Coûts et bénéfices de l'inflammation dans les relations hôte-parasite

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Résumé

Les relations hôte-parasite sont complexes et soumettent les deux protagonistes à un ensemble de compromis aussi bien plastiques qu'évolutifs. D'un côté, bien que l'immunité soit indispensable pour la lutte contre les parasites, elle peut aussi causer de nombreux dégâts à l'hôte lors de la réponse et mener à des maladies inflammatoires. De l'autre côté, les parasites, bien que dotés de mécanismes d'évasion, vont être affectés par l'environnement inflammatoire de l'hôte. Cela pose la question des coûts et bénéfices que l'interaction entre ces deux protagonistes va avoir sur chacun d'eux lors de l'apparition de troubles inflammatoires chez l'hôte. Grâce à différentes approches expérimentales et bibliographiques, j'ai pu montrer que l'immunopathologie est un trait qui perdure vraisemblablement grâce aux bénéfices de la réponse immunitaire dans la lutte contre les parasites. Par ailleurs, j'ai pu mettre en évidence qu'une inflammation altérait positivement les traits d'histoire de vie du parasite Heligmosomoides polygyrus aussi bien de façon plastique qu'après sélection expérimentale. Cependant, le parasite investit davantage dans l'immunomodulation et le camouflage lorsqu'il est confronté à cet environnement, laissant planer la question du coût de cette inflammation à long terme et sur plusieurs générations.

Mots clés : plasticité, sélection, compromis, traits d'histoire de vie, inflammation, immunomodulation, *Heligmosomoides polygyrus*, *Plasmodium yoelii*, *Mus musculus domesticus*

Abstract

Host-parasite interactions are characterized by trade-offs that involve both plastic and microevolutionary responses. On one hand, while immunity is essential to fight parasites, it can also cause damage to the host, leading to autoimmunity and inflammatory diseases. On the other hand, parasites have to cope with the immune environnement provided by the host. This raises the question of the costs and benefits of the inflammatory response for the two partners of the interaction. With different experimental and literature-based approaches, I showed that immunopathology is a trait that likely persists because of the immediate benefits of the immune response in terms of protection against parasites. Furthermore, I was able to show that inflammation positively altered the life history traits of the gastrointestinal nematode *Heligmosomoides polygyrus* both plastically or after experimental evolution. However, the parasite invested more in immunomodulation and camouflage when facing an inflammatory environment, leaving open the question of the costs associated with an inflammatory environment over the entire lifespan of the parasite and/or across generations.

Keywords: plasticity, selection, trade-off, life history traits, inflammation, immunomodulation, *Heligmosomoides polygyrus*, *Plasmodium yoelii*, *Mus musculus domesticus*

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Introduction

De la plasticité phénotypique à l'adaptation locale

Le phénotype d'un individu est déterminé par sa composante génétique, l'environnement dans lequel il se trouve et l'interaction entre sa génétique et l'environnement. Ainsi, confrontés à des environnements différents, les individus peuvent arborer des phénotypes différents. C'est ce qu'on appelle la plasticité phénotypique. Le changement de phénotype peut être d'ordre morphologique, physiologique, comportemental… La plasticité phénotypique se retrouve par exemple pendant le développement ou durant les différentes phases d'un cycle de vie où à partir d'un même génome, différents stades vont apparaitre (e.g. insectes (larve et adulte : Papillon Monarque (*Danaus plexippus*)), parasites (cycle de vie correspondant à différents stades : la douve du foie (*Fasciola hepatica*)). Ainsi, les travaux de Woltereck en 1909 ont montré que la taille du casque des daphnies (*Daphnia kladophora*) dépendait des conditions environnementales durant le développement ou encore que la couleur de la bavette des roselins familiers (*Carpomedacus mexicanus*) changeait en fonction de la richesse en caroténoïdes de leur alimentation, passant du jaune au rouge (Hill et al. 2002).

La plasticité phénotypique peut être étudiée par la pente de régression des valeurs phénotypiques sur les valeurs environnementales (Fig. 1). La courbe ainsi obtenue est appelée « norme de réaction », proposition faite par Woltereck en 1909.

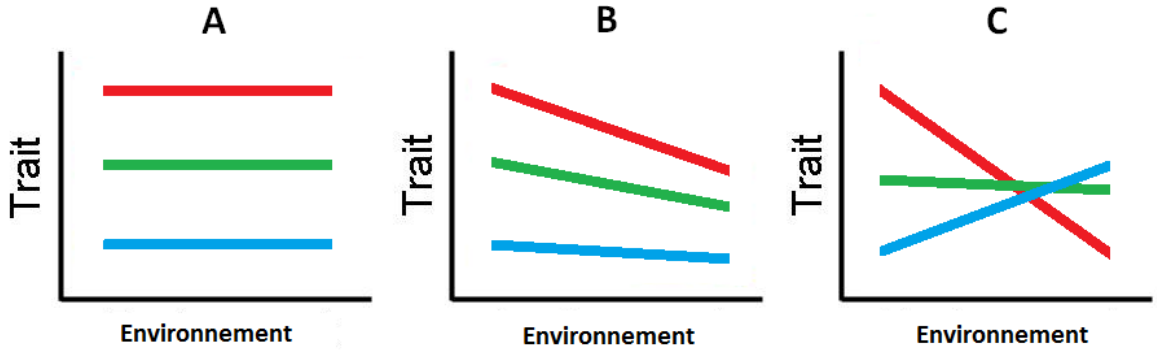

Figure 1. La plasticité phénotypique peut être définie comme étant la capacité d'un génotype à produire plus d'un phénotype quand il est exposé à différents environnements. Chacune des courbes représente la norme de réaction pour un génotype. (A) le trait ne change pas en fonction de l'environnement, il n'est donc pas plastique, mais l'expression est différentes pour les trois génotypes, (B) le trait change selon l'environnement, la pente étant plus forte pour le génotype rouge ; l'ordre des génotypes restant inchangé, (C) le trait change selon l'environnement et le signe de la pente reliant l'expression du trait à l'environnement change entre génotype ; l'ordre des génotypes change en fonction de l'environnement. Pour les cas (B) et (C) on constate une interaction génotype environnement.

Lors de l'étude de ces normes de réactions, certains traits vont avoir une pente différente de zéro, montrant une altération (positive ou négative) du trait étudié par le gradient environnemental. Cependant d'autres traits peuvent montrer une relation inverse au premier trait et ainsi compenser la variation du premier trait. C'est par exemple souvent le cas entre la fécondité et la longévité lorsque des organismes sont confrontés à un environnement dangereux : mieux vaut investir dans la reproduction plutôt que dans la survie. Un exemple classique de ce compromis est le cas de l'escargot pulmoné *Biomphalaria glabrata* exposé aux miracidies de *Schistosoma mansoni* (Minchella and Loverde 1981 ; Thornhill et al. 1986). Lorsqu'il est infecté l'escargot augmente sa fécondité en perspective d'une durée de vie raccourcie. Un autre exemple de variation d'allocation dans les traits d'histoire de vie est celui de *Heligmosomoides polygyrus* confronté à un environnement inflammatoire provoqué chez l'hôte par l'ingestion d'un sucre complexe (Donskow-Lysoniewski et al. 2013). L'inflammation provoquée par l'ingestion du produit favorise le parasite dans son développement et sa survie mais réduit sa production d'œufs.

Cependant, bien que la plasticité permette d'arborer des phénotypes différents, les phénotypes produits ne sont pas tous égaux en termes de fitness. En effet, les roselins familiers montrent une variation de la couleur de leur bavette en modifiant leur

alimentation, cependant, cette variation du phénotype est liée à la fitness des individus, les oiseaux ayant la bavette la plus rouge étant ceux qui ont la plus grande fitness (Badyaev et al. 2001). Par ailleurs, Murdock et al. (2013) évaluent la survie du moustique *Aedes aegypti* infecté par *Escherichia coli* et soumis à plusieurs températures. L'un des deux génotypes présente une norme de réaction en cloche avec un optimal de survie à l'infection à 26°C, température où la survie de ce génotype sera la meilleure. Ces optimaux de fitness ou de traits fortement reliés à la fitness sont signe d'une adaptation locale à certaines conditions présentées lors du gradient environnemental. Cela montre que la plasticité a ses limites. En effet, bien que celle-ci soit indispensable pour répondre à de changements environnementaux, elle ne permet pas d'affronter tout environnement. Un exemple classique chez l'homme est celui de l'adaptation à l'altitude. En effet, toute personne vivant au niveau de la mer souffrira du manque d'oxygène à partir d'une certaine altitude. Cependant, les populations des Andes, des plateaux de l'Himalaya ou d'Éthiopie, ont une augmentation du volume pulmonaire et de la rapidité de la respiration, des tissus dont les capacités d'échanges sont augmentées ou encore des hémoglobines plus affines à l'oxygène, caractéristiques qui leur permettent de vivre dans ces environnements (Moore et al. 1998, Moore 2001, Weber 2007).

Pour pouvoir aller au-delà des limites que la plasticité offre, seule la sélection peut œuvrer pour permettre une adaptation à ce nouvel environnement. Cependant, contrairement à la plasticité, pour qu'il y ait adaptation locale il faut que les pressions environnementales/de sélection qui mènent à l'adaptation soient relativement constantes. En effet, pour que le processus de sélection ait une prise, il faut permettre aux nouveaux allèles, conférant un avantage en fitness, de pouvoir augmenter en fréquence dans la population. La sélection directionnelle va permettre ce phénomène génération après génération (e.g. *Biston betularia* : Mallet 2004 ou les cichlidés du lac Malayi : Albertson et al. 2003).

Il est cependant rare qu'il n'y ait qu'une seule pression de sélection dans un environnement naturel. En effet, diverses pressions de sélection vont s'exercer sur les individus et ces pressions peuvent être antagonistes. Ainsi la sélection ne sera plus directionnelle mais balancée. La drépanocytose chez l'homme est une illustration courante

de ce phénomène. Les individus porteurs de la version allélique S du gène de la chaine β de l'hémoglobine à l'état hétérozygote ont l'avantage de mieux résister à l'infection par *Plasmodium falciparum* que les individus n'ayant pas cette version allélique. Cependant, à l'état homozygote les individus sont atteints de drépanocytose (anémie sévère) ce qui contre-sélectionne la version allélique S. Ainsi cette version allélique ne sera avantageuse qu'à l'état hétérozygote et maintenue par sélection balancée à une faible fréquence (Allison et al. 1956, Cavalli-Sforza et Bodmer 1999). Il se peut aussi que la sélection agisse de façon antagoniste sur des traits différents n'impliquant pas un seul et même gène. On parle alors de compromis évolutif. C'est ce qui a été observé par Ghalambor et al. (2004) chez *Poecilia reticulata*. Ils ont confronté des femelles guppy à deux environnements différents, l'un avec une forte pression de prédation, l'autre avec une faible pression de prédation. Dans l'environnement à forte pression de prédation les femelles allouent plus dans la reproduction que dans le milieu pauvre en prédateurs, ce qui a un impact sur le poids des femelles proche de la parturition, et sont aussi capables de fuir plus vite. Cependant, lors de la gestation, les compétences d'accélération et de distance parcourue sont réduites au même niveau que celui des femelles dans l'environnement avec peu de prédateurs. Ainsi les femelles sous forte pression de prédation ne peuvent pas maintenir leur investissement à la fois dans la reproduction et dans leurs capacités motrices.

Les organismes sont donc sans cesse confrontés à diverses pressions de sélection biotiques ou abiotiques aussi bien synergiques qu'antagonistes. Les relations hôte-parasite illustrent bien ce processus d'adaptation. En effet, un parasite va exploiter son hôte alors que ce dernier va tenter de l'empêcher de nuire, voire de l'éliminer. Ces objectifs opposés vont sans cesse pousser ces organismes à trouver des adaptations pouvant contrer l'exploitation par le parasite ou les défenses de l'hôte (Schmid-Hempel 2008), les menant dans une course aux armements.

Par ailleurs, les hôtes doivent faire face à de nombreux parasites, très différents les uns des autres, virus, bactéries, vers parasites, champignons, etc., ce qui nécessite d'avoir un moyen de défense très plastique et capable de s'adapter à tout nouveau parasite l'infectant (e.g. diversité du Complexe Majeur d'Histocompatibilité (Robinson et Delvig 2002), des récepteurs des cellules T et des immunoglobulines (Agrawal et al. 1998)). D'un autre côté les

parasites doivent, à chaque nouvel hôte, être capables de répondre à un nouvel environnement de par la physiologie mais surtout à cause du système immunitaire de l'hôte qui représente pour lui un environnement très variable et imprévisible.

La réponse immunitaire

Lors d'une infection par un parasite ou lors d'une blessure, le système immunitaire débute son activation en déclenchant l'inflammation. Cette inflammation est caractérisée par trois phases : la phase vasculaire, la phase cellulaire et la résolution (Sorci et Faivre 2009). Durant la phase vasculaire, les mastocytes, présents dans le tissu interstitiel, vont sécréter de l'histamine, après la phase de reconnaissance d'un corps étranger, ce qui aura pour conséquences la vasodilatation des vaisseaux sanguins et l'afflux de plasma dans le tissu interstitiel. Dans le même temps, les macrophages vont sécréter des messagers tels que le facteur de nécrose tumoral alpha (TNF-α) et les interleukines (IL) 1, 6, 8 et 12 comme chémoattractants et ainsi débuter la diapédèse, c'est-à-dire l'entrée des cellules leucocytaires (principalement monocytes et polynucléaires) de la circulation dans le tissu interstitiel, qui permettra d'enclencher la phase cellulaire. Cette phase cellulaire correspond donc à l'arrivée des leucocytes circulants sur le lieu de l'inflammation : les monocytes qui donneront de nouveaux macrophages, les polynucléaires, pour la phagocytose et la production de molécules toxiques contre le corps étranger, ainsi que les lymphocytes. Ces derniers, serviront à la mise en place de l'immunité acquise. En effet, en même temps que les mastocytes et macrophages détectent le corps étranger, les cellules dendritiques assimilent le corps étrangers puis vont passer dans le réseau lymphatique pour rejoindre les ganglions, lieu où elles pourront présenter aux lymphocytes T et B naïfs un motif appartenant au corps étranger et ainsi activer les lymphocytes pour qu'ils puissent rejoindre la zone inflammatoire et se joindre à la phase cellulaire en sécrétant des molécules cytotoxiques pour les cellules T cytotoxiques et des anticorps pour les cellules B (Delves et al. 2008).

Caricaturalement, lors de la réponse à un parasite, le système immunitaire peut emprunter deux voies qui sont les voies Th1 (cellules T helper de type 1) et Th2 (cellules T helper de type 2). Chacune des voies est spécialisée dans une réponse contre les parasites intracellulaires (Th1) et extracellulaires (Th2). Lors d'une réponse Th1, les cellules infectées

seront éliminées grâce à la phagocytose par les macrophages, à la lyse cellulaire par les lymphocytes T cytotoxiques ou encore la neutralisation (blocage de l'adhésion des bactéries), à l'opsonisation (marquage par immunoglobulines pour amélioration de la phagocytose) ou à l'activation du complément (pour provoquer la lyse des microparasites). La réponse Th2 est une réponse de type humorale de par la production d'anticorps par les cellules B pour « marquer » le parasite. Ces anticorps permettront ensuite aux granulocytes de s'arrimer au parasite et de libérer les molécules toxiques pour le détruire et aux macrophages de phagocyter les reliquats de parasite. Cette seconde voie sert aussi à remodeler les tissus de l'hôte pour encapsuler certains stades parasitaires de macroparasites, ainsi qu'à la reconstruction des tissus endommagés (Allen et Wynn 2011). Les messagers libérés lors d'une réponse de type Th1 sont principalement les cytokines IFN-γ (Interféron gamma), TNF-α (Facteur de Tumeur Nécrosant alpha) et l'interleukine-12 (IL-12), alors que l'activation de la voie Th2 est quant à elle réalisée par la libération de cytokines IL-4, IL-5 (Kidd 2003).

Une fois le parasite éliminé, la phase de résolution s'engage : c'est l'arrêt du processus d'inflammation. Cet arrêt se fait grâce à la sécrétion de cytokines anti-inflammatoires telles que l'interleukine-10 (IL-10) ou le Facteur de Croissance Transformant β (TGF-β) par les macrophages et cellules T régulatrices (Belkaid 2007 ; Sorci et Faivre 2009).

Le coût de l'immunité

Coût énergétique

Le système immunitaire sert à nous défendre contre les agents pathogènes et ainsi participe au maintien de l'organisme (Kurtz et al. 2013). Cependant le système immunitaire a longtemps été considéré comme non coûteux pour l'organisme. Pourtant, l'activation de ce dernier et la production de cellules et messagers de l'immunité pourraient avoir un coût. C'est au début du 21e siècle que les premières études ont montré le coût de l'immunité (ex : Klasing et al. 2004 ; Lee et al. 2006). Lee et al. (2006) ont montré que la composition du régime alimentaire, plus ou moins riche en carbohydrates et/ou en protéines affectait la capacité des chenilles *Spodoptera littoralis* à lutter contre le virus *nucleopolyhedrovirus*. Par ailleurs, l'augmentation de la température de l'hôte requiert une augmentation de

métabolisme de base de 10% voire jusqu'à 55% lors d'infections sévères chez l'homme (Kluger 1991 ; Hurtado et al. 2008).

