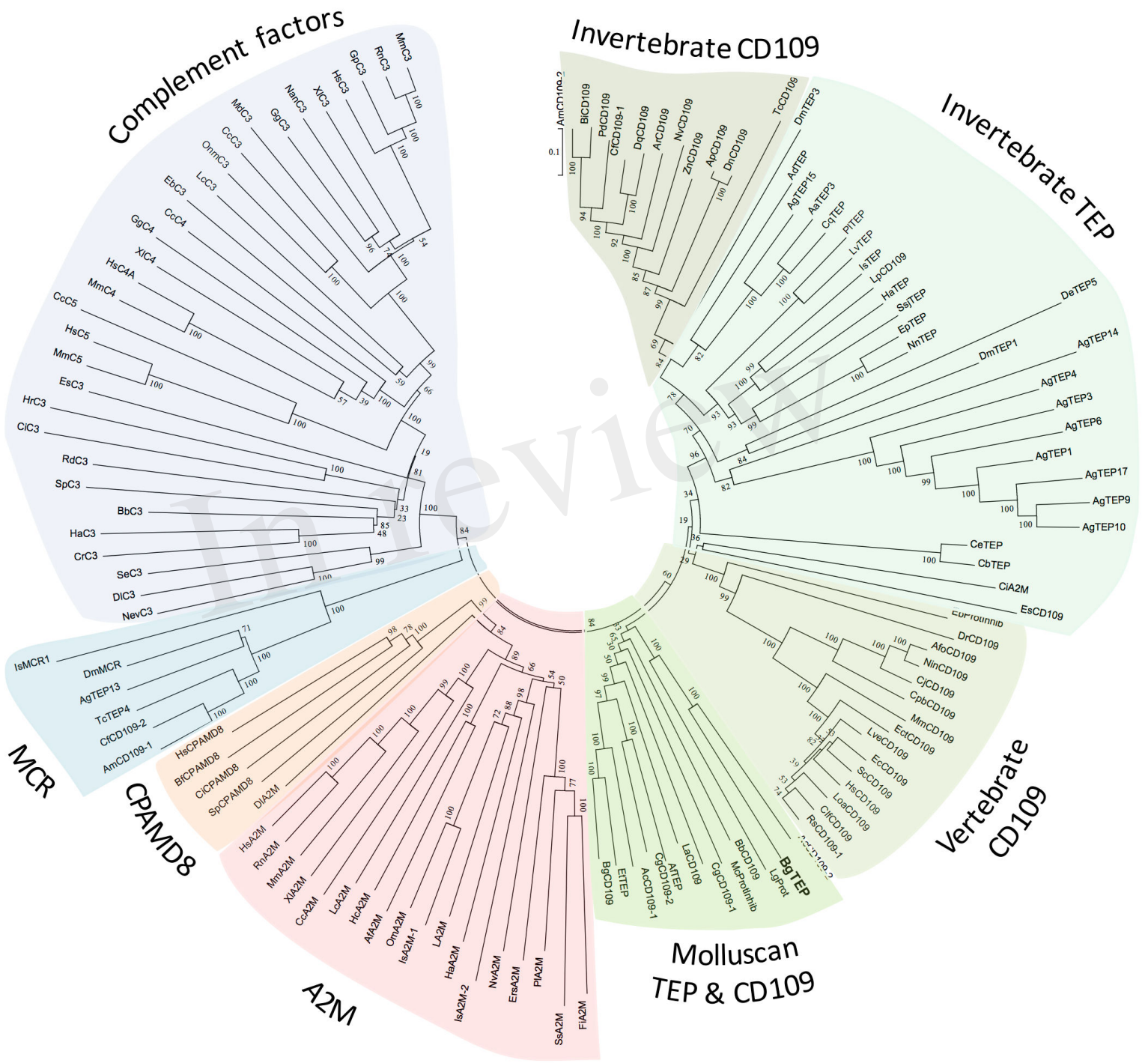


Figure 3.TIFF

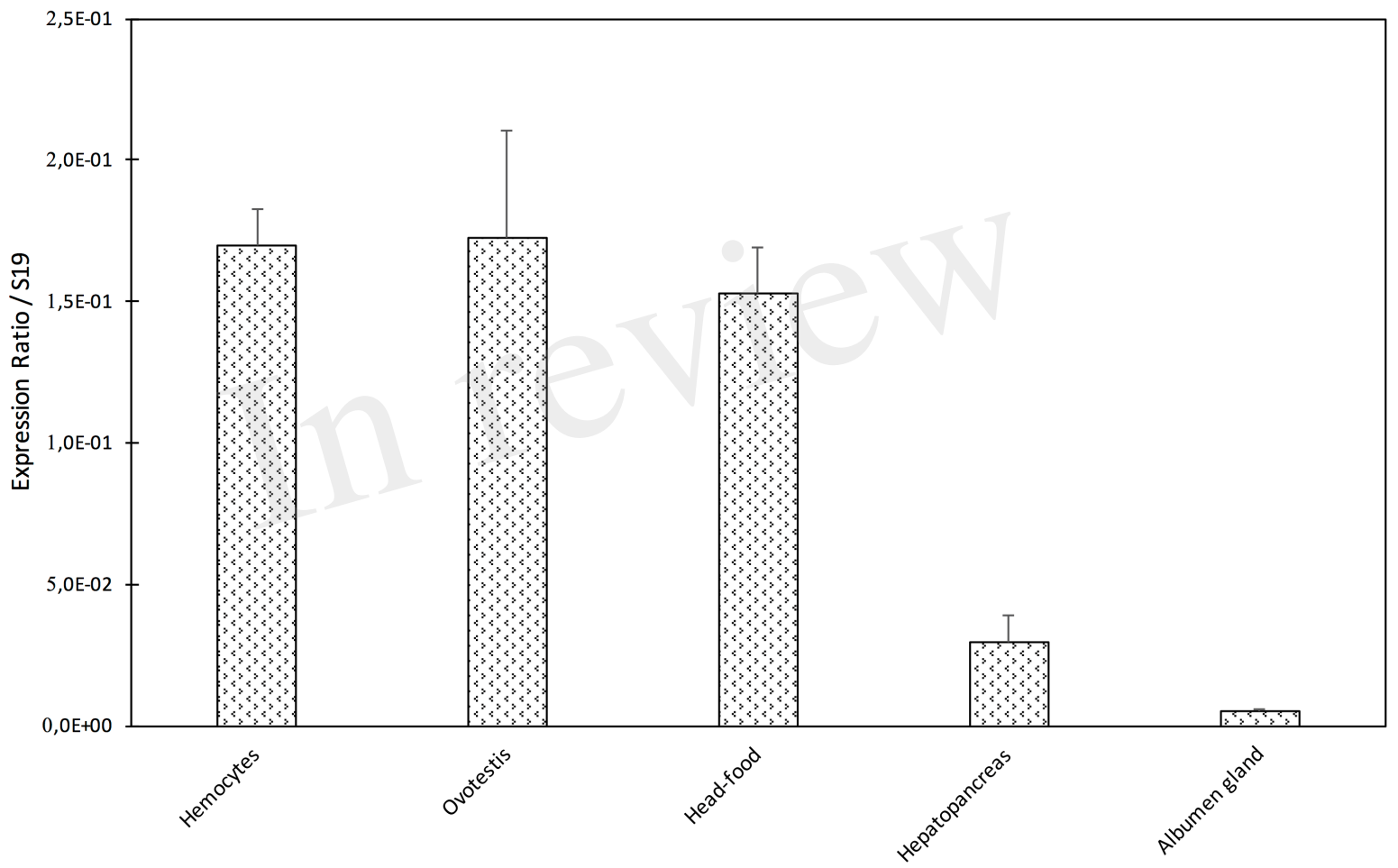


Figure 4.TIFF

Plasma



Ab

Ab + pep

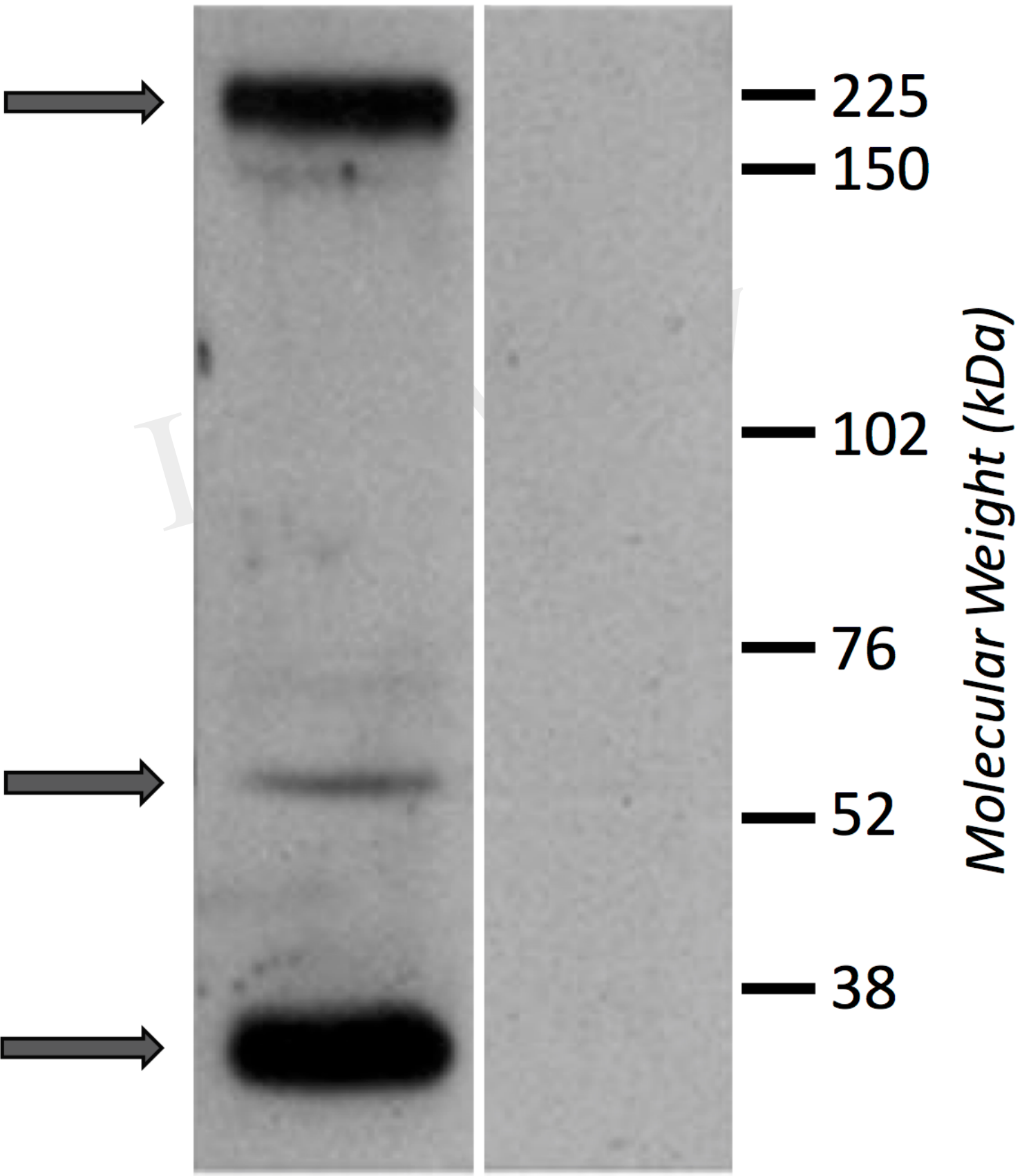
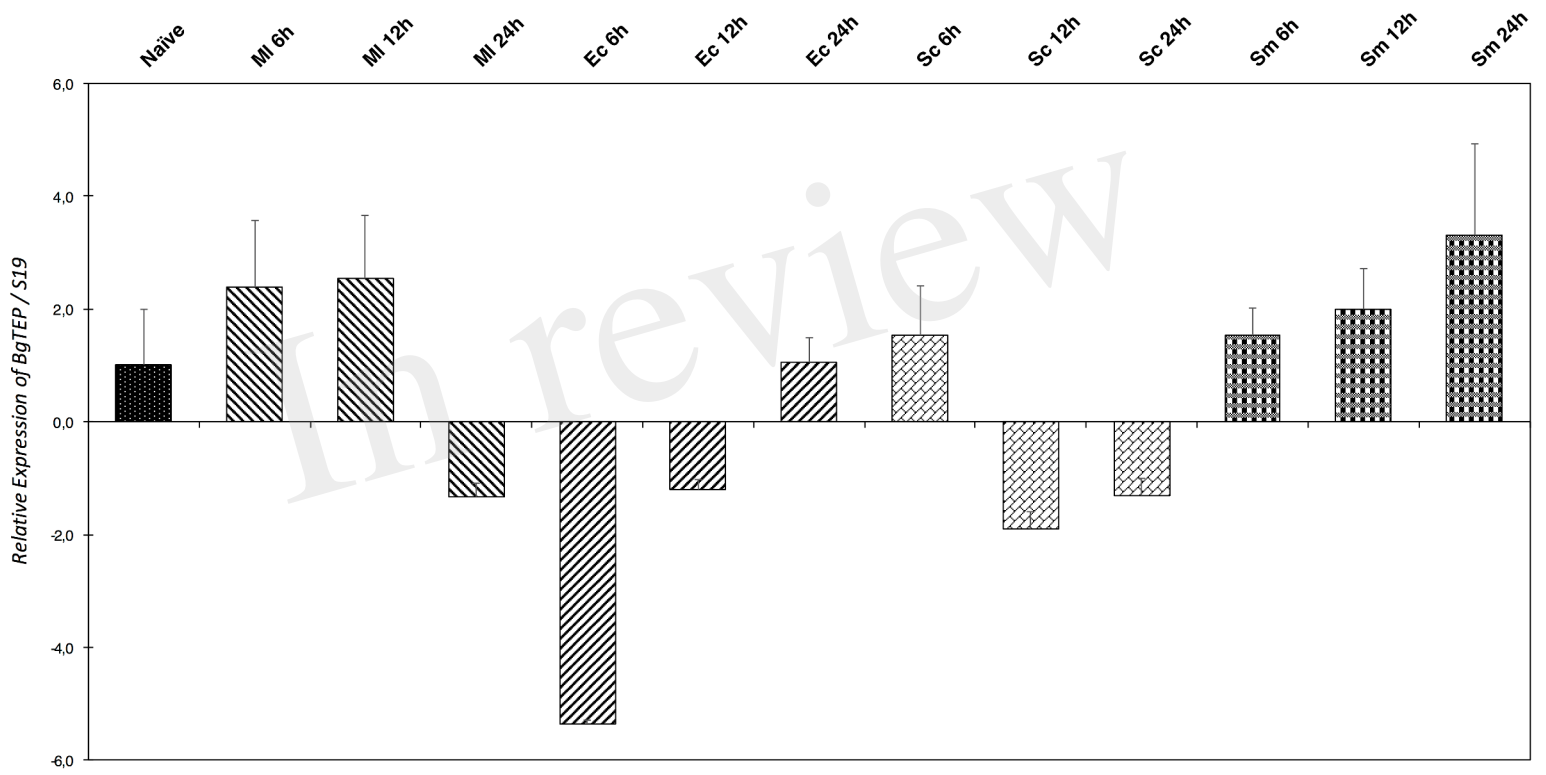