Dans un environnement où les ressources sont limitées, cette réponse immunitaire peut affecter d'autres traits d'histoire de vie (Amat et al. 2007 ; Hasselquist et Nilsson 2012). Ainsi, la réponse immunitaire peut être négativement liée à des traits d'histoire de vie comme l'effort reproductif, la fécondité, la croissance, la longévité ou encore la locomotion (Merino et al.2000 ; Moret et Schmidt-Hempel 2000 ; Gwynn et al. 2005 ; Husak et al. 2016). Par exemple, Calatan et al. (2012) ont évalué l'effet de la température et la variation du coût énergétique de l'immunité durant un challenge LPS chez les larves de Tenebrio molitor. A 30°C, contrairement à de plus basses températures, les larves de T. molitor ayant subi le challenge LPS avaient une réponse immunitaire plus forte ainsi que leur métabolisme basal plus élevé mais en contrepartie avaient une plus grande perte de poids suite à ce challenge.

Coût immunopathologique

Outre le fait que la mise en place de la réponse immunitaire soit coûteuse pour l'organisme, le système immunitaire peut aussi causer des dégâts à son hôte (Moore et al. 2003), phénomène appelé immunopathologie. Par exemple, l'infection de l'homme par un hantaan virus de campagnol roussâtre (*Myodes glareolus*) en France est quasiment asymptomatique chez certains individus alors que chez d'autres il provoque des fièvres hémorragiques qui ne sont aucunement liées à la virémie mais bien à la réaction immunitaire mise en place par l'hôte (Charbonnel et al. 2014). En effet, cela peut être dû à une réponse excessive dirigée vers le parasite (Ayres et Schneider 2012 ; Vale et al. 2014). Le meilleur exemple de réponse exacerbée qui conduit à des dégâts immunopathologiques importants est le choc septique qui apparait après le passage de bactéries dans la circulation sanguine. C'est du fait d'une production excessive et systémique de cytokine TNF-α (facteur de nécrose tumorale α), impliquée dans la réponse envers les microparasites en favorisant l'infiltration de cellules immunitaires et la fluidification du sang, que le volume sanguin va décroitre et les organes commencer à défaillir menant au choc septique (Graham et al. 2005). C'est aussi le cas de l'IL-13 lors d'une réponse de type Th2. Cette cytokine est impliquée dans la formation de granulome pour encapsuler les vers lors de

l'infection par *Schistosome sp.* Un excès de production d'IL-13 peut ainsi provoquer une fibrose hépatique pouvant conduire à la mort (Hoffmann et al. 2002).

Par ailleurs, l'immunité acquise peut donner lieu à des maladies auto-immunes et allergiques. Le système immunitaire peut prendre pour cible des corps étrangers non dangereux, voire des molécules du soi. Lors de la maturation des cellules effectrices de l'immunité acquise, plusieurs filtres existent pour éviter la reconnaissance des molécules du soi. Dans le thymus les cellules T immatures vont subir deux sélections, une positive où le système s'assure que la cellule T est bien capable d'interagir avec le CMH (complexe majeur d'histocompatibilité) et une négative où les cellules T qui seront capables de trop réagir avec des molécules du soi seront éliminées (Kurd et al. 2016). Dans la moelle osseuse, les lymphocytes B subiront une sélection négative où seules celles réagissant faiblement aux peptides du soi présentés seront sélectionnées pour continuer leur maturation (Hardy 2001). Cependant, cette phase de sélection n'est pas à 100% efficace et des erreurs peuvent être commises et engendrer des cellules T et B capables de reconnaitre des molécules du soi ou des molécules étrangères non dangereuses (e.g. Murosaki et al. 1991). Néanmoins, des mécanismes de protections contre ces cellules qui ont échappées aux filtres existent car pour que l'activation de ces cellules soit possible il faut qu'il y ait un ensemble de signaux délivrés par des cellules présentatrices d'antigènes lors de la liaison avec les molécules du CMH (Complexe Majeur d'Histocompatibilité) et co-stimulatrices et qu'il y ait production de cytokines stimulatrices (Yin et al. 2013). Ainsi, si cette reconnaissance n'est pas un succès, cela peut mener soit à l'anergie, qui empêche les lymphocytes ou les cellules NK de s'activer en les rendant non répondantes, soit à l'apoptose de ces cellules (Feig et Peter 2007). L'intervention de cellules T régulatrices est aussi responsable du blocage de l'activation des lymphocytes par des cellules dendritiques (Wing et al. 2008).

La régulation de l'immunité

La réponse immunitaire innée est non spécifique et produit des molécules toxiques appelées ROS pour Reactive Oxygen Species et RNS pour Reactive Nitric Species, deux familles de molécules oxydantes qui ne font pas la différence entre le parasite et l'hôte. Il est alors indispensable que la production de ces ROS et RNS soit certes suffisant pour lutter contre le parasite mais aussi en assez faible quantité pour éviter les dégâts que l'hôte va

subir en déclenchant cette réponse. Ce compromis dans la réponse immunitaire est régulé à plusieurs niveaux (Delves et al. 2008). Le premier est que la réponse immunitaire a besoin de la présence du parasite pour s'activer et que son absence amènera à son arrêt. Deuxièmement, une fois le parasite éliminé, une grande majorité des lymphocytes T dédiés à la réponse entreront en phase d'apoptose, les autres devenant des lymphocytes mémoires. Il y a aussi l'inhibition des lymphocytes T une fois qu'ils se sont liés aux cellules B par exemple, ce qui les empêche d'activer d'autres cellules B. Et finalement, la présence de cellules T régulatrices. Ces dernières ont la capacité d'inhiber la prolifération des autres lymphocytes effecteurs. Ces cellules sont particulières importantes pour le maintien de la tolérance immunitaire, c'est-à-dire à la reconnaissance des cellules du soi et du non soi non dangereuses. Leur action se fait par l'intermédiaire de cytokines anti-inflammatoires comme l'interleukine-10 ou le TGF-beta (Taylor et al. 2006), ou en consommant l'interleukine-2 qui permet d'activer les autres lymphocytes effecteurs (de la Rosa et al. 2004), par cytolyse directe des lymphocytes effecteurs ou par l'expression de molécules inhibitrices sur leur surface conduisant à l'apoptose des lymphocytes effecteurs (Garin et al 2007). Outre la régulation des autres lymphocytes, elles sont aussi un rôle dans la régulation de cellules présentatrices d'antigènes en les inhibant (Wing et al. 2008). Elles ont aussi la capacité d'atténuer la production de molécules toxiques par les phagocytes (Mills 2004 nature) ou encore d'augmenter le seuil auquel les cytokines de la voie th1 ou th2 sont produites (Abbas et al. 2004).

Cette régulation est essentielle au contrôle de l'inflammation pour éviter des dégâts immunopathologiques tout en assurant la mise en place d'une réponse capable de faire face au parasite. Néanmoins, elle représente aussi une faiblesse. En effet, les parasites ont mis au point un ensemble de mécanismes pour échapper au système immunitaire de leur hôte (Schmid-Hempel 2008). Parmi eux se trouve l'échappement par immunomodulation. Ce mécanisme tient à la sécrétion par le parasite de molécules interférant avec le système immunitaire dans le but de le réguler (Hewitson et al. 2009). Ce sujet sera abordé plus en détails ultérieurement dans cette introduction.

Le rôle des cytokines

Les cellules du système immunitaire communiquent entre elles par divers médiateurs chimiques que petite taille que sont les cytokines. Ces dernières en fonction de leur type vont pouvoir activer l'immunité, la désactiver et lui faire prendre des voies immunitaires différentes. Ainsi, leur production est essentielle pour combattre les parasites, comme nous le montrent un nombre très important d'études utilisant des souris génétiquement modifiées pour un gène de cytokine. Par exemple, une étude de Kurtz et al. (2013) montre que des souris C57BL/6, dont le gène de l'interleukine-6 (cytokines pro-inflammatoires) a été mis KO et infectées par *Francisella tularensis*, souffraient d'une mortalité plus importante et arboraient une charge parasitaire bien supérieure à celle observée chez les souris contrôle. Par ailleurs, un excès de production de cytokines pro-inflammatoire peut conduire à de sévères dégâts immunopathologiques voire la mort de l'individu (Annane et al. 2005; Graham et al. 2005). Ainsi le niveau de production de ces cytokines a un rôle clé dans l'efficacité de la lutte contre le parasite et dans les dommages immunopathologiques.

Par ailleurs, chaque cytokine n'est pas produite seule mais avec un ensemble d'autres médiateurs pro- et anti-inflammatoires. On peut alors supposer que si la balance entre ces différentes cytokines pro- et anti-inflammatoires penche vers le pro-inflammatoire, la réponse immunitaire provoquera des dégâts immunitaires alors que si celle dernière penche trop du coté anti-inflammatoire alors la réponse sera insuffisante pour combattre le parasite. Guerrero et al. (2013) ont montré en injectant à des souris des lipopolysaccharides d'*Escherichia coli*. En effet, ils ont mesuré la production de cytokines pro et anti-inflammatoires et ont montré que les individus qui avaient la plus grande fitness suite à cette inflammation étaient ceux qui, bien qu'ils avaient produit beaucoup de cytokines pro-inflammatoires, avaient aussi produit beaucoup de cytokines anti-inflammatoires.

Cependant une question ce pose : pourquoi la sélection ne nous a pas débarrassé de l'immunopathologie en ajustant au mieux le niveau de ces messagers et ainsi la réponse de l'immunité ? La réponse immunitaire est un trait complexe qui résulte de l'action d'un grand nombre de gènes. Par ailleurs, chaque composant de la réponse immunitaire peut avoir une activité pléiotrope entre différents mécanismes de l'immunité (Downing et al. 2010). Ainsi, en agissant sur un composant par une pression de sélection quelconque, d'autres

mécanismes peuvent être affectés. De plus, comme mentionné auparavant, les individus sont confrontés à une diversité de parasites qui ne demandent pas tous de mettre en place la même réponse immunitaire ; d'où l'observation de polymorphisme important sur les gènes de l'immunité responsable de variation dans la résistance à plusieurs parasites (Turner et al. 2011). Ainsi, dans un environnement naturel où les hôtes seront confrontés à de nombreux parasites d'espèces différentes, les pressions de sélection que ces parasites vont créer sur l'immunité peuvent être contraires et mener à des compromis sur certains gènes contrôlant l'immunité. Les cytokines sont des messagers du système immunitaire qui ont un très grand nombre d'interactions avec beaucoup d'autres composants de ce système, ce qui leur confère un rôle pléiotrope et en font ainsi un bon modèle pour étudier ces compromis (Downing et al. 2010). En effet, certains locus responsables de la régulation de l'immunité sont sous sélection balancée. C'est le cas du gène de la cytokine CXCL10 (Guo et al. 2013) ou encore des gènes de l'interleukine-1 et -2 (Turner et al. 2012) pour lesquels un signal de sélection balancée a été mis en évidence.

Immunité et âge

D'autre part le système immunitaire, au travers de l'inflammation, est aussi lié à l'apparition de maladie chez l'homme à un âge avancé tels que maladies cardiovasculaires, l'athérosclérose, l'obésité ou les maladies neurodégénératives (Franceschi et al. 2007 ; Cevenini et al. 2013 ; Okin et Medzhitov 2013). De plus, avec l'âge le système immunitaire est de moins en moins performant (e.g. Lavoie et al. 2007), c'est ce qu'on appelle l'immunosénescence (Weiskopf et al. 2009). Cependant, on observe que chez les individus âgés, contrairement aux juvéniles ou jeunes adultes, les effecteurs pro-inflammatoires augmentent (e.g. IL-6, TNF-α : Vasto et al. 2007) alors que les effecteurs anti-inflammatoires diminuent (e.g. IL-10) conduisant à un déséquilibre dans la régulation de l'inflammation et conduisent à une inflammation chronique délétère pour l'individu (Goto et Makoto 2008). Ce phénomène est appelé « inflammaging » et peut-être vu comme un effet pléiotrope antagoniste de l'immunité.

Une composante pléiotrope antagoniste ?

Des traits ou fonction qui favoriseraient la fitness à un jeune âge pourraient-être sélectionnés en dépit du coût qu'ils engendrent à un âge avancé, âge auquel la sélection

naturelle a le moins de prise. C'est ce qu'a proposé George C. Williams en 1957 avec sa théorie de la pléiotropie antagoniste. Ces traits appelés pléiotropes sont portés par des gènes appelés gérontogènes. L'un des exemples de gérontogène est glp-1 chez *Caenorharbditis elegans*. En effet, des mutations sur ce gène produit des individus avec des problèmes de fertilité, voire stériles, mais qui ont une vie prolongée (Arantes-Oliveira et al. 2002). D'autres exemples concernant des gènes de l'apoptose, de la lutte contre les radicaux libres ou de protéines de choc thermique sont discutés dans Leroi et al. 2005. Par ailleurs, d'autres compromis entre des traits de vie tôt dans la vie et à des âges avancés ont été étudiés en laboratoire aussi bien qu'*in natura* (revue dans Lemaitre et al. 2015) et s'illustrent le plus souvent par des relations négatives entre la reproduction tôt dans la vie et la survie adulte.

Malgré son évidente nécessité dans la lutte contre les agents pathogènes, le système immunitaire arbore, de par le coût de la mise en place de la réponse, le coût immunopathologique, le déclin de ses performances avec l'âge et son importance dans l'apparition de maladie inflammatoire avec l'âge, toutes les caractéristiques laissant penser qu'il pourrait très bien s'agir d'une fonction avec des effets de pléiotropie antagoniste (Franceschi 1999). Malgré ce constat évident, peu d'études ont abordé le sujet. En effet, dans une revue de Lambeth et al. (2007) les ROS impliquées dans la réponse immunitaire innée, capables d'endommager l'ADN, les protéines et les lipides, sont souvent liés à des maladies chroniques qui apparaissent avec l'âge et abrègent la vie des patients. Ils semblent donc être un bon exemple de l'action de pléiotropie antagoniste du système immunitaire. Par ailleurs, Ungewitter and Scrable en 2009 postulent que la protéine p53 pourrait avoir un effet de pléiotropie antagoniste chez l'homme. En effet, cette protéine est impliquée dans la protection contre les cellules cancéreuses en interrompant la prolifération cellulaire mais peut aussi interférer avec celle des cellules souches indispensables au renouvellement de l'organisme. Ils ont mis en évidence que l'augmentation de cette protéine p53 dans l'organisme réduisait le risque de développer des tumeurs mais augmentait le risque de vieillir plus vite. Cependant, ces deux études n'utilisent pas directement l'expérimentation pour tester cette hypothèse et rare sont celles qui abordent le sujet par l'expérimentation (e.g. Pursall et Rolff 2011).

Cependant certaines maladies inflammatoires chroniques peuvent se manifester tôt dans la vie sans attendre la mise en place d'une inflammation à bas bruit avec l'âge. En effet, les maladies inflammatoires chroniques de l'intestin (ou MICI) regroupent la maladie de Crohn et la recto-colite hémorragique. Elles se caractérisent toutes les deux par une inflammation de la paroi d'une partie du tube digestif et sont diagnostiquées le plus souvent chez des adolescents ou jeunes adultes (Desreumaux mai 2011 - dossier INSERM). Bien que des prédispositions génétiques à ces maladies existent, comme celle liée au gène NOD2/CARD15 qui augmente le risque par 40 de développer la maladie de Crohn (Desreumaux 2005), ces maladies pourraient avoir une forte composante environnementale (Ananthakrishnan 2015).

La recrudescence de maladies inflammatoires

Depuis le milieu du 20^{e} siècle dans les pays les plus développés/industrialisés, la prévalence des maladies d'origine inflammatoire, telles que la maladie de Crohn, le diabète de type 1 et les allergies n'a cessé d'augmenter (Bach 2002).

Bien que ces maladies aient un déterminisme génétique (e.g. gène NOD2/CARD15 dans la maladie de Crohn), la brutale augmentation de ces troubles inflammatoires laisse plutôt penser à un facteur environnemental. En effet, des facteurs comme fumer, avoir subi une ablation de l'appendice, l'alimentation, le stress et la dépression ainsi que les contraceptifs hormonaux augmenteraient le risque de contracter une telle maladie (Ananthakrishnan 2015). Cependant ce sont plutôt des résultats épidémiologiques qui mettent le doigt sur une potentielle cause environnementale puisque ces maladies sont majoritairement observées dans les pays occidentaux les plus développés (Hovde et Moum 2012). Par ailleurs, ces maladies inflammatoires, à l'instar d'autres maladies auto-immunes, sont un problème de santé récent dont la prévalence a fortement augmenté dans les dernières décennies (Bach 2002 ; Hovde et Moum 2012). Cette augmentation en prévalence est corrélée avec la diminution de maladies infectieuses comme la tuberculose, l'hépatite A, la rougeole ou les oreillons dans nos sociétés occidentales (Bach 2002). De plus, sur l'ensemble des pathogènes auxquels nous avons été exposés durant notre évolution, seuls les helminthes ont complètement disparus des populations des sociétés occidentales (Rook 2013). De ces observations est née l'hypothèse de l'hygiène (Strachan 1989) suivie de celle des « vieux amis » qui postule que le fait d'avoir été exposé durant notre évolution à une

multitude de micro-organismes, a modelé notre système immunitaire pour y faire face ou les tolérer et qu'actuellement les conditions d'hygiène de nos sociétés modernes, bouleversant notre environnement, font que notre système immunitaire est mal adapté à cette rareté de de micro-organismes (Rook 2010) et ainsi conduit aux désordres immunitaires observés.