Figure 5.TIFF



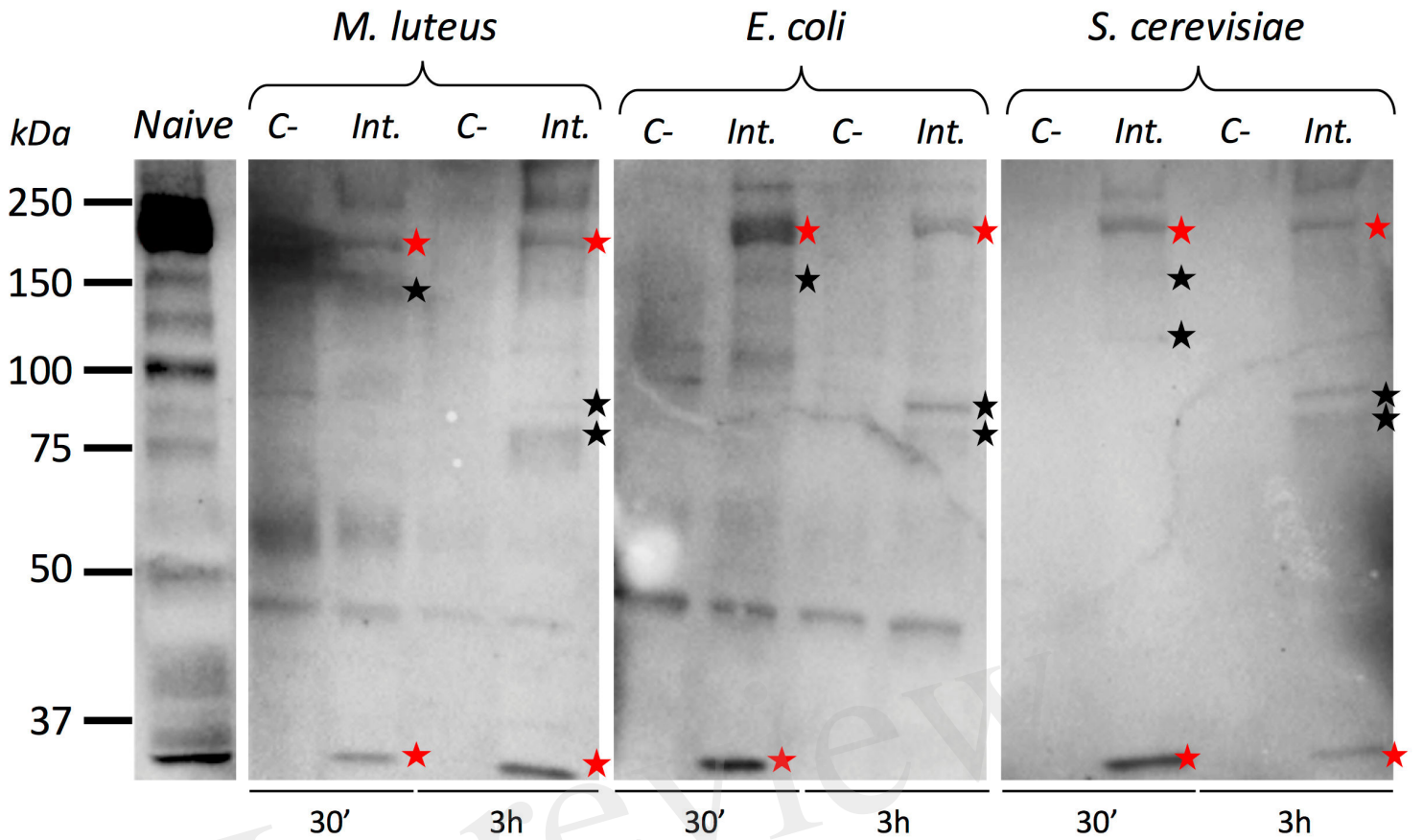
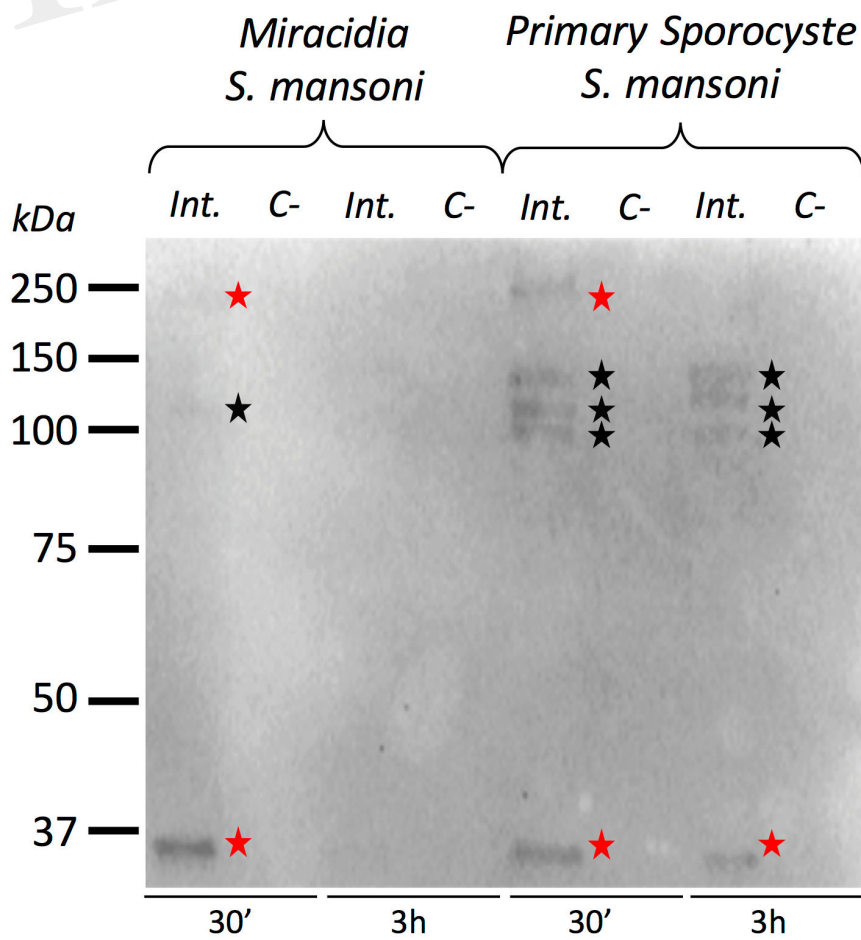
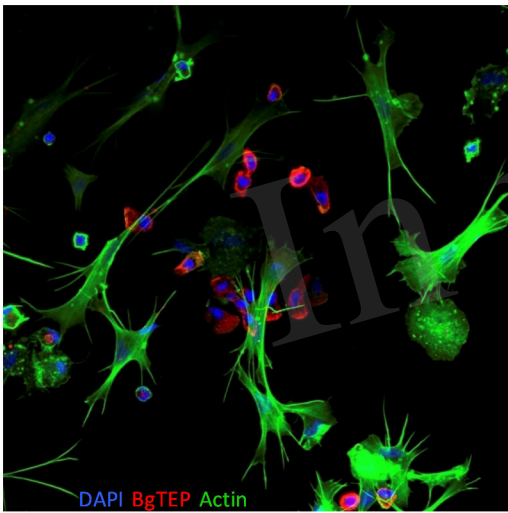
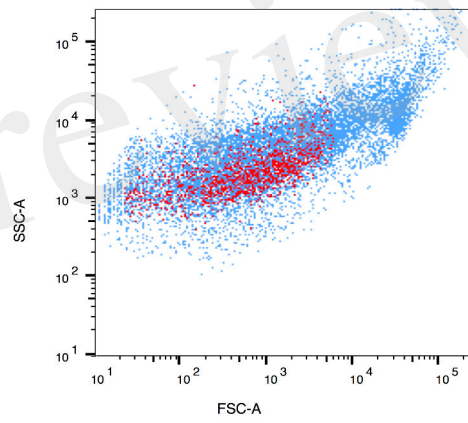
A**B**

Figure 7.TIFF

A



B



C

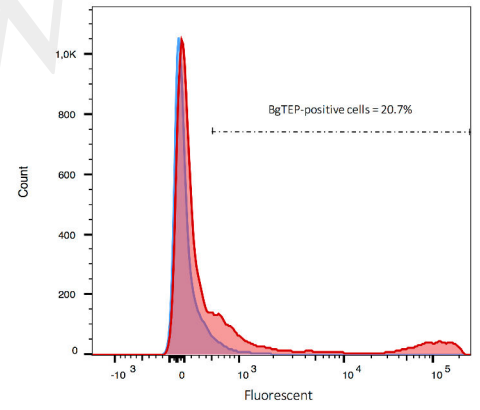
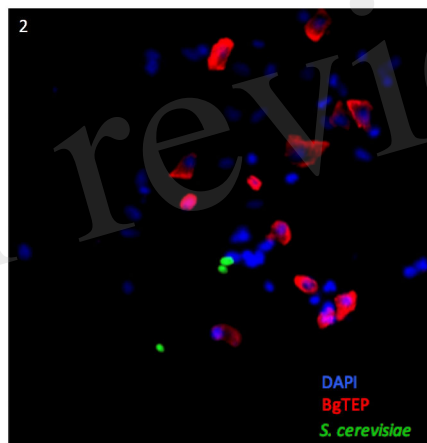
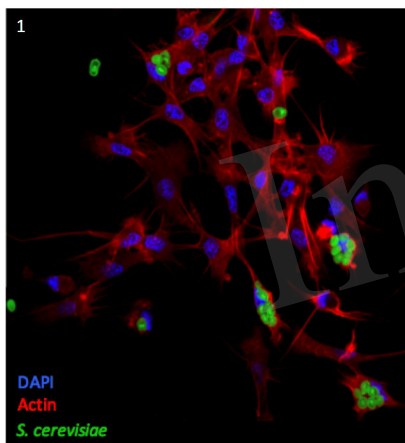
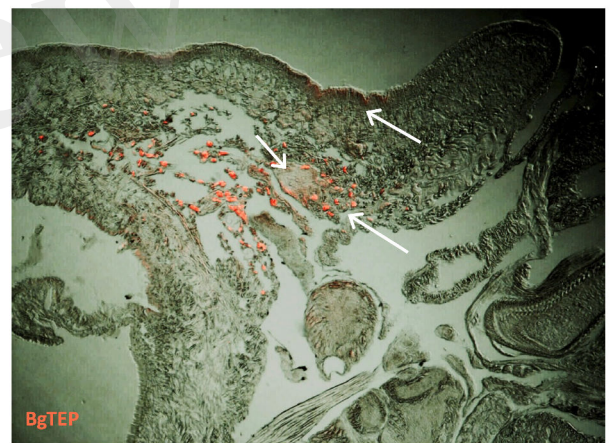


Figure 8.TIFF

A



B



VALORISATION

➤ **A shift from Cellular to Humoral Responses Contributes to Innate Immune Memory in the Vector Snail *Biomphalaria glabrata*.**

Pinaud S, Portela J, Duval D, Nowacki FC, Olive MA, Allienne JF, Galinier R, Dheilly NM, Kieffer-Jaquinod S, Mitta G, Théron A, Gourbal B.