Nos « vieux amis » et leur capacité d'immunomodulation

Les organismes parasites sont inévitablement confrontés aux défenses de l'hôte dans lesquels ils vont effectuer tout ou une partie de leur cycle. Certains d'entre eux peuvent même rester plusieurs années à infecter leur hôte (Dreyer et al. 2005). Cela requiert donc de lutter contre le système immunitaire de leur hôte dans la durée. Pour ce faire un certain nombre d'entre eux ont des stratégies pour échapper au système immunitaire comme le fait de changer les protéines de surfaces exprimées, méthodes retrouvées chez les trypanosomes (Horn 2014 ; pour plus de stratégies d'évasion du système immunitaire voir Schmid-Hempel 2008).

Par ailleurs, le fait que le système immunitaire ait un ensemble de mécanismes de régulation est une faille que les parasites peuvent exploiter. En effet, les helminthes sont considérés comme les rois de la manipulation de l'immunité (McSorley et al. 2012, 2013 ; Finlay et al. 2014) et interviennent à plusieurs niveaux de l'immunité pour se protéger de l'hôte. Ces derniers sont dotés de protéines, peptides, glycanes, lipides ou de petites molécules organiques capables d'interférer avec l'immunité. Ces molécules sont connues sous l'acronyme « ES » pour « Excretory-Secretory products » (Hewitson et al 2009). Bien qu'une réponse de type Th2 soit la voie pour combattre les parasites extracellulaires et donc néfaste pour les helminthes, certains ES sont capables d'induire une réponse Th2, comme l'ES-62 chez *Acanthocheilonema viteae* (Al-Riyami et al. 2012), oméga-1 ou encore IPSE/alpha-1 retrouvés parmi les antigènes des œufs de *Schistosomes sp*. (Carvalho et al. 2009). Ces molécules ont la capacité d'interagir avec les cellules présentatrices d'antigènes et cellules T pour induire une réponse Th2. Cette activation de la voie Th2 est en fait une activation Th2 modifiée qui conduit à la prolifération de cellules Th2, de macrophages alternativement activés, de lymphocytes T régulateurs et à la production d'IL-10 (McSorley et Loukas 2010). Cette voie Th2 sert également à la réparation de blessures et il a été suggéré qu'une réponse Th2 pouvait être un mécanisme de tolérance

aux helminthes en contribuant à la réparation des blessures engendrées par ces derniers (Gause et al. 2013).

Une autre possibilité de manipulation de l'immunité est de jouer sur la régulation de celle-ci. Certains ES comme les cystatines (inhibiteur de cystéines protéases) peuvent réduire l'expression de molécules co-stimulatrices des cellules dendritiques ou interférer avec le CMH de type II et ainsi inhiber la reconnaissance entre cellules dendritiques et cellules T (Klotz et al. 2011 ; Manouri et al. 2001). De plus, cette altération peut se faire par l'activation de la prolifération de cellules T régulatrices qui à leur tour vont inhiber aussi bien la réponse de type Th1 que Th2 comme chez *Heligmosomoides polygyrus* (Finney et al. 2007). Les ES responsables de cette altération sont des dérivés du TGF-β ou induisent la production de TGF-β (cytokines anti-inflammatoire) (McSorley et al. 2012, 2013 ; Finlay et al. 2014). Finalement des ES comme Sm16 de *Schitosoma mansoni* sont capables de bloquer la production de cytokines pro-inflammatoires et/ou de type Th1 lors de la présentation de PAMPs (Pathogen-Associated Molecular Pattern) aux cellules dendritiques et macrophages (Brannstrom et al. 2009).

Bien que cette immunomodulation permette aux parasites d'améliorer l'environnement hôte dans lequel ils se trouvent en diminuant la réponse inflammatoire ou en la dirigeant vers une voie qui leurs est favorable, elle peut aussi leur être délétère. En effet, diminuer la réponse immunitaire peut abaisser les défenses générales de l'hôte et le rendre susceptible à d'autres parasites opportunistes (Qureshi et al. 2005 ; Nacher 2008 ; Chen et al. 2012 ; Mendez-Samperio 2012 ; Paterson 2013 ; Cotton et al. 2015). Par exemple, Nacher en 2008 montre que l'infection par des helminthes chez des patients atteints par *Plasmodium falciparum* augmentait par deux la charge parasitaire mais diminuait les cas de malaria cérébrale et de déficiences de la fonction rénal chez ces patients. Cependant, ces études s'intéressent au parasite opportuniste et à l'implication de cette coïnfection dans la maladie pour l'hôte mais ne s'interrogent que peu sur l'impact que cette coïnfection peut avoir pour les parasites immunomodulateurs. En effet, d'une part, ces parasites opportunistes pourraient conduire à la mort de l'hôte et donc à une diminution drastique de la fitness du parasite immunomodulateur. D'autre part, ils peuvent aussi agir par compétition direct ou indirect avec ce parasite et ainsi diminuer sa fitness. Les études de coïnfections montrent

qu'en effet l'interaction de deux parasites dans un hôte modifie les traits d'histoire de vie de ces derniers (Kondo et al. 2005 ; Lohr et al. 2010 ; Knowles et al. 2013 ; Griffiths et al. 2014). Ainsi cette fonction d'immunomodulation doit être sous pressions de sélection balancées entre la modulation de l'immunité hôte, tout en assurant de ne pas le démunir contre d'autres parasites opportunistes. A ma connaissance, peu études se sont penchées sur les compromis auxquels les parasites immunomodulateurs doivent faire face (e.g. Cornet et Sorci 2010) et laisse le champ libre pour de futures perspectives de recherche.

Outre le fait que ces ES soient de première utilité pour le parasite qui les sécrète, cette immunomodulation peut aussi avoir des effets bénéfiques chez leurs hôtes (revue dans Heylen et al. 2014). C'est le cas d'un bon nombre d'helminthes comme le nématode *Necator americanus* chez l'homme (Croese et al. 2006) ou encore *Heligmosomoides polygyrus* chez la souris (Leung et al. 2012). Lorsque des souris traitées avec une molécule provoquant chez elles une inflammation aigue de l'intestin, génétiquement modifiées ou co-infectées avec des bactéries induisant une inflammation de l'intestin, l'infection simultanée par ces nématodes réduit significativement l'inflammation et ainsi les symptômes associés à ces troubles inflammatoires (Chen et al 2006 ; Blum et al 2012 ; Adisakwattana et al. 2013).

Il en est de même chez l'homme avec des parasites tels que *Trichuris suis* dont les œufs ingérés par un sujet atteint de la maladie de Crohn ou de colite ulcérante voient leur symptômes atténués et peut même conduire à la rémission de la maladie (Summers et al 2003, Sandborn et al 2013).

Nos « vieux amis » à la rescousse !

De par leur capacité d'immunomodulation, les helminthes sont de bons candidats pour la mise en place de thérapies pour combattre des troubles inflammatoires. La première étude expérimentale a été menée en 2001 sur un modèle de souris dont l'inflammation du colon provoquée par l'ingestion de Dextran Sulfate Sodium (DSS), un sucre complexe provoquant une inflammation du colon, s'est améliorée avec l'infection par le cestode *Hymenolepis diminuta* (Reardon et al. 2001). De nombreuses autres études expérimentales ont prouvé la capacité de plusieurs helminthes à améliorer voire supprimer les symptômes liés à l'ingestion de produits provoquant une inflammation de l'intestin ou sur des souris KO déclenchant spontanément une inflammation de l'intestin (revue par Heylen et al. 2014 ;

Elliott et Weinstock 2012). S'appuyant sur ces résultats encourageants, Summers et ses collaborateurs ont tenté l'expérience chez l'homme en 2005 avec deux études sur 29 patients atteints de la maladie de Crohn et avec 54 patients atteints de colite ulcérante. Dans le premier cas, en donnant 2500 œufs de *Trichuris suis* toutes les 3 semaines pendant 12 semaines, 79% des patients ont constaté une amélioration des symptômes (Summers et al. 2005a). Dans la seconde étude, cette fois ci avec un contrôle placebo, 43% des patients ont constaté une amélioration de leurs symptômes contre 17% dans le groupe placebo. D'autres essais cliniques sont à ce jour en cours et s'annoncent tout aussi prometteurs (Helmby 2015).

Toutes ces études s'intéressent à l'hôte et à la rémission de ses symptômes mais sans porter attention aux répercussions sur les traits d'histoire de vie du parasite. A ma connaissance, seule une étude de Donskow-Lysoniewska et ses collaborateurs en 2013 s'est penchée sur le sujet pour le nématode *Heligmosomoides polygyrus*, l'un des nématodes les plus prometteurs lors des études sur l'animal (Elliott et Weinstock 2012) mais aussi celui dont on connait le mieux les produits ES (McSorley et al. 2012). *Heligmosomoides polygyrus* est un nématode intestinal qui a la capacité de réguler le système immunitaire de l'hôte par la production d'ES, produits d'excrétion et de sécrétion, comportant entre autres un homologue du TGF-β, cytokine anti-inflammatoire (Filbey et al. 2014). L'équipe de Donskow-Lysoniewska a donc mesuré divers traits d'histoire de vie du parasite après que les hôtes (souris BALB/c) aient été exposés à une dose de DSS à 5% pendant 8 jours, 3 jours avant l'infection et 5 après. Ils ont ainsi pu montrer que la production d'œufs du parasite a été diminuée, que la survie des larves L4 et des adultes a été augmentée surtout pour les parasites mâles. D'autre part et comme attendu, l'infection améliore les symptômes de l'hôte, voire les fait disparaitre. Ces résultats démontrent l'impact qu'a le statut inflammatoire de l'hôte sur les traits d'histoire de vie du parasite.

Cependant, l'environnement varie, et les conditions que rencontre un parasite ne sont pas toujours les mêmes lors de son infestation. Par exemple la coïnfection avec d'autres parasites (Behnke et al. 2005), l'âge (Lavoie 2007), la résistance (Reynolds 2012) ou le régime alimentaire de l'hôte (Kristan 2007, 2008) sont des paramètres qui influencent les traits d'histoire de vie des parasites. Par ailleurs, les expérimentations animales ont montré que le

traitement par des helminthes avait aussi bien un pouvoir curatif que préventif (voir Heylen et al. 2014), ce qui place le parasite dans des environnements inflammatoires différents qui peuvent potentiellement altérer différentiellement ses traits d'histoire de vie. Il semble donc essentiel d'aborder la plasticité des traits d'histoire de vie du parasite dans un environnement inflammatoire variable, ainsi que celle de ses capacités de régulation. Outre l'adaptation plastique, des parasites constamment confrontés à un environnement inflammatoire sur plusieurs générations pourraient aussi montrer des adaptations non plastiques à cet environnement.

Problématique

La course aux armements entre les parasites et leurs hôtes a doté chacun des camps de caractéristiques de défenses et d'évitements acquises au cours de leur coévolution. Néanmoins, malgré l'évidente nécessité du système immunitaire dans la lutte contre les agents pathogènes, ce dernier arbore des coûts aussi bien énergétiques qu'immunopathologiques, mais aussi sur le long terme avec l'apparition de maladie inflammatoires avec l'âge, montrant que l'immunité résulte d'un ensemble de compromis évolutifs. Mais dans quelles mesures les bénéfices de l'immunité surpassent ses coûts et expliquent le maintien de cet ensemble de troubles immunitaires observés de nos jours ?

De l'autre côté du front, les parasites comme les helminthes montrent des capacités d'évasion de l'immunité très poussées qui leur permettent de faire face à l'immunité de leur hôte dans la durée et d'être souvent favorables à leur hôte lors d'affections inflammatoires chroniques. Cependant, l'environnement inflammatoire de l'hôte va avoir un impact sur les traits d'histoire de vie du parasite. Ainsi, nous pouvons nous poser la question des coûts et bénéfices pour les parasites immunomodulateurs dans cet environnement inflammatoire.

L'environnement inflammatoire auquel les parasites doivent faire face est très variable de par la plasticité de l'immunité et de l'état inflammatoire de l'hôte à leur arrivée mais aussi tout au long de l'infection durant laquelle des modifications de cet état inflammatoire peuvent survenir. Par ailleurs, les parasites contre lesquels les hôtes doivent lutter ne sont pas tous identiques et arborent de nombreuses tactiques d'échappement de l'immunité.

Ainsi, étudier les relations hôte-parasites avec une approche écologique et évolutive, autrement dit (1) en faisant varier les environnements des hôtes et parasites par une approche de normes de réaction, ou de variations temporelles de l'environnement, (2) mais aussi en terme de coûts et bénéfices pour chacun des protagonistes, est essentiel à la compréhension des compromis liés à l'immunité et à sa régulation aussi bien pour l'hôte que pour le parasite.

Les modèles biologiques utilisés

Lors de mes travaux j'ai utilisé des souris de laboratoire de différentes souches : SJL, BALB/c CBA et B6CBAF1 femelles pour leurs divergences immunitaires envers le nématode intestinal parasite *Heligmosomoides polygyrus* (Reynolds 2012). Seuls quelques mâles ont servi à la reproduction lors d'une étude. L'ensemble des souris ont été achetées au fournisseur JANVIERLABS. Mis à part pour le chapitre 6 concernant la pléiotropie antagoniste où j'ai utilisé *Plasmodium yoelii*, toutes les souris infectées l'ont été par le nématode *Heligmosomoides polygyrus*.

Heligmosomoides polygyrus (bakeri)

Heligmosomoides polygyrus, aussi connu sous le nom de *Nematospiroides dubius*, est un nématode intestinal parasite à cycle direct de muridés (Ehrenford, 1954). Plusieurs sous espèces sauvages existent et sont appelées *H. polygyrus polygyrus* infectant le mulot (*Apodemus sylvaticus*) en Europe, *H. polygyrus corsicus* retrouvée chez la souris domestique (*Mus musculus domesticus*) en Corse et *H. polygyrus americanus* chez le campagnol des bruyères (*Phenacomys intermedius*) en Amérique du nord (Behnke, 1991). *Heligmosomoides polygyrus* est phylogénétiquement proche de parasites de ruminants (e.g. *Heamonchus contortus*) mais aussi de l'homme (*Ancylostoma duodenale* et *Necator americanus* ; de Bellocq et al. 2001).

La sous-espèce d'*Heligmosomoides polygyrus* maintenant utilisée à travers le monde en laboratoire est connue sous le nom de *Heligmosomoides polygyrus bakeri*. Dans ce manuscrit nous utiliserons le nom de d'*Heligmosomoides polygyrus* comme référence au modèle de laboratoire *Heligmosomoides polygyrus bakeri* (pareillement à la majorité des publications à son sujet).

Cycle de vie

Le stage L3 des larves de ce parasite est le stade infectieux. Ces larves translucides d'un demi millimètre, alors libres dans l'environnement, sont ingérées par l'hôte lors de son alimentation. Elles vont ensuite suivre le tube digestif et vont s'enkyster dans la paroi de l'intestin grêle juste après ou quelques centimètres après l'estomac. Là, ces larves vont muer deux fois pour atteinte le stade adulte sexué, d'environ un (pour les mâles) à deux (pour les femelles) centimètres et d'une coloration rouge, et ressortir dans la lumière tube digestif au

bout 7 à 8 jours d'infection. Les adultes s'accrochent aux villosités intestinales, aussi source de nourriture pour eux (Bansemir et Sukhdeo, 1994), et s'y reproduisent. Au huitième jour post-infection les femelles commencent à pondre leurs œufs que l'on retrouve dans les fèces des souris à peine 24h après. Ces œufs contiennent 8 à 16 cellules à la sortie de l'hôte et qui vont donner les larves au stade L1 qui vont éclore 2 jours plus tard et après 24h de plus les L1 muent en larves de stade L2, qui se nourrissent des bactéries de l'environnement. Après 4 jours, les larves L2 vont muer en larve L3, le stade infectieux.

A l'état naturel, le mulot (*Apodemus sylvaticus*) a une charge parasitaire de 12 vers par souris en moyenne et cette charge peut monter jusqu'à 250 (Gregory 1990).

Actuellement, de nombreuses souches de souris domestiques ont été utilisées pour cultiver mais aussi étudier ce parasite et montrent un gradient de résistance-susceptibilité à *Heligmosomoides polygyrus* (Reynold et al. 2012).

Résistance et susceptibilité à Heligmosomoides polygyrus

De façon similaire aux autres nématodes, les infections avec *Heligmosomoides polygyrus* induisent l'activation des cellules T helper CD4+ vers une réponse de type Th2 (cellules T helper de type 2 - Allen and Maizels 2011; Maizels et al. 2012a) caractérisée par la production de cytokines Th2, principalement l'interleukine-4 (Urban et al 1991) accompagnée des interleukines 9, 5, 13. L'IL-4 a été identifiée comme un marker de résistance dans les modèles expérimentaux d'infection par des nématodes (Urban et al. 1998; Turner et al. 2003; Finkelman et al. 2004; Hayes et al. 2004; Anthony et al. 2007). Les cytokines de la voie Th2 comme l'IL-4 et IL-13 activent aussi les macrophages M2 et provoquent l'expulsion du parasite en induisant la production de mucus par les cellules en gobelet et une hypercontractilité des muscles lisses (Zhao et al. 2008). A l'inverse, le manque d'IL-4, d'origine génétique ou environnemental, est associé à une réponse de type Th1 et mène à la mise en place d'une susceptibilité à *H. polygyrus* (Ing et al. 2000; Maizels et al. 2012b; Filbey et al. 2014).

Capacités d'immunomodulation

Comme mentionné auparavant, les helminthes comme les nématodes et cestodes, ont la capacité d'interférer avec l'immunité de leur hôte pour améliorer leur propre fitness en

sécrétant divers composants. Chez *Heligmosomoides polygyrus* ces composés sont appelés HES pour « *Heligmosomoides polygyrus* Excretory-Secretory products ».

Ces composés agissent principalement en polarisant les cellules T naïves vers une maturation en cellules T régulatrices productrices d'IL-10 capablent d'atténuer la réponse de type Th1 et Th2 (Finney et al. 2007 ; Setiawan et al. 2007; Rausch et al 2008 ; Grainger et al. 2010). Ces HES sont aussi capables d'inhiber la réponse des cellules dendritiques suite à l'interaction ligand-TLR (Segura et al 2007) ou de réduire la sécrétion d'IL-12 (Massacand et al. 2009). Une synthèse des composés retrouvés dans les HES est présentée dans le tableau ci-joint repris de McSorley et al. 2012.

Tableau 1. Les familles de protéines contenus dans les produits d'excrétion et de sécrétion de *Heligmosomoides polygyrus.* Extrait et traduit de McSorley et al. 2012.

Famille de protéine	*Effets immunologiques*	*Références*
Apyrase	Dégradation de l'ATP inflammatoire	Idzko et al. 2007
	Supprime les pathologies liées à l'asthme	
	Induction de la voie Th17 dans le tube digestif	Atarashi et al. 2008
Chitinase	La chitin promeut la voie Th2	Sutherland et al. 2009 et Da Silva et al. 2010
	La chitinase inhibe la voie Th2 ?	
Cystatine	Inhibition du processus de création de déterminants antigéniques	Manoury et al. 2001
	Supprime les pathologies liées à l'asthme et à la colite ulcerante	Schnoeller et al. 2008 et Jang et al. 2011
Galectine	Apoptose des cellules T activées, principalement Th1	Kim etal. 2010 et Liu et al. 2012
Lysozyme	Dégradation de la paroi des bactéries	Dommett et al. 2005
	Provoque un changement dans la balance des commencaux ?	Walk et al. 2010
Retinol-ligand	Le retinol induit les cellules Treg et supprime la voie Th17	Esteves et Ehrlich 2006, Elias et al. 2008 et Kim et al. 2012
	Réduit la réponse immunitaire en cas de déficience en rétinol	
Serpine	Inhibition des sérines protéases	Zang et al. 1999, Molehin et al. 2012 et Lefrançais et al. 2012
	Inhibition de l'activation par l'IL-33	
VAL/ASP/SCP	Allergisants	Diemert et al. 2012
	Candidats comme vaccins	Asojo et al. 2005
	Attraction des neutrophiles	Bower et al. 2008
	Promeut la voie Th1	He et al. 2009
Mimic du TGF-β	Supprime la prolifération Th1 et Th2	Grainger et al. 2010
	Induit les cellules Treg Foxp3+	
	Supprime la maturation des cellules présentatrices d'antigènes	
	Empêche de transduction du signal des TLR	

Treg = cellules T régulatrices; VAL : Venom-allergen-like; ASP : Protéines sécrétées par *Ancylostoma* ; SCP : protéine du manteau spermatique ; TLR : récepteur toll-like

Le genre plasmodium

Les espèces du genre *Plasmodium* sont des protozoaires de l'embranchement des apicomplexes. Ces parasites dixènes (à deux hôtes durant leur cycle parasitaire) ont pour hôtes un diptère du genre *Culex, Anopheles, Culiseta, Mansonia* ou *Aedes* et un second hôte mammifère primate (dont l'homme : e.g. *P. falciparum*) ou non primate (rongeur : e.g. *P. chabaudi* ou *yoelii*), oiseau (e.g. *P. ashfordi*) ou reptile (e.g. *P. iguanae*). Chez l'homme, ces parasites, principalement *P. falciparum*, provoquent des symptômes allant de fièvre élevée accompagnée de douleurs diffuses (maux de tête, courbatures) et de troubles digestifs (nausées, diarrhées) à des troubles de la conscience, ictères et atteintes de la fonction rénale, voire la mort. D'après le CDC (le centre de prévention et de contrôle des maladies aux Etats Unis), environ 214 millions de personnes ont contracté la malaria à travers le monde et 438 000 personnes en sont mortes en 2015 ce qui en fait l'un des plus gros fléaux sanitaires à l'échelle mondiale actuellement.

Cycle de vie

Le genre *Plasmodium* infecte successivement deux hôtes pour boucler son cycle, l'un étant un diptère et le second un mammifère, oiseau ou reptile (cycle parasitaire fig. 2 : *Plasmodium* humain). Le diptère est considéré comme son hôte définitif car la phase sexuée se produit chez ce dernier. Les gamétocytes, microgamétocytes (gamètes mâles) et macrogamétocytes (gamètes femelles) sont ingérés par le diptère lors du repas de sang sur l'hôte. Dans l'estomac du diptère, les microgamétocytes vont pénétrer les macrogamétocytes pour former le zygote diploïde. Le zygote devient alors un ookinete et va pénétrer la paroi de l'intestin du diptère et s'y enkyster pour se multiplier pour former un oocyste rempli de sporozoïtes. Une fois que l'oocyste se rompt, les sporozoïtes migrent vers les glandes salivaires du diptère où ils seront transmis à l'hôte secondaire lors du prochain repas de sang. Cette phase du cycle chez le diptère se nomme le cycle sporogonique. Une fois dans la circulation sanguine de son hôte, les sporozoïtes infectent les cellules du foie et y maturent en schizontes. A la rupture des schizontes, des mérozoïtes sont relâchés. Ces derniers peuvent soit infecter de nouvelles cellules hépatiques ou infecter des globules rouges. C'est le cycle extra-érythrocytaire. Une fois dans un globule rouge, le parasite entame une reproduction asexuée : c'est le cycle érythrocytaire. Les mérozoïtes se transforment en trophozoïtes immatures puis maturent et se reproduisent pour former un

schizonte comportant de nouveaux mérozoïtes qui infecteront de nouveaux érythrocytes. Au stade trophozoïtes immatures, ces derniers peuvent se transformer en gamétocytes qui infecteront un diptère lors de son repas de sang.

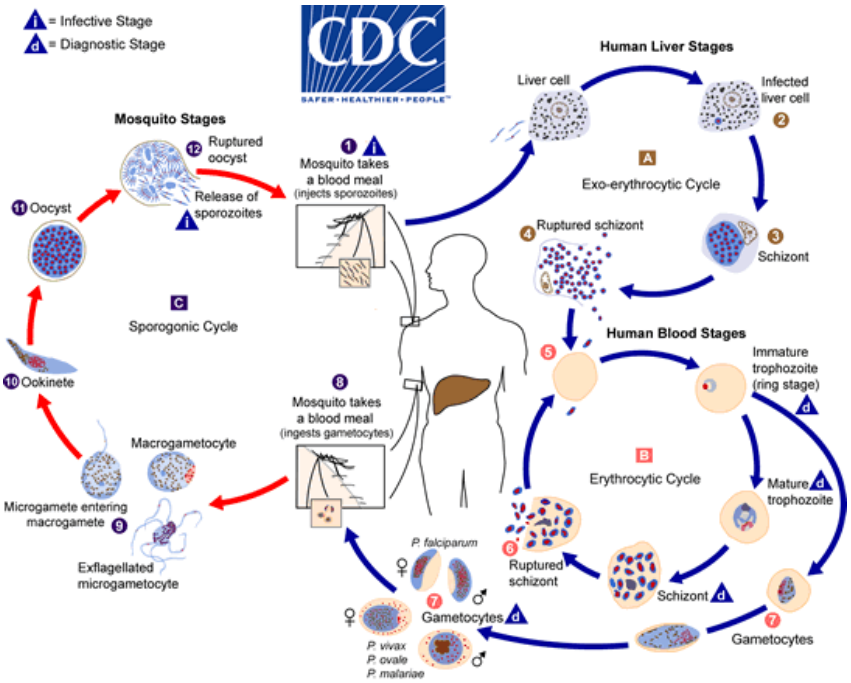

Figure 2. Cycle de vie de *Plasmodium falciparum.* Récupéré du site web du CDC. http://www.cdc.gov/malaria/about/biology/index.html

Réponse immunitaire mise en place lors d'une infection à plasmodium

Lors d'un premier contact la réponse immunitaire est plutôt une réponse de type Th1, avec production de cytokines inflammatoires IL-2, IL-12, TNF-α et IFN-γ. La mise en place d'une réponse humorale démarre plus tardivement (pendant la diminution de la parasitémie lors du pic initial : deuxième semaine d'infection) avec la production IgM et IgG2a pour finir avec la production d'IgG1 et IgE lors de pics secondaires si une infection chronique s'installe (Taylor-Robinson 2010).

Plasmodium yoelii

Durant mes travaux j'ai utilisé un plasmodium de rongeur (*Plasmodium yoelii – souche 17XNL)* pour pouvoir travailler sur l'aspect pléiotropique antagoniste de l'immunité (chapitre 5). Cette espèce est originaire d'Afrique centrale et a été isolée de muridés du genre *Thamnomys*.

Plan

Dans un premier temps j'aborderai la plasticité phénotypique des deux protagonistes lors de variations environnementales aussi bien pour le parasite que l'hôte au travers d'une approche en normes de réaction (chapitre 1) puis lors de variation temporelle de l'état immunitaire de l'hôte à différents stades du cycle de vie du parasite (chapitre 2) ou encore la plasticité de l'immunomodulation du parasite confronté à un environnement inflammatoire (chapitre 2) ou à une régulation immunitaire inhibée (chapitre 3).

La plasticité phénotypique est attendue dans ces interactions, cependant, comme abordé précédemment, la plasticité est limitée et seule l'adaptation sur plusieurs générations à un environnement donné peut permettre un ajustement du phénotype par une adaptation locale sur un plus ou moins grand nombre de générations. Ainsi dans un second temps j'aborderai les possibilités d'évolution des traits d'histoire de vie du parasite confronté à un environnement inflammatoire mais aussi l'évolution de ses capacités d'immunomodulation (chapitre 3 et 4).

Par ailleurs, la sélection peut aussi mener à la fixation de traits délétères à un âge avancé car ces derniers peuvent avoir un effet pléiotrope antagoniste en favorisant l'individu qui les porte à un jeune âge, âge où la sélection a le plus de prise. Ainsi l'immunité est essentielle pour la lutte contre les parasites alors qu'elle provoque des dégâts à l'organisme et des troubles inflammatoires à un âge avancé. Ainsi je me suis intéressé au possible rôle pléiotrope antagoniste de l'immunité, domaine resté jusqu'à maintenant plutôt théorique (chapitre 5).

Par ailleurs, bien que la coévolution avec nos parasites et nos symbiotes ait modelé notre immunité pour maximiser notre fitness, l'adaptation à un environnement peut aussi nous démunir lors d'une altération brutale de cet environnement. Ainsi l'augmentation actuelle des maladies inflammatoires pourrait en partie être attribuée à un changement dans notre environnement parasitaire et symbiotique qui fait de nous des individus mal-adaptés à notre environnement. J'aborderai ce sujet plus en détails lors du chapitre 6.

Au-delà des problèmes de mal-adaptation à un changement brutal d'environnement, les compromis évolutifs envers nos différents parasites ont mené à des messagers du système

immunitaires (cytokines) pouvant être à la fois bénéfiques ou délétères en fonction de la situation. Ainsi dans le chapitre 7 j'aborderai ce sujet avec une approche globale en utilisant une analyse bibliographique d'infections chez des individus dont une cytokine a été mise KO pour tenter de démêler les compromis sous-jacents liés aux coûts et bénéfices qu'elles représentent aussi bien pour l'hôte que pour le parasite en abordant la question de la place de l'immunopathologie.

Finalement, je ferai une synthèse de mes recherches en confrontant mes résultats issus de plusieurs de mes travaux entre eux et avec la littérature et conclurai en apportant des pistes de recherche pour de futurs travaux.

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Chapitre 1 : Normes de réactions de l'immunité et de la fitness de l'hôte et des performances du parasite dans l'interaction souris – nématode intestinal.

Title: Reaction norms of host immunity, host fitness, and parasite performance in a mouse–intestinal nematode interaction

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Abstract

The outcome of the encounter between a host and a parasite depends on the synergistic effects of the genetics of the two partners and the environment (sensu lato) where the interaction takes place. Reaction norms can depict how host and parasite traits vary across environmental ranges for different genotypes. Here, we performed a large scale experiment where three strains of laboratory mice (SJL, BALB/c and CBA) were infected with four doses of the intestinal nematode Heligmosomoides polygyrus. Increasing infective dose can be considered as a proxy of the environment-dependent risk to contract the infection. We looked at the fitness traits of hosts and parasites, and we assessed the underlying immunological functions likely to affect the observed pattern of resistance/susceptibility/tolerance. We found that infective dose had a strong effect on both host fitness and parasite performance. Interestingly, for most traits, host genotypes did not rank consistently across the increasing infective doses and according to the expected pattern of strain-specific resistance/susceptibility/tolerance. Analyses of cytokine production allowed to better understanding the mechanistic basis underlying variation in fitness-linked traits. Infective dose affected the shape of the reaction norms of the cytokines IL-4, IL-10 and IL-6. Dose-dependent variation in cytokine production explained, moreover, the strain-specific pattern of infection cost, host resistance and parasite performance. As long as the infective dose increased, there was a marked shift towards a pro-inflammatory status in the SJL strain that was positively correlated with cost of the infection and parasite performance. Overall, our study strongly suggests that the notion of host resistance is labile and depends on the environmental conditions where the interaction takes place. Moreover, integrating information on fitness-linked traits and the underlying mechanisms seems essential for a better understanding of host and parasite adaptations across variable environments.

Introduction

The negative impact of parasites on host fitness can arise as the consequence of multiple factors. Parasites can divert metabolic resources that are no longer available for the vital functions of the host. Parasite multiplication, reproduction or migration within the host can cause direct harm to host tissues. Pathogens can also release toxic compounds (e.g. botulinum toxin, cholera toxin, *or enterotoxins*). Finally, the immune response mounted against an invading parasite can also induce damage if it targets host cells and tissues or if it is over-expressed. Whatever the mechanism underlying the cost of infection (metabolic, disruption of tissues, toxin release, or immunopathology), its magnitude is likely to depend on the interaction between host genes, parasite genes and the environmental conditions encountered by the two partners (Tetard-Jones et al. 2007; Zouache et al. 2014).

Reaction norms are a powerful way to depict how host and parasite fitness varies as a function of the genetic background across environmental gradients. A reaction norm refers to the capacity of a given genotype to express different phenotypes when exposed to varying environmental conditions, and can be assessed as the slope of the regression of phenotypic values over environmental values. If the slope is different from zero the phenotypic trait is plastic (its expression does depend on the environmental conditions). Genotypes may also differ in their capacity to respond to environmental changes and their relative fitness within each environmental type can change as long as the environment changes (Scheiner 1993; Wolinska and King, 2009). This can give rise to non-parallel reaction norms, with the phenotypic expression depending on the interaction between the genotype and the environment. Genotype by environment interactions indicate that genotypes might be more or less adapted to local environmental conditions and that phenotypic plasticity is itself under genetic control (Lazzaro and Little 2009).

Characterizing environmental conditions can be challenging since several biotic and abiotic factors contribute to define environmental quality. An experimental approach is therefore indispensable to tease apart the effect of different environmental features. Several studies have investigated the effect of biotic and abiotic factors on the outcome of the infection, the pattern of resistance/susceptibility and the specificity of host and parasite genotypes (Bedhomme et al. 2004; Lazzaro et al. 2008; Sadd 2011; Murdock et al. 2013). For

instance, the diatom *Asterionella formosa* infected with the chytrid parasite *Zygorhizidium planktonicum* shows a genotype- and temperature-dependent pattern of susceptibility, with some genotypes being resistant at certain temperatures while becoming susceptible at others (Gsell et al. 2013). Similar patterns have been reported for a range of organisms. Lazzaro et al. (2008) studied the resistance of fruit flies (*Drosophila melanogaster*) from three geographic regions (one in Africa and two in North America) to bacterial infection across three different temperatures. They found that bacterial load was the lowest at high temperature for host genotypes coming from the African population, whereas the same genotypes suffered the highest bacterial load at low temperature. Unfortunately, however, the underlying immunological mechanisms were not investigated.

While temperature and resource availability have been the most consistently studied environmental traits when investigating reaction norms, the environment can also be explicitly described by biotic interactions that the host experiences with other organisms (predators, competitors, pathogens) sharing its habitat. Environments can spatially (Caceres et al. 2006) and temporally (Altizer et al. 2006) differ in their abundance of pathogenic organisms or vectors that can transmit infectious diseases, and this usually induces a difference in parasite burden across host populations living in different habitats (Froeschke et al. 2010; Loiseau et al. 2013). One way to approach this environmental variability, independently from other environmental traits, is to experimentally infect hosts with different inoculum sizes; a high inoculum size representing a risky environment, a low inoculum size (or a zero inoculum size) a safer environment (Ben-Ami et al. 2008).