PLoS Pathogens, 2016 doi: 10.1371/journal.ppat.1005361

➤ **Integrated Multi-Omic Analyses In *Biomphalaria-Schistosoma* Dialogue Reveal The Immunobiological Significance Of FREP-SmPoMuc Interaction.**

Portet A, Pinaud S, Tetreau G, Galinier R, Cosseau C, Duval D, Grunau C, Mitta G, Gourbal B.

Developmental and Comparative Immunology, 2017 doi: 10.1016/j.dci.2017.02.025

➤ **A Multistrain Approach To Studying The Mechanisms Underlying Compatibility In The Interaction Between *Biomphalaria Glabrata* And *Schistosoma Mansoni*.**

Galinier R, Roger E, Moné Y, Duval D, Portet A, Pinaud S, Chaparro C, Grunau C, Genthon C, Dubois E, Rognon A, Arancibia N, Dejean B, Théron A, Gourbal B, Mitta G.

PLoS Neglected Tropical Disease, 2017 doi: 10.1371/journal.pntd.0005398.

➤ **First Characterization Of Viruses From Freshwater Snails Of The Genus *Biomphalaria*, The Intermediate Host Of The Parasite *Schistosoma Mansoni*.**

Galinier R, Tetreau G, Portet A, Pinaud S, Duval D, Gourbal B.

Acta Tropica, 2017 doi: 10.1016/j.actatropica.2016.12.021

↻ **Invertebrate Soluble Proteins In Pathogens Sensing, A Medzhitov's Experiment.**

Tetreau G, Pinaud S, Portet A, Galinier R, Gourbal B, Duval D.

Soumis dans Frontiers Immunology, Juillet 2017

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

Portet A, Pinaud S, Chaparro C, Charriere G, Allienne JF, Duval D, Galinier R, Gourbal B.

Soumis dans in Molecular Biology and Evolution, Juillet 2017

Polymorphic sets of immune receptors support specificity in the innate immune memory of the Schistosomiasis vector snail, *Biomphalaria glabrata*.

Pinaud S, Portet A, Portela J, Allienne JF, Saint-Beat C, Arancibia N, Galinier R, Duval D, Gourbal B.

En preparation pour Molecular Ecology, Été 2017

Biomphalysin expansion, from bacterial β -PFT to immune weapon in Schistosomiasis vector snail *Biomphalaria glabrata*.

Pinaud S, Tetreau G, Portet A, Chaparro C, Galinier R, Gourbal B, Duval D.
En preparation pour Genome Research, 2017

Trained Immune Cells Support Innate Immune Memory In Invertebrate.

Pinaud S, Chaparro C, Lebron R, Cosseau C, Portet A, Grunau C, Netea M, Duval D & Gourbal B.
En preparation pour Cell Host & Microbe

Arm Race Between Endogenous miRNAs From Host And Circulating Xeno-Mirna Of Parasites.

Pinaud S, Lassalle D, Chaparro C, Portet A, Allienne JF, Gourbal B, Duval D.
En preparation, 2017

The BgTEP, a key factor of the *Biomphalaria glabrata* immune cellular response?

Portet, A., Galinier, R., Pinaud, S., Portela, J., Gourbal, B. and Duval, D.
En préparation pour Journal of Innate Immunity, 2017

Alcool absorption in human must increase *Schistosoma* transmission

Aliaga B 🍏, Pinaud S 🍏, Portet A 🍏, Rognon A, Duval D.
En preparation, 2017

Trained Immunity: evolutive success in eukaryote.

Gourbal B 🍏, Pinaud S 🍏, Conrath U, Van der Meer J, Netea M.
Invitation dans Seminar Immunology, Décembre 2017

🍏 premier auteur, ➤ article publié, ↻ article soumis

Il n'y a pas de simplicité véritable. Il n'y a que des simplifications.

Léon-Paul Fargue (1876-1947)

Plus que Merci ?

La constitution d'un jury de thèse est pleine de sens, parce que celui-ci va devoir décortiquer, comprendre et juger le travail entrepris depuis plusieurs années. Les sensibilités des différents membres va alors déterminée la dynamique et la teneur des discussions durant la défense de cette thèse. Parce que je souhaitais que chaque aspect de mon travail puisse faire face à un spécialiste reconnu, chacun des membres choisis par Benjamin, David et moi-même représente pour nous une source d'inspiration scientifique très importante dans notre travail. Les impressionnantes présentations au congrès Society for Invertebrate Pathology de Tours en 2016 de Dominique Ferrandon (Strasbourg) et Jean-Michel Drezen (Tours) n'ont fait que confirmer mon souhait qu'ils puissent rapporter mon manuscrit de thèse. Avec leurs réponses rapidement positives, le choix des examinateurs à suivi la même démarche et exigence. C'est ainsi que David Duneau (Toulouse), José-Juan Lopez-Rubio (Montpellier), Yannick Moret (Dijon) et Jean-Marc Deragon (Perpignan) ont accepté de participer à ce jury hétéroclite où je souhaite que chaque sensibilité et personnalité puisse enrichir nos discussions.

Pour avoir accepté de siéger dans ce jury, de vous investir lors ma soutenance de thèse, et enfin de m'avoir permis d'améliorer grandement la qualité finale de ce manuscrit : **un grand merci.**

Je souhaiterais tout d'abord remercier Benjamin Gourbal (@Chef) sans qui ma formation universitaire n'aurait pas été ce qu'elle est aujourd'hui. C'était la rencontre entre deux jeunes étudiants de Licence bien candides et empotés comme il faut. Mais nous étions sincères et motivés, suffisamment pour nous faire progresser dans ce feu Axe2 qui nous a tant appris. J'ai démarré comme le pénible du fond qui posé des questions qui n'intéressaient que lui, pour finir en pénible du fond qui adore ce qu'il fait.

Licences 3, Master I, Master II puis 3 ans (et 18 (\pm 1) jours de thèse en moyenne) aux côtés de ce beau groupe de travail qui mérite d'avoir plus de courage dans ses ambitions. Nous n'étions pas parfaits, ça tombe bien vous non plus, ça montre que nous pouvons encore progresser, mais chacun de son côté dorénavant, à moins que des projets futurs réussissent à nos rassembler à nouveau.

L'immunité mémoire innée (Priming pour les intimes) ne va pas se caractériser seul.

Sincèrement merci. Le scientifique que j'essaie d'être au jour le jour va essayer d'emporter avec lui, au moins les qualités que vous lui avez tous transmises. Pour les défauts, j'en ai suffisamment pour récupérer aussi les vôtres, n'exagérons rien. Nous n'étions parfois pas d'accord dans ce qui fait la vie et la science d'une équipe ou d'un laboratoire, mais j'espère que cela n'aura eu de conséquence que de nous améliorer respectivement troquant un peu mon rôle envers mon chef de parasite, pour un brin de mutualisme.

Le terme de 'Supervisor' anglo-saxon a pour moi parfaitement caractérisé notre interaction. Les venus dans ce bureau tropical (quand je me souvenais de la raison qui m'avait poussé à m'y rendre) densifiaient d'autant nos réflexions que le calendrier des expériences pour y répondre. J'espère que l'immunité des Lophotrocozoaires (titre en construction) continuera à croiser la route de multiples projets pour aller de plus en plus loin. Aussi, je ne pourrai être qu'admiratif de ces dizaines, centaines et milliers d'heures passées à pratiquer un art qui n'est plus de la science, mais qui permet à tous les gens qui ont pu graviter autour de son groupe d'avoir un stage, une thèse, un salaire, un projet pour travailler. Le tout avec trop peu de reconnaissance à mon goût.

Merci pour ce moment, et pour les prochains.

Ma thèse a eu la chance d'être très bien encadrée parce qu'en plus d'un directeur, j'ai eu la chance d'avoir un co-directeur de thèse. David Duval (@Chef). Il n'est pas l'archétype de ce que j'imagine d'un biologiste en 2017, c'est un taiseux, entêté et acharné. Quelques années à ses côtés révèlent un homme clairvoyant et spirituel qui puise dans un travail acharné un sens aigu de l'intuition scientifique. Si l'apport professionnel de David sur mon travail est évident, il m'aura appris bien plus que cela. Je souhaite à la famille Duval un calendrier toujours aussi chargé, mais un avenir empli de sérénité.

Nos compétences mais aussi nos sensibilités scientifiques, sans s'affronter frontalement, réussissent à se compléter et c'est la raison qui explique, je pense, notre plaisir à travailler ensemble. ~~Parfois il demeure encore impossible de travailler ensemble ou de se comprendre (cf entêté?)~~. À l'avenir, 'in order to' travailler ensemble avec plaisir, il faudra imaginer des projets incluant à la fois de la microscopie à force magnétique (bonjour Mr Duval), des toxines, du CRISPR, des lapins, du Saint-Nicolas de Bourgueil et plein de truc en ..seq et en ..omic. Ça parait facile. Merci pour tout !

Rien n'est pour autant une raison suffisante pour mettre des chaussettes avec des sandales.

@Chef et @Chef, ma thèse n'aurait pas été la même sans l'un de vous deux, vous m'avez énormément appris et j'en suis très reconnaissant. Vous formez un beau couple improbable, si je réussis une carrière scientifique vous en serez tenu pour responsable.

Je tiens à présenter aux familles Gourbal et Duval mes plus plates excuses pour l'accaparement de vos époux pendant de longues journées et nuits. Vous comprendrez malgré tout que, certes, ils n'ont pas vécu une sinécure à mes côtés, mais je doute pour autant que mon départ diminue leur passion pour le travail nocturne.

Connaissant cher Chefs, vos outrecuidances pour la démonstration sentimentale au boulot, je m'arrête là. Je suis un thésard de plus, mais vous resterez mes uniques Chefs durant ma thèse.

Je vous souhaite le meilleur.

#Axe2 #Shift #IIMII #Pasteur #Turku #SIP

J'ai eu la chance dans ces 3 ans de formations (+2 de masters), de ne pas être entouré que par ces deux énergumènes, mais par bien d'autres personnalités.

Le bureau étrange où @Richard Galinier et Jean-François (@Jeff) Allienne exerce est probablement le pèlerinage obligatoire pour tout être vivant au laboratoire. La moindre question, technique, technologique, scientifique, stratégique et acrobatique (il manquait une rime en ique) trouve très souvent sa réponse en quelques minutes. Bien plus que cela, c'est aussi des

pilliers humains de ce laboratoire qui m'ont apporté bien plus que leurs juridictions leurs exigées. Jeff est un phare dans la biologie moléculaire du laboratoire, nous guidant au travers des produits et des techniques, pour nous permettre de travailler dans l'efficacité, la sérénité et la sécurité. Richard (en plus de sa qualité de directeur général mondial de la formation scientifique translationnelle et autre biologie moléculaire) est un altruiste, préférant souvent le rôle de coéquipier de luxe, de celui qui amène dans le brouillard le sprinter vers le maillot jaune, le tout sans jamais monter sur un vélo. Il est la prépondérante troisième des quatre roues du carrosse de l'axe2, gageons que son épopée virale lui donne des envies d'enfiler de nouvelles tuniques. C'est aussi pour son attachement à démêler la hiérarchie supposée qu'il aime à se mêler souvent au bureau du fond et désamorcer parfois les tracas du quotidien. Un grand merci pour l'ensemble de son oeuvre.

Une petite dernière ? Le chercheur est fatigué après une belle thèse.

@L'axe2 du laboratoire 2EI qui m'a recruté en thèse s'est alors fondu dans l'équipe BMC puis ÉcoÉvl (Écologie et Évolutions des Interactions) du laboratoire IHPE emmenée par @Christoph Grunau. Le travail de structuration de cette grande équipe de travail n'est pas de tout repos et pourtant cette équipe prend de plus en plus de sens et de poids au fur et à mesure de son existence. Cette équipe est certes perfectible, mais les gens commençant à jouer le jeu, les publications à venir parleront en sa faveur. Merci d'en être cet artisan.

Une thèse, c'est une cinquantaine d'heure au laboratoire par semaine, mais c'est aussi autant d'heures en dehors, où l'on a plaisir de retrouver ceux qui partagent ou non, nos labeurs scientifiques.

@Guillaume Tetreau a débarqué en PostDoc pendant ma thèse, il venait de Cornell si je me souviens bien, j'en suis plus bien sur... et il a libéré (délivré?) un son nouveau à notre groupe de travail ; bien présent, entre 16 et 16 000 Hz, trop. Si ses compétences sur notre escargot préféré se sont restreintes à les observer dans nos mains, normal, il fût recruté pour travailler sur un ténébrion qu'il ne verra pas beaucoup plus (heureusement pour le projet...), sa sensibilité scientifique et son amour pour la bière, BroForce et la découpe du saucisson nous rapprocha irrémédiablement. Quel honneur d'avoir partagé la vie d'un structuraliste !! Il devrait être

candidat à un Ignobel d'ici peu si il arrive à montrer que le pickleball est un sport, c'est pas gagné. En attendant, on remonte sur notre cheval et je lui dit ...

Merci mec. Force et honneur.