If parasite abundance is among the most important components of host environment, from the perspective of the parasite, the immune system of the host probably represents the principal environmental feature it has to cope with when entering the host. Exposing the parasite to different hosts (either different genotypes or different engineered phenotypes) with different immune functioning is therefore a useful way to manipulate the environment the parasite faces within the host.

The aim of the present article was to investigate the reaction norms of host immunity, host fitness and parasite performance over environmental gradients. To this purpose, we used the association between lab strains of house mice and the intestinal nematode

Heligmosomoides polygyrus bakeri, a natural parasite of rodents with a direct life cycle (Ehrenford 1954). As for other nematode parasites, infection with *H. polygyrus* induces the activation of $CD4^+$ T lymphocytes (T-helper) towards a Th2 profile (Allen and Maizels 2011; Maizels et al. 2012a) characterized by the production of Th2 cytokines, interleukin-4 (IL-4) being one of the hallmarks of the Th2 response (Urban et al. 1991). IL-4 has indeed been shown to be a marker of resistance in experimental models of infection with nematodes (Urban et al. 1998; Turner et al. 2003; Finkelman et al. 2004; Hayes et al. 2004; Anthony et al. 2007). Th2 cytokines, such as IL-4 and IL-13, also activate M2 macrophages and promote parasite expulsion by inducing fluid secretion in the gut, smooth muscle contraction and thickening, and intestinal hypercontractility (Zhao et al. 2008). On the contrary, a lack of IL-4, genetically or environmentally driven, associated with a Th1 response has been shown to lead to susceptibility to *H. polygyrus* (Ing et al. 2000; Maizels et al. 2012b; Filbey et al. 2014).

We infected three lab strains of house mice (SJL/JRj, BALB/cJRj, and CBA/JRj) with 4 doses of infective larvae (0, 200, 400, and 600) of *H. polygyrus*. The three mouse strains have been described as having different patterns of resistance/susceptibility to the infection with the nematode, SJL and BALB/c being considered as resistant (expelling adult parasites within four to six weeks) and CBA as susceptible (the infection persisting for months) (Reynolds et al. 2012). CBA mice should probably also be considered as tolerant to the infection. Tolerance is a concept routinely used by phytopathologists that has been recently adapted to animal infections (Raberg et al. 2007) and refers to individuals that withstand the infection paying small or no costs at all (Schneider and Ayres, 2008). Even though immunological tolerance and tolerance to the infection are not strict synonymous terms, tolerance to the infection often involves the activation of immunological effectors that prevent immunopathology (Medzhitov et al. 2012). We therefore provided a different immune environment to the parasite, and exposed the host to different environmental risk using different infective doses that simulate a more or less safe environment. We then assessed fitness-linked traits for both partners of the interaction (host body mass and survival, parasite egg count) and the underlying immunological mediators. We assessed IL-4, the main Th2 cytokine involved in the expulsion of the parasite, the pro-inflammatory cytokine IL-6, and the anti-inflammatory cytokine IL-10.

We expected that cytokine production should be strain specific (Filbey et al. 2014) because of different resistance/susceptibility/tolerance to the parasite. In particular, we predicted that resistant host strains should produce more Th2 cytokines as long as the infective dose increases (as previously shown for rats infected with the nematode *Strongyloides ratti*, (Bleay et al. 2007), whereas the susceptible/tolerant strain should have a flat reaction norm for Th2 cytokines, resulting in a strain by environment interaction. Concerning host fitness, we predicted that resistant host strains should have higher fitness at low parasite dose and a shallower fitness decrease with increasing parasite dose compared to the susceptible/tolerant strain, resulting in non-parallel reaction norms. However, this simple prediction might be challenged if resistance is coupled with an over-reacting immune response and the associated immunopathology cost (Graham et al. 2005; Colditz 2008; Long and Boots, 2011; Guerreiro et al. 2012) across the gradient of infective doses. Concerning parasite fitness, we predicted that fecal egg count should be higher in the more permissive environment (the CBA strain) compared to the resistant strains and that the slope of egg count on infective dose should be steeper for the permissive strain across the gradient of infective doses.

Materials and methods

Ethics statement

All animal experiments were approved by the Comité d'Ethique de l'Expérimentation Animale Grand Campus Dijon (CNREEA n° C2EA – 105) and by the "Ministère de la Recherche et de l'Enseignement Supérieur" (project N°01867.01) according to the national guidelines ("Charte nationale portant sur l'éthique de l'expérimentation animale", Ministère de la Recherche et de l'Enseignement Supérieur) on the use of animals for research purposes.

Mice, parasites and treatments

CBA, BALB/c and SJL female mice were purchased from JanvierLABS (JRj) and housed (five individuals per cage 18.5 x 38 x 22.5cm enriched with shelters) at the Université de Bourgogne. They were maintained under constant temperature (24°C) and photoperiod (12L:12D). They were fed with standard mouse pellets *ad libitum* and had *ad libitum* access to filtered tap water. At the age of 7 weeks, mice were infected with L3 stage larvae of the intestinal nematode *Heligmosomoides polygyrus bakeri*. Five mice per strain received by

gavage 200, 400, or 600 larvae in 0.1 ml of drinking water, using a feeding needle on an insulin syringe of 1 ml. Control, non-infected, individuals received the same volume of water with no larvae. We therefore had 12 experimental groups (3 strains x 4 infective doses) each containing five mice (total sample size = 60). At the end of the experiment, mice were euthanized by cervical dislocation (or before the end of the experiment if they reached the limit point).

L3 larvae were obtained from B6CBAF1/JRj mice, as maintaining hosts. Freshly collected feces were mixed with distilled water and coal in powder, spread on a Whatmann paper (grade 40) kept wet by daily humidification in a Petri dish at ambient temperature. Stage 3 larvae were harvested 8 days after feces collection.

Host fitness

Mice were monitored daily for mortality until day 32 post-infection. Body mass (precision 0.1 g) was measured at day 0, 7, 11, 14, 18, 20, 25, 28, 32 post-infection. At day 7 post-infection we took a blood sample for cytokine analysis. Blood samples (approximately 100 µl) were collected from the maxillary vein using a sterile lancet needle. Blood was immediately centrifuged at 4° C and 4,000 g for 10 minutes. Plasma was immediately stored at -80°C for further analysis.

Parasite fitness

We used fecal egg count as a proxy of parasite fitness. Fecal egg count combines parasite fecundity and the adult parasite population size. High values of egg count therefore indicate hosts that harbored large parasite populations and/or highly fecund worms. At day 11, 14, 18, 20, 25, 28, 32 post-infection, each mouse was transferred at 9 am on a grid in an individual cage with a humidified towel paper at the bottom to prevent feces desiccation. Mice were left four hours in these cages and then put back in their shared cages. Feces produced during this four-hour period were collected and 200 mg were mashed and suspended in 5 ml of water. Thereafter, 10 ml of salted water (75% of saturation) were added to allow eggs to float. After agitation, a fraction of this suspension was transferred into a McMaster chamber for egg count. We performed two counts per sample and used the mean values. Fecal egg count is expressed as number of eggs per mg of feces. In addition to

daily egg count, we also computed the total egg production as the sum of each fecal egg count throughout the study.

Cytokine assay

We used the Luminex technology on Bio-plex200 instrument (Biorad®) to assess the concentration of plasmatic cytokines. We used Milliplex® Map kit (MCYTOMAG-70K, Millipore) to assess the concentration of five cytokines: IL-4, IL-6, IL-10, TNF-α and IFN-ɣ. We used a total of 25µl of plasma for the five cytokines following manufacturer kit protocol. The flow cytometry runs, based on standard curve, were carried out using Bio-Plex software (Biorad). The values below the standard curve were considered with conservative estimation corresponding to the lower values of the standard curve of each cytokine. However, for TNF-α and IFN-ɣ this concerned the vast majority of samples, which therefore prevented us to use these values in the statistical analyses. Cytokine analyses were thus restricted to IL-4, IL-6, and IL-10.

Statistical analyses

Body mass was analyzed using a generalized linear mixed model with a normal distribution of errors. Mouse identity was declared as a random factor to take into account the repeated nature of the data. Fixed factors included in the model were: time, mouse strain, infective dose, and all the interactions. Degrees of freedom were estimated using the Satterthwaite method.

Mortality was analyzed using the status of each individual at the end of the study period (dead or alive) and entered into a generalized linear model with a binomial distribution of errors. Explanatory variables were host strain, infective dose and the interaction between the two.

Fecal egg count was log-transformed to normalize the data and analyzed using a generalized linear mixed model with a normal distribution of errors. Mouse identity was declared as a random factor. Fixed factors were time, *z*-transformed squared-time, host strains and infective dose. Degrees of freedom were estimated using the Satterthwaite method.

We also analyzed total egg output during the study period using a generalized linear model as described above with the exception that the random effect was removed from the model (there was only one observation per individual mouse).

Cytokine concentration was also log-transformed to normalize the data and analyzed with a generalized linear model where host strains and infective doses were the independent variables. Graphical inspection of the data indicated a possible non-linear relationship between cytokines and infective dose. Therefore a *z*-transformed quadratic term was also included in the model (and the interaction between squared dose and strain).

We also explored the possible correlation between cytokine production and parasite fitness. This was achieved by using IL-4, IL-6 and IL-10 production (log-transformed) at day 7 and fecal egg count (log-transformed) at day 11 (in a generalized linear model). We also computed the ratio between log-transformed values of IL-6 and IL-10 plasmatic concentrations to check whether a shift toward a pro-inflammatory status incurred a cost for the host, in terms of mortality. This was achieved by adding the IL-6/IL-10 ratio to the generalized linear model (with a binomial distribution of errors) that was used to analyze mouse mortality. The model had then the status of each individual at the end of the study period (dead or alive) as the dependent variable and host strain, infective dose, and IL-6/IL-10 ratio as explanatory variables.

For each model we started with the full set of interaction terms and simplified it by removing the highest interactions when not significant.

Variation in sample size reflects missing values for some variables (for instance, for some animals blood samples were too small to allow cytokines measurements), and animals that were euthanized during the course of the experiment because they reached the limit point.

All statistical analyses were performed with SAS (version 9.2).

Results

Host mortality

The model exploring the effect of strain and dose on host survival showed a strong effect of infective dose (mortality substantially increased as long as the dose increased, $\chi_1^2 = 13.14$, p = 0.0003) and an effect of strain (SJL having a lower survival compared to the other two strains, $\chi_2^2 = 9.48$, p = 0.0087) (Fig 1). We could not directly test the significance of the interaction between dose and strain because the model did not converge when including the interaction term, certainly because of quasi-complete separation of data points across cells. We, however, compared the AICs of models containing only dose, only strain, strain + dose and the additive and interactive effects and found that the additive model (strain + dose) had the lowest AIC (30.35), suggesting that the interaction was not statistically significant (AIC = 33.46).

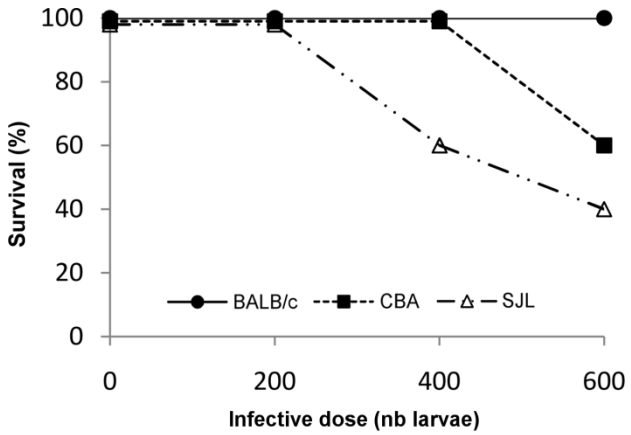

Figure 1. Host mortality at day 32 pi as a function of infective dose and strain. Full circles and full line refer to BALB/c, full squares and pointed line refer to CBA, empty triangles and dashed line refer to SJL.

Host body mass

We ran a generalized linear mixed model to explore the variation of body mass during the study period. The model included time post-infection, strain, dose and the two- and three-way interactions. The three-way interaction between time, strain and dose was statistically significant (Table 1). Overall, mice tended to gain body mass over time, but for BALB/c and SJL the gain in body mass was greater for the highest infective doses (Fig 2). We also ran three independent models for each strain to check whether the slopes relating body mass with time differed among the four infective doses. The results showed that the interaction

between time post-infection and dose was indeed significant for BALB/c and SJL but not for CBA (Table 2).

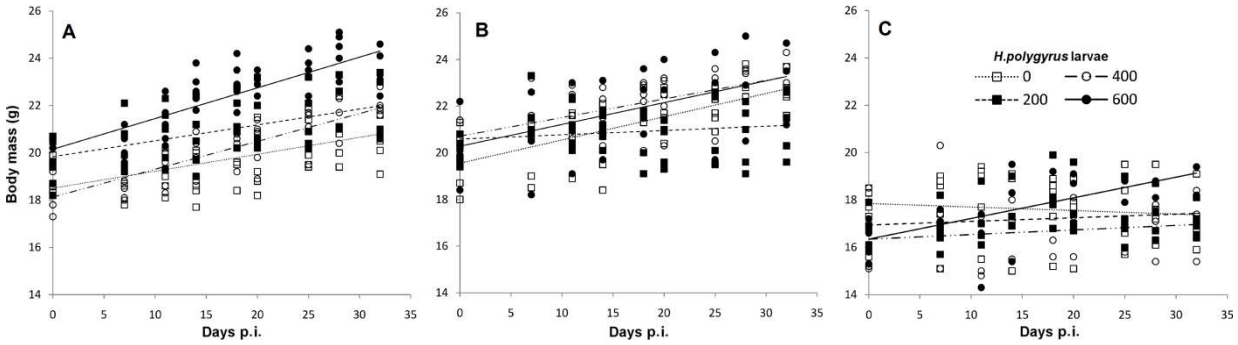

Figure 2. Body mass (g) trajectories from day 0 to day 32 pi according to the infective dose and host strain. Each panel represents a mouse strain: (A) BALB/c, (B) CBA, (C) SJL. Each line represents the linear regression of body mass over time for each infective dose. Control: empty squares and pointed line; 200 larvae: filled squares and dashed line; 400 larvae: empty dots and dashed pointed line; 600 larvae: filled dots and full line.

Table 1.Generalized linear mixed model exploring the effect of strain and dose on variation in body mass (g) over the period covered by the study (32 days). Mouse identity was included in the model as a random factor to take into account the repeated measurements.

Sources of variation	df	F	P
Time	1,427	57.65	<0.0001
Strain	2,67.6	7.13	0.0015
Dose	1,68.7	0.05	0.8325
Time*Strain	2,427	24.63	<0.0001
Time*Dose	1,437	29.50	<0.0001
Strain*Dose	2,68.6	3.59	0.0330
Time*Strain*Dose	2,436	5.46	0.0046
Random effect		Z	P
Mouse ID		4.85	<0.0001

Table 2. Generalized linear mixed model exploring the effect of infective dose on variation in body mass (g) over the period covered by the study (32 days) by strain. Mouse identity was included in the model as a random factor to take into account the repeated measurements.

Strains	BALB/c			CBA			SJL		
Sources of variation	df	F	P	df	F	P	df	F	P
Time	1,158	82.84	<0.0001	1,146	33.26	<0.0001	1,124	2.63	0.1077
Dose	1,21.2	2.05	0.1672	1,32.3	1.26	0.2692	1,19.9	3.41	0.0798
Time*Dose	1,158	36.98	<0.0001	1,151	0.15	0.6974	1,127	26.23	<0.0001
Random effect		Z	P		Z	P		Z	P
Mouse ID		2.92	0.0018		2.65	0.0041		2.84	0.0022

Cytokine response

We explored the host response in terms of production of five key cytokines at day 7 pi: IL-4, IL-6 and IL-10, TNF-α and IFN-γ. However, TNF-α and IFN-γ had values that were below the detection threshold for most of the individuals which prevented us to use them in the statistical analyses.

We tested the interaction between host strain and infective dose for each cytokine. For both IL-4 and IL-6, there was some evidence supporting the idea that the relationship between cytokines and dose differed among strains in a non-linear way, even though the interaction was slightly above the significance threshold for IL-4 (squared dose x strain interaction, IL-4: $F_{2,47} = 2.97$, $p = 0.0632$; squared dose x strain interaction, IL-6: $F_{2,47} = 4.59$, $p = 0.0162$) (Fig 3).

IL-10also showed a strong non-linear relationship with infective dose (squared dose, $F_{1,43} = 11.20$, $p = 0.002$); however, for IL-10 the shape of the relationship was similar for the three host strains (dose x strain interaction, $F_{2,43} = 1.57$, $p = 0.2219$, Fig 3).