@Cécille Saint-Béat, bibiche a débarqué sur ses petites ballerines roses en essayant de pas faire trop de vagues. Pourtant, ce sera un raz-de-marée pour nombreux d'entres nous. Si tu ne t'étais pas endormis tous les midi a table, on aurait certainement pu avoir de conversations très intéressantes sur le cinéma et la vie ...

Merci d'avoir tenu en vie les centaines de souris impliquées dans mes expériences pendant des longues heures dans ta grotte avant de la refiler à @CyndAïE. Du restes, tu es partie vers de nouvelles aventures bien plus excitantes scientifiquement, mais on va te garder avec nous pendant encore un petit moment. #dedimogwai

@Julien KS, CALMEZ-VOUS! Depuis notre séjour marseillais, nos relations ne seront plus jamais les mêmes pour les raisons que tu connais autant que moi : les chipirones. T'as la chance d'avoir un sujet génial entre les mains et t'es en train d'en faire un truc encore mieux. Garde le cap malgré les tempêtes, on a jamais vu un Sud-Africain blanc de Bordeaux lâcher l'affaire. T'es un bon! Je te laisse la garde de Damien Lassalle (de Muscuu). Essaie de le canaliser le petit sinon il va startupiser tout le labo, les gens ne sont pas prêt. D'ailleurs @Damien (je sais que tu vas lire les remerciements juste pour te trouver dedans alors que le reste de la thèse pourrait être beaucoup plus intéressant pour ta suite) tu récupères un sujet génial que j'ai moi même récupéré de Julien P qui l'avait lui même récupéré de Yves M qui l'avait lui même récupéré d'une bactérie Clostridium. Je te confie David et mon ordi, prends en soin. Si tu prends le bon train (blague de cheminots), il y a l'opportunité de faire un truc géant, faisez pas les cons les mecs!

Bonne continuations les enfants !

@Cristian Chaparro, c'est le Chilien du groupe, pour les quotas. En plus de l'extraordinaire vitesse de réflexions Cristian est doté du petit plus typique des latins qui nous donne envie d'ouvrir une autre bière belge, un autre bourbon et une autre cachaca. Si tu parlais moins de langues tu serais beaucoup moins énervant. J'espère que j'aurais quelques scripts à optimiser dans l'avenir qu'on puisse continuer à bosser ensemble. À

l'occasion, je t'expliquerais comment analyser deux trois trucs avec le terminal sur Mac. En attendant, tu es toujours la personne qui crois en nous et qui nous remet dans le droit chemin. J'espère qu'ils vont prendre soin de toi, tu le mérites. Dank u Cristiano!

@Nathalie Arancibia, avec un seul r, c'est la Chilienne du groupe, pour les quotas. Désolé de t'avoir demandé tant et tant de mollusques toutes ces années, mais sache que sans ton travail, le mien serait bien moins garni. Merci beaucoup. Quand t'ouvres ton restaurant d'ampanadas je viens en acheter, ce sera l'occasion de chanter en écoutant RTL2.

@Julien Portela a eu la tâche d'initier bons nombres de projets autour du priming puis il s'est accroché (c'est peu de le dire) à cette thématique qui lui tenait tant à coeur. Tu as toujours été une oreille attentive, dommage que tu sois si nul à PES. Aujourd'hui ParaDev mérite de réussir et toi avec, je vous souhaite de vous diversifier et de vous agrandir. Au passage, l'idée de relâcher des miracidia de Schisto en Corse en profitant des vacances de Jérôme, juste pour voir si ça pouvait marcher : brillant. À quand la Têt ?

@Olivier Rey, le Dude du labo. Quel plaisir de constater que les nouvelles générations qui vont remplir nos laboratoires vont l'être par ta génération d'enseignants-chercheurs, entre fraîcheur, rigueur et bienveillance. Une superbe pioche pour le laboratoire. Bonne continuation. Je compte sur toi pour battre @Rémi à la pétanque tous les ans jusqu'à sa retraite pour venger mon honneur sali un vendredi moite de juillet 2017.

@Ronaldo Augusto de Carvalho, Annia Alba, Mariana Lima, est-ce qu'il y a une loi en Amérique latine et centrale qui oblige à avoir des Noms et Prénoms qui finissent par la même lettres ? Vous avez été supers, de superbes rencontres qui nous font adorer notre travail. J'espère avoir dans ma vie l'occasion de découvrir le Brésil et Cuba et vous y retrouver. J'espère vous avoir convaincu que les Français sont des cons comme les autres, que Paris n'est pas la France et que le fromage, c'est bon. See you dudes!

@Maxime, la semaine d'intégration banyulinc fût notre rencontre (CHUT!) et on s'est bien marré depuis à Perpignan, Montpellier ou ailleurs. Tristan, Etienne, Aude j'ai rien à dire sur vous parce que vous être trop sérieux en congrès (OK, sauf Etienne) ! Sachez que Aude a une boîte à côté de son bureau d'une tradition qu'elle a ramenait de chez vous, elle met des pièces plusieurs fois par jours, mais personne ne comprend, vous nous

expliquerais. Bref ! On a partagé pour le coup la même galère et pourtant, on en a trouvé des moments de franche camaraderie (Malraux et al, 1962) vous avez été TOPs. (#bisous)

@Jérôme Boissier, le seul statisticien bio au monde, il fait tout son travail avec des bouliers faits-mains dans un petit atelier de ré-insertion d'ancien narco-trafiquant en Cerdagne. La fraîcheur avec laquelle il aime enseigner (des fois) nous a prouvé, à moi et des générations de jeunes biologistes, que la recherche pouvait ne pas être si austère. La pertinence avec laquelle il traite les problématiques scientifiques au jour le jour reste impressionnante pour moi. Merci Professeur Boissier de Corse.

@Di(Ane) Merce-dat le fameux duo magique (et un peu Irma). Rare sont les paperasses que je vous ai envoyées qui étaient correctement remplies du premier coup. J'ai essayé autant que faire ce peu de pas vous compliquer plus votre travail que ce qu'il est déjà. Merci de nous permettre de bosser.

@Marion Picart, @David Roquis, @Rodolpho Rondon, @Sara Fneich les vieux de la vielle. On s'est croisés et maintenant que sa touche à la fin, on comprend les sentiments étranges qu'imposent la fin de thèse. Vous nous avez permis de nous intégrer dans ce labo qui nous impressionnait tant. Vos petites attentions nous ont permis de nous sentir un peu chez nous dans notre bureau en P2. Aujourd'hui vous vazez à d'autres avens et je ne peut que vous en souhaiter des encore meilleurs. Courage ! Vos successeuses (@Camille UO, @Manon Falllletttte, @Stephen Murano (ZZ) et @Nelia Muliana (attendez, on m'indique Muliano dans mon oreillette) sont dans les starting-blocks, qui de *Biomphalaria*, *Schistosoma* ou de *Crassostrea* va gagner ce sprint effréné?