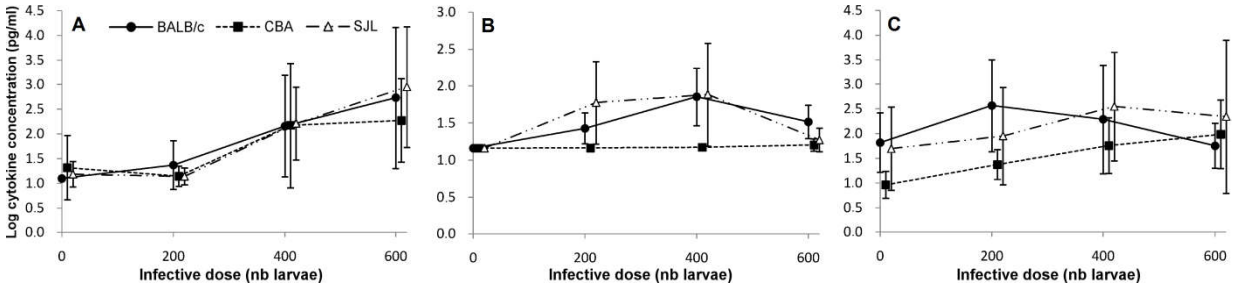

Figure 3. Reaction norms of circulating cytokines over a gradient of infective doses for three host strains. (A) IL-6 (log pg/ml), (B) IL-4 (log pg/ml), (C) IL-10 (log pg/ml). We report mean values (± SE) for each infective dose and host strain (full dots with full line: BALB/c; full squares with pointed line: CBA; empty triangles with dashed line: SJL).

Parasite performance

Due to a mistake of sample labeling, egg count was missing for CBA mice infected with 200 larvae at day 18 pi. No eggs were found at day 7 post-infection as expected from the timing of adult emergence in the intestinal lumen. At day 11, fecal egg counts were higher in CBA and lower in SJL and BALB/c mice. Over time, the number of eggs shed tended to decrease for BALB/c and SJL infected with 200 and 400 larvae, whereas the infection was stable for CBA. However, for the highest infective dose (600 larvae), fecal egg count remained constant over time for the three host strains. The statistical model showed that the three-way interactions between i) time post-infection, strain and dose, and ii) squared-time post-infection, strain and dose were non-significant, albeit the p values were close to the significance threshold (Table 3, Fig 4).

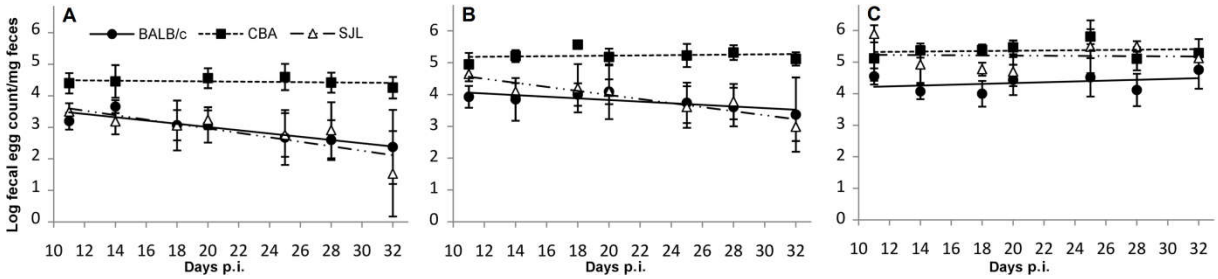

Figure 4. Fecal egg count over time (from day 11 to day 32 pi).The three panels refer to the three infective doses (A, 200 larvae; B, 400 larvae; C, 600 larvae). Full dots and full lines refer to BALB/c, full squares and pointed lines refer to CBA, empty triangles and dashed lines refer to SJL.

Table 3. Generalized linear mixed model exploring the effect of host strain and infective dose on variation in fecal egg count of *H. polygyrus* over the period covered by the study (32 days). Mouse identity was included in the model as a random factor to take into account the repeated measurements.

Sources of variation	df	F	P
Strain	2,199	0.01	0.9937
Dose	1,200	2.64	0.1056
Time	1,214	38.62	<0.0001
Time²	1,212	10.63	0.0013
Dose*Strain	2,200	1.20	0.3020
Time*Strain	2,213	7.62	0.0006
Time²*Strain	2,212	1.90	0.1522
Dose*Time	1,217	17.62	<0.0001
Dose*Time²	1,213	6.70	0.0103
Dose*Time*Strain	2,216	2.59	0.0771
Dose*Time²*Strain	2,213	2.83	0.0614
Random effect		Z	P
Mouse ID		3.08	0.0010

We also looked at the total fecal egg output during the study period by adding the values at day 11, 14, 20, 25, 28, 32 pi, which therefore concerned only the individuals that were alive at day 32 pi. Given that egg count was missing for one group at day 18, we removed these values for all strain/dose combinations to make the data comparable. We found a statistically significant interaction between strain and dose on total fecal egg count (dose x strain interaction, $F_{2,31}$ = 5.62, p = 0.0083). This interaction was due to different slopes relating total fecal egg count and dose for the different strains (Fig 5). In particular, whereas SJL strongly differed from CBA in the number of eggs shed in the feces when infected with a low number of larvae, the difference tended to vanish for the highest infective dose (Fig 5).

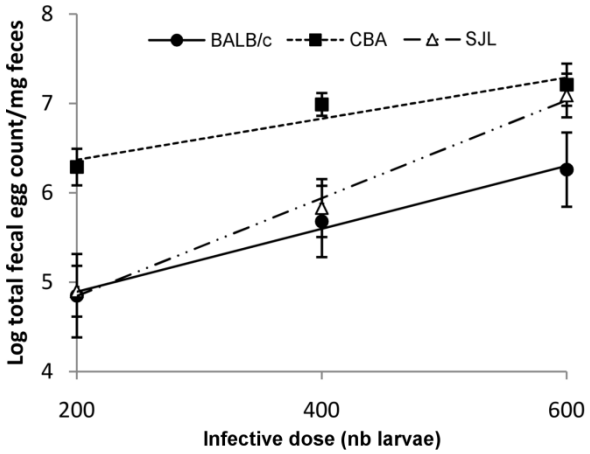

Figure 5. Total fecal egg count from day 11 to day 32 pi over the gradient of infective doses and host strains. Full circles and full line refer to BALB/c, full squares and pointed line refer to CBA, empty triangles and dashed line refer to SJL.

Cytokines as predictors of host and parasite fitness

We wished to investigate the idea that the observed increase in mortality of SJL mice exposed to the highest infective dose was due to the shift towards a pro-inflammatory status. To this purpose, we computed the ratio between IL-6 and IL-10. High values of this ratio indicate a poorly regulated inflammatory response and can be considered as a marker of a pro-inflammatory status. This ratio has been previously used in clinical literature to predict, for instance, the severity of systemic inflammatory response syndrome (Taniguchi et al. 1999). We included the ratio between IL-6 and IL-10 in the model that explored host mortality. In agreement with the prediction, we found that high values of the ratio were positively associated with mortality (χ^2_1 = 9.00, p = 0.0027), while infective dose (χ^2_1 = 22.29, p < 0.0001) and strain (χ^2_1 = 20.70, p < 0.0001) were still associated with host mortality.

We also investigated the possible role of cytokine production as a predictor of parasite fitness. Given that cytokines were measured at day 7 post-infection, we decided to focus on the number of eggs shed at day 11. We found a statistically significant interaction between IL-6 and strain (Table 4), showing that the slope of the regression between fecal egg count at day 11 pi and IL-6 production at day 7 pi differed among strains (Fig 6). IL-4 ($F_{1,27}$ = 0.10, p = 0.7512) and IL-10 ($F_{1,26}$ = 0.00, p = 0.9829) were not good predictors of fecal egg count; the interactions between strain and IL-4, and strain and IL-10 were not statistically significant (all p's > 0.05, Fig 6).

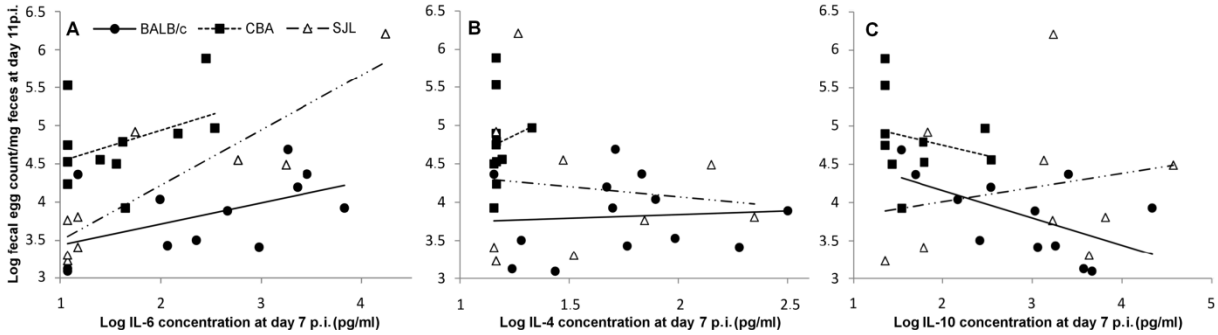

Figure 6. Fecal egg count at day 11 pi as a function of plasmatic cytokines. The tree panels refer to IL-6 (A), IL-4 (B) and IL-10 (C). Full dots and full lines refer to BALB/c, full squares and pointed lines refer to CBA and empty triangles and dashed lines refer to SJL.

Table 4. Analysis of covariance investigating the effect of IL-6, dose and strain on fecal egg count at day 11 pi.

Source of variation	df	F	P
log IL-6	1,25	0.83	0.3700
Dose	1,25	18.15	0.0003
Strain	2,25	5.86	0.0082
log IL-6 * Strain	2,25	3.49	0.0462

Discussion

Overall, we found that, even though infective dose had a strong effect on both host fitness and parasite performance, the shape of the reaction norm was host-strain specific for many traits and in particular for immune effectors (cytokine production).

We observed a rise in host mortality as long as the infective dose increased. Inoculum size is generally tightly correlated with the cost paid by the host (Ebert et al. 2000). This cost can obviously come from the direct spoliation/exploitation effect due to parasites that divert resources away from the host vital functions. Alternatively, or in addition to, an over-reacting immune response has been shown to incur high costs, in terms of immunopathological damage (Graham et al. 2005). Understanding the mechanisms underlying the cost of infection is important if we want to have reliable definitions of host resistance, susceptibility and tolerance (Medzhitov et al. 2012). For instance, available information on the relative susceptibility or resistance of mouse strains to *H. polygyrus* shows that CBA mice are susceptible to the infection while SJL are considered as resistant (Reynolds et al. 2012). This definition is based on the persistence of the parasite within the host (SJL mice expelling the worm rapidly, whereas CBA mice keep the parasites for months)

(Reynolds et al. 2012). However, when one allows the infective dose to vary, it can be noticed that the fitness cost paid by the different mouse strains provides a strikingly different picture, with SJL being the strain paying the highest toll to the infection. We suggest that this is probably due to the exacerbated immune activation of SJL over the gradient of infective doses.

To the best of our knowledge, this is one of the few studies that explicitly used a reaction norm approach to investigate cytokine production over a gradient of infective doses (Paterson et al. 2008). Previous work on wistar rats infected with the nematode *Strongyloidesratti* has shown that Th2 cytokines (IL-4 and IL-13) were significantly up-regulated when rats were infected with 150 or 750 infective larvae (compared to 6 or 30) (Bleay et al. 2007), showing a plastic response to the infectious environment. However, this study only included a single host strain which prevented to test the genotype by environment interaction.

Even though *H. polygyrus* is a model system in parasitology and immunology, the vast majority of studies have focused on the molecular mechanisms underlying the persistence or the expulsion of the parasite and its capacity to interfere with the host immune response. Our results are consistent with previous findings (Filbey et al. 2014) since we showed that CBA mice did not produce detectable levels of circulating IL-4, whatever the number of *H. polygyrus* larvae they were exposed to. The reaction norm for CBA mice was flat. We found that so-called resistant strains (SJL and BALB/c) increased their IL-4 production when exposed to *H. polygyrus* (as compared to non-infected mice). However, the rate of increase of IL-4 production over the infective dose was not linear. A similar non-linear relationship between cytokine production and dose was actually found for the two other cytokines considered here, IL-10 and IL-6. However, while IL-10 showed a bell-shaped function that was similar for the three host strains (even though it tended to be flatter for CBA mice), we found statistical support for a squared-dose by strain interaction for IL-6.

The dynamics of fecal egg count was drastically different across infective doses and host strains. When looking at the lowest infective dose, we found that CBA hosts provided a favorable ground for the persistence of the parasite since fecal egg count did not decline over the first month post-infection while BALB/c and SJL mice tended to rapidly expel the

parasite. Interestingly, however, the dynamics of fecal egg count dramatically changed when the infective dose increased, especially for the two so-called resistant strains (BALB/c and SJL). These results show that host susceptibility/resistance is actually a labile trait that depends on the environmental conditions encountered during the infection. In particular the well-known difference in susceptibility between CBA and SJL totally vanished when mice were exposed to 600 larvae. In terms of the underlying mechanisms, this view is consistent with the observation that IL-4 production tended to decrease for the highest infective doses.

Finally, we wished to investigate the role of cytokine production as a predictor of host fitness and parasite performance. We found evidence suggesting that a poorly regulated pro-inflammatory response can indeed cause deleterious effects on host health, promoting immunopathological damage in the association between *H. polygyrus* and its rodent hosts. A shift toward a pro-inflammatory response was also associated with a better parasite performance in terms of fecal egg count at day 11 pi, even though there were substantial differences among host strains. Fecal egg count at day 11 pi is the product of two traits: the number of female larvae that successfully managed to emerge into the intestinal lumen and the individual fecundity. We cannot tease apart the two and therefore we cannot establish whether the observed positive correlation between IL-6 and fecal egg count does result from a better larval survival, a better individual fecundity or both.

Other studies using KO models have provided consistent results in line with the view that pro-inflammatory effectors are important for parasite fitness. IL-6 KO mice clear the infection at a much faster rate than WT individuals (Smith and Maizels, 2014) as do IL-1β KO individuals (IL-1β being another major pro- (Th1) inflammatory cytokine) (Zaiss et al. 2013). How the parasite benefits from pro-inflammatory effectors is currently not fully understood, even though one might speculate that the antagonistic effect between Th1 and Th2 effectors might help the parasite when the Th1 response is over-expressed at the expenses of the Th2 one.

Overall, our findings provide partial support to the predictions. In particular we expected that resistant host strains should have higher fitness at low parasite dose and a shallower slope of fitness decrease with increasing parasite dose. However, contrary to our initial prediction the strain that suffered the most from the exposure to increasing number of

parasites was not the susceptible strain (CBA) but the resistant one (SJL). This result strongly suggests that there is a decoupling between the resistance (the capacity to take control of parasite population) and the cost of infection (the fitness cost paid by infected hosts). As in many other model systems, the cost of infection with *H. polygyrus*, while clearly being dose-dependent, mostly arises from the deleterious effect of an over-reacting immune response.

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Chapitre 2 : Ajustements des traits d'histoire de vie d'un nématode parasite confronté à une inflammation intestinale

Title: Life-history adjustments to intestinal inflammation in a gut nematode

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Abstract

Many parasitic nematodes establish long term, chronic infections. This implies a finely tuned interaction with the host immune response in order to avoid infection clearance. While a number of immune interference mechanisms have been described in nematodes, how parasites adapt to the immune environment provided by their hosts remains largely unexplored. Here, we used the gastrointestinal nematode *Heligmosomoides polygyrus* to investigate the plasticity of life history traits and immunomodulatory mechanisms in response to intestinal inflammation. We adopted an experimental model of induced colitis and exposed worms to the inflammatory environment at two different developmental stages (larvae and adults). We found that *H. polygyrus* responded to the inflammatory environment by up-regulating the expression of a gene supposedly involved in the interference with the host immune response. Worms infecting colitis mice also had shorter developmental rate, early fecundity and adult survival suggesting that an inflamed intestine might provide a favorable environment for the parasite.

Introduction

The interaction between hosts and parasites is based on an intimate molecular and physiological dialog between the two partners (Allen and Maizels 2011). By definition, hosts and parasites have conflicting interests, with parasites exploiting the resources provided by the hosts and the hosts avoiding the cost of the infection. Avoiding the cost of infection can be achieved by several mechanisms. Hosts might behaviorally reduce the risk to contract the infection (Villa et al. 2016), might mount a vigorous immune response and clear the infection (Hansen et al. 2013), might tolerate the infection (Schneider and Ayres 2008), or might adjust their life history traits (Agnew et al. 2000). Obviously, parasites have evolved counter-responses to each of these host-defense strategies. With this respect, the capacity of parasites to interfere with the host immune response is among the most prevalent exploitation strategies (Schmid-Hempel 2008). This makes sense, since to a certain extent, the immune response can be seen as the main source of external mortality for the parasite and any strategy limiting the impact of the immune response on parasite density or survival should confer a selective advantage in terms of transmission potential.

Helminthic parasites, such as nematodes, have been described as having very sophisticated mechanisms of immune evasion and immune interference (Maizels and McSorley 2016). The reasons that have likely promoted the evolution of such immune evasion strategies are multiple. Helminths are large metazoan presenting many antigenic targets to the host immune system. Given their size they also mechanically damage host tissues during their migration from the “entrance” site to the targeted organ, which also contributes to the activation of the host immune response (Allen and Wynn, 2011). Helminthic parasites usually establish long lasting, chronic infections and their life history traits usually reflect a strategy of long-term exploitation with long pre-patent (the period preceding reproduction) and patent (the period during which the parasite reproduces within the hosts) periods (Morand and Sorci 1998). Therefore, a successful parasite has to cope with the host immune pressure during relatively long periods of time, during different developmental stages and in different host tissues.

As for free-living organisms, parasite adaptations to the environment provided by their hosts can be achieved by plastic responses to environmental variation or by genetic selection. Phenotypic plasticity usually evolves when the environment varies unpredictably (Fusco and Minelli 2010). Since heterogeneous host immune response is a pervasive phenomenon, helminths likely face variable immune selection within and between hosts, making such organisms well suited for the study of phenotypic plasticity (Viney and Diaz 2012, Lippens et al. 2016).

In this study we investigated the plasticity of life history and immunomodulatory traits in the intestinal nematode *Heligmosomoides polygyrus*, in response to an inflammatory environment. *Heligmosomoides polygyrus* is a rodent parasite that has become a model organism for the study of the molecular dialog between hosts and parasites (e.g., Urban et al. 1991; Shea-Donohue et al. 2001, Ince et al. 2009 ; Hang et al. 2010, Reynolds et al. 2012). The persistence of the parasite (and therefore its transmission potential) depends on the capacity of the mouse host to mount a Th2 immune response (Reynolds 2012). Among-host variation in the capacity to mount an effective immune response against *H. polygyrus* results in extensive variation in parasite fitness; some host strains providing a very favorable ground for the parasite, while others expel the nematode after a few days (Reynolds et al 2012). Interestingly, *H. polygyrus* has been described to interfere with the host immune response (Maizels and McSorley 2016). This interference includes the up-regulation of T-regulatory lymphocytes (Treg cells) lymphocytes which dampen both Th1 and Th2 immunity (Setiawan et al. 2007; Grainger et al. 2010). At the molecular level, it has been shown that *H. polygyrus* secretes and excretes a number of effectors that are involved in the process of immunomodulation (Maizels et al 2012a). These effectors include a mimic of the TGF-β cytokine and a cysteine protease inhibitor (McSorley et al. 2013). Interestingly, the production of the TGF-β mimic has been shown to be stage specific, being minimal for L1 larvae and progressively increasing to reach maximal production in adults (McSorley et al. 2010). This result suggests that the fitness consequences of immunomodulation might also be stage specific. In particular, early stages of the infection might benefit from an up-regulated Th1 pro-inflammatory status because i) Th1 response tends to inhibit the more harmful Th2 response (Abbas et al. 1996) and ii) it might help larvae to disrupt the intestinal wall and allow their penetration. Whether an

inflammatory environment has different effect on age-specific stages of the nematode remains unknown.

In addition to the production of immunomodulatory compounds, parasites can adapt to the immune environment by plastically adjusting their investment into different life history traits, as suggested by theory on life history evolution (Stearns 1992; Cavaleiro and Santos 2014). Recent work has shown that *H. polygyrus* exposure to an inflammatory environment induced a series of changes in the expression of life history traits. For instance a systemic inflammation induced by a LPS injection produced an enhanced egg shedding a few hours after the raise in pro-inflammatory cytokines (Guivier et al. under review). Other studies have used local inflammatory stimulation to assess the nematode response. Donskow-Lysoniewska et al. (2013) induced an inflammatory response in the colon with a drug and found that more adult parasites were recovered in the intestinal lumen compared to the control situation. These results corroborate the idea that an inflamed intestinal environment might provide a favorable ground for the establishment of *H. polygyrus*.

Here, we extend this previous work by specifically addressing the question of the stage-dependency of the plasticity of life history traits. To this purpose, we used a rodent host model where colonic inflammation was induced by the ingestion of dextran sulphate sodium (DSS). We designed an experimental set up that allowed us to test the effect of the inflammatory environment on adult worms (the DSS treatment started when adult nematodes emerged in intestinal lumen), and on larvae (hosts where infected with *H. polygyrus* larvae when already facing the inflammatory treatment). We therefore measured worm life history traits and the expression of two candidate genes involved in the immunomodulatory process (*Hp–tgh2*, *rHp–CPI*) in experimental and control groups.

Based on the finding that immunomodulatory products are stage-specific in *H. polygyrus*, we predicted that exposure to an inflammatory environment should have positive fitness consequences at the larval stage. We also predicted that immunomodulatory genes should be upregulated in adults exposed to an inflammatory environment.

Materials & Methods

Ethics statement

All animal experiments were approved by the Comité d'Ethique de l'Expérimentation Animale Grand Campus Dijon, France (CNREEA n C2EA – 105) and by the ''Ministère de la Recherche et de l'Enseignement Supérieur" (project N01867.01) in accordance with the national guidelines (''Charte nationale portant sur l'éthique de l'expérimentation animale", Ministère de la Recherche et de l'Enseignement Supérieur) on the use of animals for research purposes.

Animals

145 Balb/c mice were purchased from JanvierLABS (Laval, France) and housed (five individuals per cage - 18.5 x 38 x 22.5 cm - enriched with shelters) at the Université de Bourgogne, France. Mice were maintained under a constant temperature (24°C) and a photoperiod of 12:12 (light:dark). They were fed with standard mouse pellets ad libitum and had ad libitum access to filtered tap water. Mice were weighed (± 0.1 g) every two days.

At the age of 7 weeks, mice were infected by gavage with stage 3 larvae (120 larvae in 0.1 ml of water) of the intestinal nematode *H. polygyrus bakeri.* L3 larvae were obtained from B6CBAF1 mice as the maintenance hosts. Upon ingestion, L3 larvae penetrate and encyst in the serosa of the duodenal wall where they undergo a couple of molts before emerging in the intestinal lumen approximately at day 8 post-infection.

Treatments

One hundred forty mice were randomly allocated to one of five different experimental groups. Mice infected with *H. polygyrus* were treated with dextran sulfate sodium (DSS) in the drinking water (at 2.5%) during 8 days. In one group, mice (n = 20) were exposed to DSS three days before the *H. polygyrus* infection (in this group worms faced an inflammatory environment during the early phase of the infection). In the other group, mice (n = 30) were exposed to DSS at day 8 post-infection, when the first adults emerge in the intestinal lumen (in this group, therefore, adult worms faced the inflammatory environment). Another group of mice (n = 40) was infected with the same number of *H. polygyrus* larvae but was not treated with DSS, and served as a first control group. The other two control groups were

formed by non-infected mice treated with DSS (8 days at 2.5%, n = 20), and non-infected non-DSS treated mice (n = 30).

Mice were euthanized by cervical dislocation at different time points to collect spleens and adult worms in the intestine. Spleens were immediately frozen in liquid nitrogen and then stored at -80°C. Adult worms were collected from the intestinal lumen and either immediately frozen in liquid nitrogen for gene expression analysis either stored in individual tubes for further counting.

Parasite life history traits

Adult worms were collected from the intestinal lumen and counted under a binocular loupe.

To assess fecal egg count, mice were transferred at 9 am on a grid in an individual cage with a humidified towel paper at the bottom to prevent feces desiccation. Mice were left four hours in these cages and then put back in their shared cages. Feces produced during this four-hour period were collected and 350 mg were smashed and suspended in 2.5 ml of water. Thereafter, 5 ml of salted water (75% of saturation) were added to allow eggs to float. After agitation, a fraction of this suspension was transferred into a McMaster chamber for egg count. We performed two counts per sample and used the mean values. Fecal egg count is expressed as number of eggs per mg of feces. We also computed the per capita fecundity as the ratio between the fecal egg count and the number of female worms recovered in the intestine.

Nucleic acids extraction and Real-time PCR

Total mRNA was extracted from about twenty *H. polygyrus* adults per mouse and from the spleen ($3mm^3$) using TRIzol/ chloroform protocol as recommended by the manufacturer (Invitrogen, Carlsbad, CA, USA). In both cases, RNA was precipitated using isopropanol, washed with 75% ethanol, and re-suspended in 50 µl of RNase free water. We eliminated DNA contamination using DNAse Treatment and Removal Reagents Ambion kit (Thermo Fisher scientific, Waltham, MA USA) following the manufacturer protocol, except for spleen samples because for each pairs of primers, at least one overlapped two exons being therefore RNA-specific. RNA extractions were electrophoresed on 2% agarose gels and

visualised using ethidium bromide staining. Degraded samples of RNA extractions were eliminated. Then, total RNA concentration was estimated using a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). We normalized concentrations to 250 ng/µl with RNase free water and stored at -80°C. cDNAs bank was generated from 2 µl of extracted RNA (500 ng/reaction) in a 20 µl reaction using Improm-II Reverse Transcription System kit (Promega, Madison, WI, USA), following the manufacturer protocol.

We quantified *Hp-Tgh2*, *Hp-CPI* and mouse-*TGF-β* expression by performing a real-time PCR (RT-PCR) using an Applied Biosystems StepOne Plus thermocycler (Applied Biosystems), in triplicate for each sample and gene. The PCRs were run on a total volume of 20 µl including 10 µl SYBR Green Master Mix 2X (Applied Biosystems), 0.5 µM of each primer, 1 µl of cDNA diluted 20 times and RNAse/DNase-free water to complete the total volume. Negative controls produced at each step of the procedure: RNA isolation, cDna bank construction and RT-PCR (water) were added on each plate to ensure the absence of any contamination. The PCR amplification was as follows: a first denaturation step at 95°C for 10 minutes, then 40 PCR cycles including denaturation and annealing step at 95°C for 15 seconds and elongation at 60° C for 1 minute. To check the specificity of the assay, melting curves for all reactions were determined. This procedure consisted of incubations for 15 seconds at 95°C, 60 seconds at 60°C and a final slow heating with a rate of 0.3°C per second up to 95°C with continuous fluorescence measurement. A negative control (water) was added on each plate to ensure the absence of any contamination. We used the Hp beta-actin as advocated by McSorley et al. (2010) and the mouse beta-actin as housekeeping genes.

Hp primers used were the same as in Guivier et al. (submitted) designed or retrieved from McSorley et al. (2010) : *Actin*-F : 5'-TGA GCA CGG TAT CGT CAC CAA C , *Actin*-R : 5'-TTG AAG GTC TCG AAC ATG ATC TG (171bp). Hp-Tgh2-F: 5'-CGG TGT GTC TGC CTG AAG AT, *Hp-Tgh2*-R: 5'-CGT TGT ATT TGT GCG GTG CA (108 pb). Hp-CPI-F: 5'-CTC GCC TCG TCT ATC GTC AC, *Hp-CPI*-R: 5'-CCG CCT TCC ATG CTT TTT CC (100bp). Mouse primers were designed from the *Mus musculus domesticus* beta-actin (NM_007393.5) and the Tranforming Growth Factor-β1 (TGF-β) (NM_011577.2) ARN sequences using the primer designing tools of the NCBI platform (www.ncbi.nlm.nih.gov/tools/primer-blast/). Mouse-*Actin*-F: CGA GCA CAG CTT CTT

TGC AG, mouse-*Actin*-R: CAT CCA TGG CGA ACT GGT G (65bp), mouse-TGF-β-F: GAC CGC AAC AAC GCC ATC and mouse-TGF-β-R: GGG ACA GCA ATG GGG GTT C (112bp).

Hp-Tgh2, *HP-CPI* and TGF-β mRNA relative expression levels were estimated for each sample using the method developed by Pfaffl (2001). We calculated Qn = [(ETar + 1)-CpTar]/[(ERef + 1)-CpRef] with ETar, ERef, CpTar and CpRef being, respectively, the mean efficiencies and the crossing points of the target and endogenous reference genes. The mean PCR efficiencies were estimated using the LinRegPCR software (Ruijter et al. 2009). Crossing point values were estimated for each sample using the second derivative maximum method implemented in StepOne software (Thermo Fisher scientific, Waltham, MA USA). We estimated, and considered in the analysis, Cp mean corresponding to the average of triplicate RT-PCR reactions for target and reference genes.

Statistical analyses

The log transformed *rHp–CPI*, *Hp-tgh2 and TGF-β* gene expression relative to the β-actin expression was analyzed using an ANCOVA model with time post-infection, treatment and their interaction.

The number of adult worms recovered in the intestinal lumen was analyzed using an ANCOVA model with time post-infection, treatment and their interaction.

The log transformed parasite fecal egg count was analyzed using a mixed linear model with mouse identity as a random factor (to account for the repeated nature of the data) and time post-infection, treatment and their interaction as fixed factors. Degrees of freedom were calculated using the Kenward-Roger method. For the treatment where adult worms were exposed to the inflammatory environment, fecal egg count was monitored over 6 different days which allowed including squared-time as a fixed factor to model a non linear effect.

The per capita fecundity was analyzed with an ANCOVA model with time post-infection, treatment and their interaction.

Mice body mass was assessed using a mixed linear model with mouse identity as a random factor and time post-infection, squared time post-infection, treatment, and their

interactions as fixed factors. Degrees of freedom were calculated using the Kenward-Roger method

For all analyses we started with the full model (with all possible interactions) and we subsequently dropped non-significant terms. We report the results of the full and the minimal models.

All analysis were performed with the R software (version 3.0.3) including “lme4” and “car” package respectively for linear mix model and type 3 analysis of variance.

Results

Immunomodulatory genes

We assessed the expression of two candidate genes involved in the process of immunomodulation. We compared the gene expression of worms experiencing a control environment and worms that experienced an inflamed environment either as larvae or as adults. When worms were exposed to the inflammatory environment as larvae, we did not find any difference in the expression of *Hp-thg2* and r*Hp-CPI* genes between control and inflammatory environments (table 1, fig. 1A). As predicted, we found that worms that experienced the inflammatory environment as adults had a higher expression of *Hp-thg2* gene (the mimic of the *TGF-*β) compared to the control environment. While at day 12 p.i., freshly emerged worms had similar *Hp-thg2* expression in the two treatments, by day 16 and 20 p.i., worms in the inflammatory environment had a much higher gene expression. This resulted in a statistically significant treatment by time interaction (table 1, fig. 1B). The expression of r*Hp-CPI* (a cysteine protease inhibitor that interferes with the antigen presenting process) did not vary between treatments (table 1).

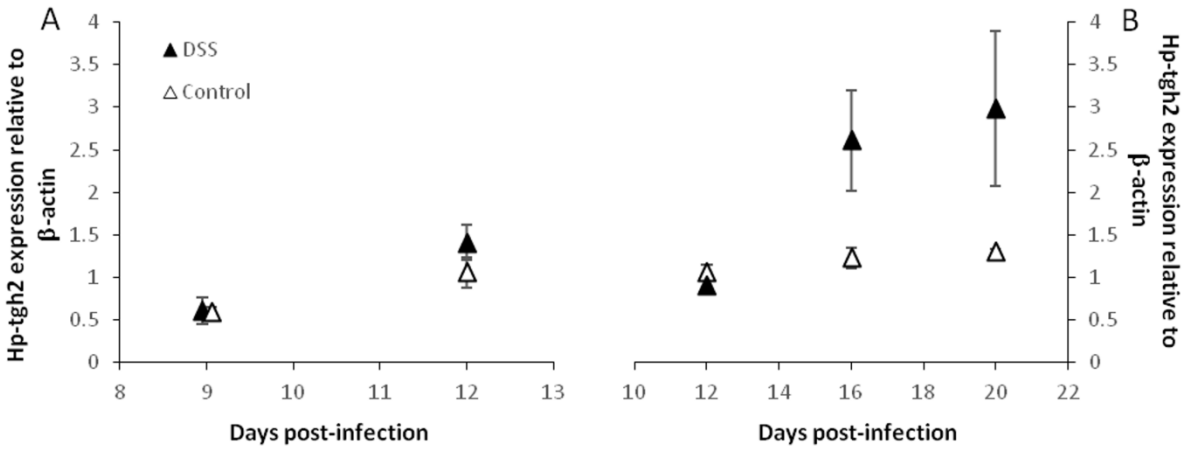

Figure 1. *Hp-tgh2* expression relative to *β-actin* as a function of days post infection in *H. polygyrus* either exposed to an inflammatory environment (full triangles) or a control environment (empty triangles). Means ± SE are reported. A) Larvae were exposed to the inflammatory environment; B) adult worms were exposed to the inflammatory environment.

Table 1. ANCOVA model on the expression of *rHp-CPI* and *Hp-thg2* genes relative to *β-actin* in *H. polygyrus* exposed to DSS either as larvae or adults. The model included time post-infection, treatment (exposed to DSS or not exposed) and the interaction between time p.i. and treatment.

		rHp-CPI			*Hp-thg2*		
Stage exposed to DSS	**Sources of variation**	F	df	p	F	df	p
Larvae	Time p.i.	2.3215	1,10	0.1586	36.8348	1,10	0.0001
	Treatment	0.0613	1,10	0.8095	1.6243	1,10	0.2313
	Time p.i.*Treatment	0.1836	1,10	0.6774	2.3089	1,10	0.1596
Adult	Time p.i.	0.0396	1,16	0.8464	1.0873	1,16	0.3126
	Treatment	2.1711	1,16	0.16	4.9057	1,16	0.0416
	Time p.i.*Treatment	2.3807	1,16	0.1424	8.5277	1,16	0.0100

Parasite abundance

We assessed the effect of the inflammatory environment on the number of adult worms recovered in the intestinal lumen. In the group where larvae were exposed to inflammation, we counted parasite numbers at day 9 and 12 p.i. In the group where adult worms were exposed to the inflammation, we counted parasite numbers at day 12, 16 and 20 p.i. In

agreement with the prediction, we found that parasites exposed to the inflammatory environment emerged earlier than the control group as shown by the higher number of adults at day 9 p.i. This difference vanished at day 12 p.i., resulting in a statistically significant interaction between treatment and time p.i. (table 2, fig. 2A). This suggests that the two groups did not differ in larval and/or adult survival but rather in developmental rate.