@Kelly Brenny-Raffely, tu es exactement dans la même galère que nous, et là, pendant que j'écris ces quelques vers, tu écrit un paragraphe passionnant sur la plasticité phénotypique. Tu vas être une super agrégée et tu vas leur apprendre plein de trucs, surtout si tu met un col-roulé. Guides tes ouailles vers le sinueux chemin du savoir, telle une shamane de l'Alaska guidant Homer Simpson vers la ville, ils t'en remercieront.

@André Théron, quel plaisir d'avoir pu travailler avec vous ne serait-ce que quelques mois. J'ai adoré le pragmatisme qui vous habitez pour expliquer des phénomènes écologiques. J'espère avoir la même passion que vous

après une si belle carrière. Je vous souhaite (si vous faites l'erreur de lire ces remerciements) maintenant une belle retraite que vous avez mérité.

@Anne Rognon, on en a passé des longues heures ensemble dans la salle du vivant. Comprendre à tes côtés les rudiments de l'expérimentation animale dans ce modèle de choix Biomph/Schisto fût un plaisir. Merci de cette transmission. Longue vie à la Guadeloupe (même sans Schisto).

@Eve (Toulza), (à lire avec la voix de Wall-E c'est plus marrant), je voulais simplement te dire que je ne suis absolument pas d'accord avec ton pesage de la pastèque. (1) Parce que tu n'as pas amené de balance accréditée par le bureau des poids et des mesures sur les lieux de la compétition et (2) que tu as décidé de favoriser par tes pratiques matriarcales, une candidate féminine au détriment de la vérité masculine. Je ne te félicite pas. Merci en tout cas de m'avoir appris, entre autre, que l'utilisation d'un pain de savon de Marseille était l'unique pré-requis pour de la biologie cellulaire. Et puis les Moocs en salle de réunion/repas... J'ai beaucoup aimé ta coque Légo®.

@Gabi, Hélène, même si votre bureau est un peu un pays étranger pour moi, j'ai toujours apprécié votre clairvoyance et votre intérêt lorsque nos sujets se croisaient. Je vous souhaite plus de sérénité dans votre avenir, dont des beaux résultats sur la chronobiologie entre autres.

@Caroline, Delphine, Guillaume C, Jean-Luc R, Jean-Michel E., Julien DL, Yannick G. J'ai été ravi de découvrir des thématiques comme les vôtres et je sais qu'à l'avenir les ponts entre Perpignan et Montpellier vont encore se raccourcir pour rapprocher nos thématiques. Je n'ai pas eu la chance d'aussi bien vous connaître aussi bien que mes collègues Perpignanais, mais en tout cas ce fût toujours un plaisir d'échanger. Nos passages à Lille, Lyon ou Cambridge nous auront permis de mieux nous connaître, en attendant peut-être encore plus dans le futur.

@Guillaume Mitta, le grand chef. C'est sous ton giron que j'ai fait mes premiers pas en thèse. Ton accompagnement pendant mon comité de suivi de thèse m'a fait comprendre à quel point le métier que j'apprenais était différent du tien et ainsi comprendre le chemin qu'il me restait à parcourir. Le travail que tu fournis pour ce laboratoire est infernal, mais nous permet de travailler sereinement. Bonne continuation.

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#loyauté #labodethèsepourtoujours #CBETM #UMR5244 #2EI #IHPE

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Références bibliographiques

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Résumé : Le clade des invertébrés cristallise en 2017 de grandes problématiques sociétales à la fois économiques et sanitaires. En effet un certain nombre des organismes présent dans ce groupe phylétique sont des vecteurs des grandes pandémies infectieuses telles que le paludisme (*Anopheles sp*), Zika, Chikungunya, Fièvre jaune, etc (*Aedes sp*), Chagas (*Triatoma sp*) ou encore la bilharziose (*Biomphalaria sp*, *Bulinus sp*). La compréhension du système immunitaire de ces organismes vecteurs doit aider la communauté scientifique à proposer des solutions pour réduire la transmission de toutes ces maladies sur le terrain. *Biomphalaria glabrata* est le vecteur unique de la Bilharziose intestinale (*Schistosoma mansoni*) en Amérique Latine. Depuis un premier cas de résistance induite par une première infection en 1998, de nombreux travaux ont exploré la réponse immunitaire mémoire innée de cet escargot tropical d'eau douce. Dans le cadre de ce travail de thèse, différents aspects de cette immunité (également appelé priming, résistance acquise) ont été explorés, de la mise en place phénotypiques, aux bases moléculaires et cellulaires. En premier lieu, nous avons pu démontrer qu'elle était dépendante d'une bascule phénotypique (d'une réponse cellulaire d'encapsulation à une réponse humorale) et transcriptomique qui lui permet de mieux répondre lors d'une seconde infection. La spécificité de cette réponse est portée par la production de répertoire complexe de récepteurs et d'effecteurs immunitaire spécifiques qui sont capables de différencier jusqu'aux différents stades de développement parasitaire d'une même espèce de parasite. Nous avons également pu montrer que cette interaction dépendait de microARN circulants ainsi que de Biomphalysines, des β -PFT acquises par transferts horizontaux depuis le monde bactérien. Enfin, cette résistance semble posséder une proximité avec l'immunité mémoire entraînée des cellules immunitaires innées des vertébrés en particulier sur la base des mécanismes moléculaires sous-jacent qui seraient liés chez *Biomphalaria* comme chez les Vertébrés à une reprogrammation épigénétique des cellules du système immunitaire innée.

Mots clé : *Biomphalaria*, *Schistosoma*, immunité mémoire innée, immunité entraînée.

Abstract: Invertebrates focus in 2017 among the major economical and societal issues across Earth. Some members are vectors of important infectious pandemic as malaria (*Anopheles sp*), Zika, Chikungunya, Yellow fever, etc (*Aedes sp*), Chagas (*Triatoma sp*) and trematodes (*Biomphalaria sp*, *Bulinus sp*). Comprehension of immune system of these vectors has to help scientist to decrease transmission on endemic area. *Biomphalaria* exposed first failure to be reinfected following first infection as soon as 1998. In my thesis we explore this immune priming (innate immune memory) and describe an immune shift from cellular to humoral immune response both in phenotype and transcriptomic response. A specificity is handle by specific immune receptor and effector repertoire to distinguish up to different developmental stage of same parasite species. This interaction is also dependent of mRNAs and Biomphalysin, a β -PFT coming from bacterial kingdom. Finally, this resistance seems to look alike the trained immune memory of innate cells in vertebrates.

Keywords : *Biomphalaria*, *Schistosoma*, innate immune memory, trained immunity.