The inflammatory environment also had a strong effect on parasite numbers when adults were exposed to the inflammatory response. While at day 12 and 16 p.i., there was no difference between the control group and the inflammatory environment, adult numbers substantially dropped in the control group at day 20 p.i. while staying constant in the inflammatory environment (table 2, fig. 2B), resulting in a statistically significant interaction between group and time p.i. This shows that the inflammatory environment had a positive effect on adult survival.

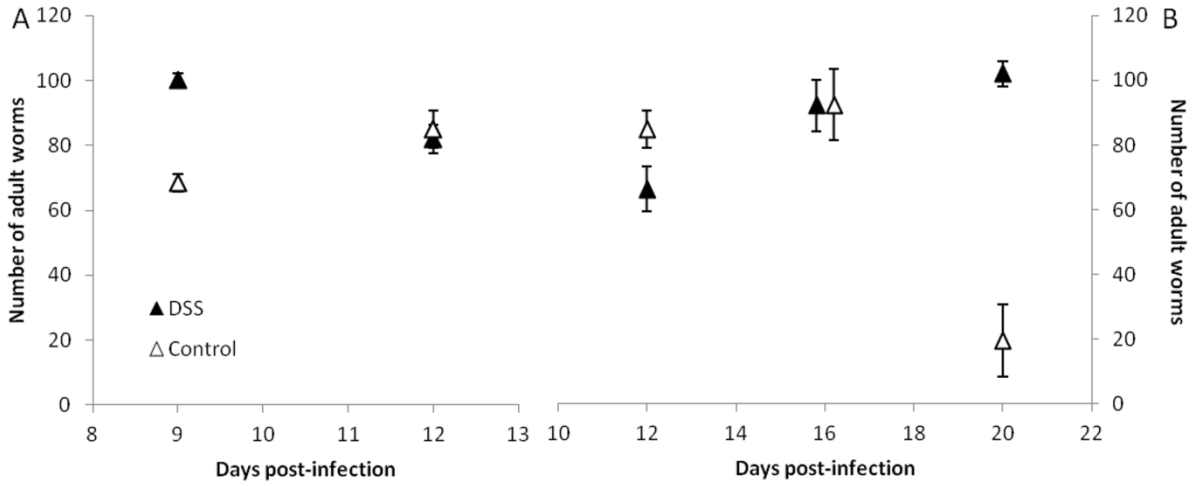

Figure 2. Number of adult worms harvested from the intestinal lumen as a function of days p.i. in *H. polygyrus* either exposed to an inflammatory environment (full triangles) or a control environment (empty triangles). Means + SE are reported. A) Larvae were exposed to the inflammatory environment; B) adults were exposed to the inflammatory environment.

Table 2. ANCOVA model on the number of adult *H. polygyrus* exposed to DSS either as larvae or adults. The model included time post-infection, treatment (exposed to DSS or not exposed) and the interaction between the two.

	Stage exposed to DSS					
	Larvae			*Adults*		
Sources of variation	F	df	p	F	df	p
Time p.i.	9.0265	1,15	0.0089	21.4819	2,21	<0.0001
Treatment	27.4922	1,15	<0.0001	2.3148	1,21	0.1431
Time p.i.*Treatment	17.4257	1,15	0.0008	13.0966	2,21	0.0002

Parasite fecal egg count

Larval exposure to the inflammatory environment consistently improved fecal egg count at day 9, 10 and 12 p.i., as a consequence the interaction between time and treatment was not significant whereas the treatment had a significant effect on fecal egg count (table 3, fig. 3A).

When adult worms faced the inflammatory environment, we also found an improvement of fecal egg count, even though the difference between the inflammatory and control group appeared at day 16 p.i. Whereas fecal egg count of the control group showed the typical bell-shaped dynamics, fecal egg count of worms in the inflammatory group remained high up to day 20 p.i. This resulted in a statistically significant interaction between group and time p.i. (table 4, fig. 3B).

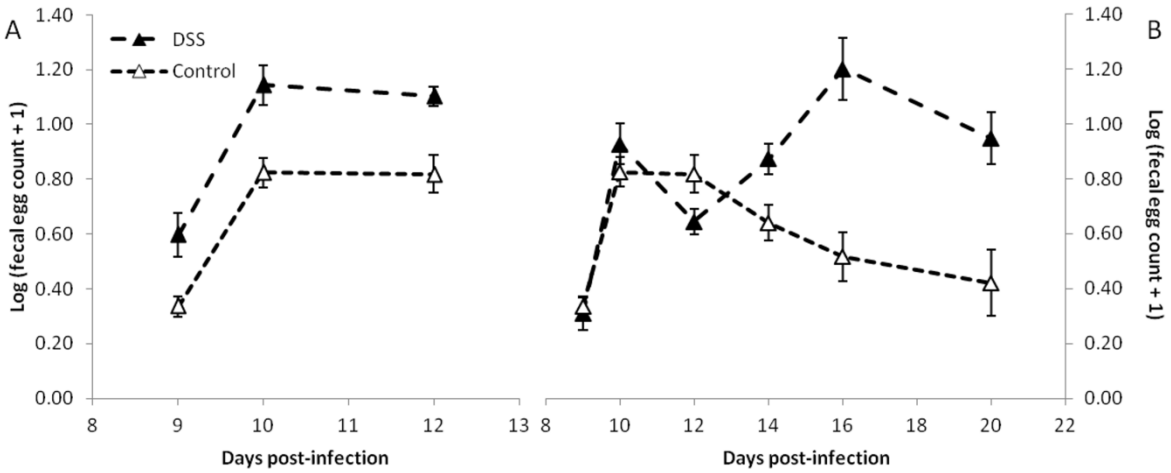

Figure 3. Fecal egg count (log number of eggs/mg of feces) as a function of days post infection in *H*. *polygyrus* either exposed to an inflammatory environment (full triangles) or a control environment (empty triangles). Means ± SE are reported. A) Larvae were exposed to the inflammatory environment; B) adults were exposed to the inflammatory environment.

Table 3. Linear mixed model of fecal egg count in *H. polygyrus* that were exposed to the inflammatory environment as larvae. A) full complete model; B) selected model.

Sources of variation			
A) full model	F	df	p
Time p.i.	12.0293	1,47.95	0.0011
Treatment	0.0013	1,46.30	0.9717
Time p.i.*Treatment	0.1683	1,47.20	0.6835
B) selected model	F	df	p
Time post-infection	37.5631	1,46.86	< 0.0001
Treatment	13.2282	1,23.40	0.0014

Table 4. Linear mixed model of fecal egg count in *H. polygyrus* that were exposed to the inflammatory environment as adults. A) full complete model; B) selected model.

Sources of variation			
A) full model	F	df	p
Time p.i.	14.8669	1,111.33	0.0002
Squared time p.i.	15.0579	1,110.68	0.0002
Treatment	0.0017	1,109.89	0.9676
Time p.i.*Treatment	0.0278	1,109.97	0.8679
Squared time p.i.*Treatment	0.3164	1,110.85	0.5749
B) selected model	F	df	p
Time p.i.	19.8578	1,112.39	<0.001
Squared time p.i.	20.3032	1,111.58	<0.001
Treatment	5.2721	1,128.94	0.0233
Time p.i.* Treatment	8.5533	1,123.23	0.0041

Parasite per capita fecundity

Differences in the fecal egg count between groups can obviously arise because of differences in individual fecundity and/or population size. To take into account the observed difference in the number of adult worms between treatments, we computed the per capita fecundity. Larval exposure to the inflammatory environment did not improve per capita fecundity compared to the control group (table 5, fig. 4A), suggesting that the effect on fecal egg count was essentially due to variation in population size.

Similarly, when adult worms were exposed to the inflammatory environment, their per capita fecundity did not differ between treatments or over time (table 6, fig. 4B).

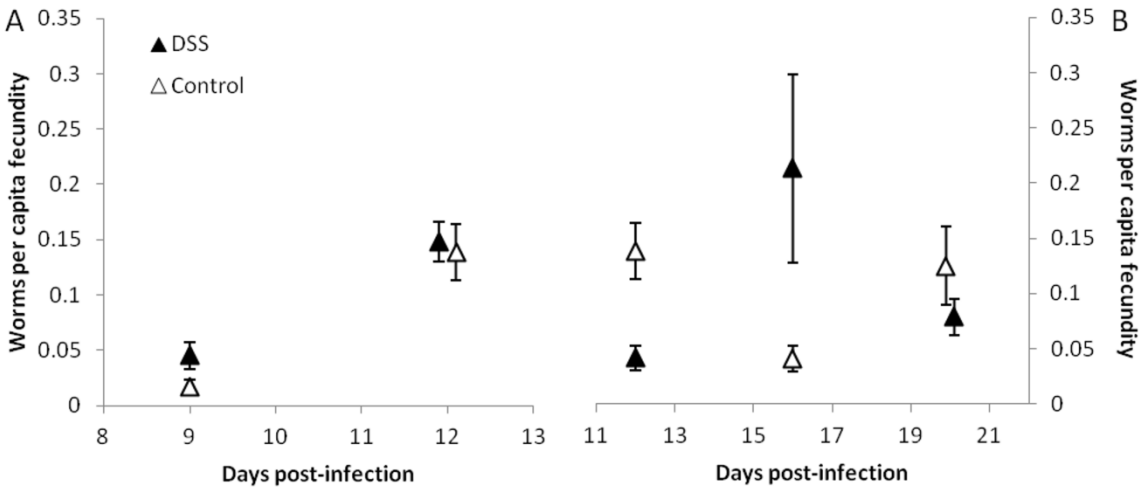

Figure 4. Per capita fecundity as a function of days post infection in *H. polygyrus* either exposed to an inflammatory environment (full triangles) or a control environment (empty triangles). Means ± SE are reported. A) Larvae were exposed to the inflammatory environment; B) adults were exposed to the inflammatory environment.

Table 5: ANCOVA model of per capita fecundity of *H. polygyrus* exposed to the inflammatory environment as larvae. A) full model; B) selected model.

Sources of variation			
A) full model	F	df	p
Time p.i.	13.3095	1,14	0.0026
Treatment	0.4005	1,14	0.5371
Time p.i.*Treatment	0.2577	1,14	0.6196
B) selected model			
Time p.i.	39.9073	1,15	<0.001
Treatment	0.9434	1,15	0.3468

Table 6. ANCOVA model of per capita fecundity of *H. polygyrus* exposed to the inflammatory environment as adults. A) full model; B) selected model.

Sources of variation			
A) full model	F	df	p
Time p.i.	0.0586	1,21	0.8111
Treatment	1.0598	1,21	0.3150
Time p.i.*Treatment	1.1372	1,21	0.2984
B) selected model			
Time p.i.	0.2747	1,22	0.6054
Treatment	0.0044	1,22	0.9478

Inflammation severity

The inflammatory status induced by the DSS treatment produces a series of symptoms on the hosts and we used changes in body mass as an integrative index of the severity of colitis. To this purpose, we had additional groups of mice that were only exposed to DSS but not infected by *H. polygyrus*, and a group of control mice (not exposed to DSS and not infected). As expected, while control and *H. polygyrus* infected mice gained body mass over the course of the study, exposure to the inflammatory drug induced a temporary lost in body mass (tables 7 and 8, figs 5A and 5B). The dynamics of body mass loss in the three inflammatory groups were very similar, suggesting that the infection had very little effect on colitis severity, at least based on body mass.

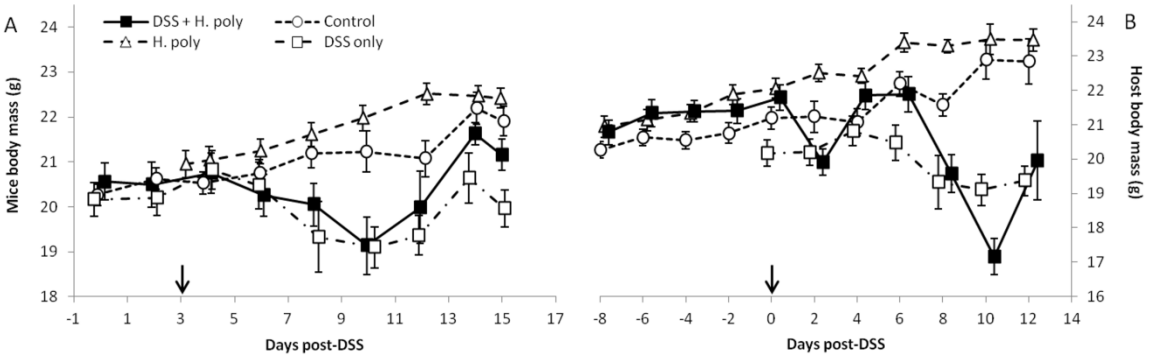

Figure 5. Host body mass as a function of time post-DSS exposure. The two panels refer to the treatment where *H. polygyrus* was exposed to the inflammatory environment as larvae (A) or as adults (B). The arrow indicates the day when mice were infected with *H. polygyrus*. Means *+* SE are reported.

Table 7. Linear mixed model of host body mass when *H. polygyrus* was exposed to the inflammatory environment as larvae.

Sources of variation	F	df	p
Time post-DSS	8.5652	1,449.30	0.0036
Squared time post-DSS	2.0116	1,449.42	0.1568
Treatment	0.4735	3,123.88	0.7013
Time post-DSS*Treatment	11.7652	3,448.46	<0.001
Squared time post-DSS*Treatment	5.7849	3,448.76	<0.001

Table 8. Linear mixed model of host body mass when *H. polygyrus* was exposed to the inflammatory environment as adults.

Sources of variation	F	df	p
Time post-DSS	6.1402	1,513.46	0.0135
Squared time post-DSS	0.2835	1,512.62	0.5946
Treatment	2.7341	3,170.98	0.0453
Time post-DSS*Treatment	3.8164	3,510.85	0.0101
Squared time post-DSS*Treatment	13.9656	3,511.29	<0.001

Host TGF-β gene expression

We finally assessed the expression of *TGF-β* in the spleen of mice in the different groups. *TGF-β* expression was up-regulated in mice exposed to the inflammatory treatment alone, compared to the other groups (tables 9 and 10, fig. 6A and 6B). Infected and DSS-exposed mice had similar expression of *TGF-β* gene to the control group, whatever the parasite stage that was exposed to the inflammatory environment. This result suggests that *TGF-β* in DSS treated mice was down-regulated when concomitantly infected with *H. polygyrus*.

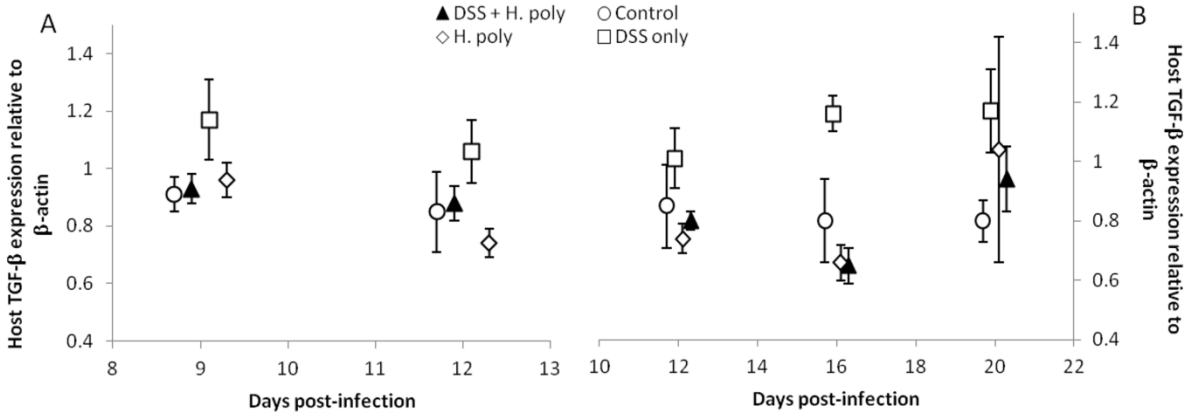

Figure 6. Host *TGF-β* expression relative to *β-actin* as a function of time. The two panels refer to the treatment where *H. polygyrus* was exposed to the inflammatory environment as larvae (A) or as adult (B). Means ± SE are reported.

Table 9. ANCOVA model on host *TGF-β* expression relative to *β-actin* when *H. polygyrus* larvae were exposed to the inflammatory environment. A) full model; B) selected model.

Sources of variation			
A) full model	F	df	p
Time p.i.	0.1924	1,29	0.6642
Treatment	1.8874	3,29	0.1538
Time p.i.*Treatment	0.3264	3,29	0.8062
B) selected model			
Time p.i.	3.1906	1,32	0.0835
Treatment	3.8046	3,32	0.0194

Table 10. ANCOVA model on host *TGF-β* expression relative to *β-actin* when *H. polygyrus* adults were exposed to the inflammatory environment. A) full model; B) selected model.

Sources of variation			
A) full model	F	df	p
Time p.i.	0.0327	2,42	0.9678
Treatment	0.4755	3,42	0.701
Time p.i.*Treatment	0.5647	6,42	0.7558
B) selected model			
Time p.i.	0.8807	2,48	0.2381
Treatment	3.3335	3,48	0.027

Discussion

We found that an inflammatory intestinal environment had profound effect on the life history traits of the gut nematode *Heligmosomoides polygyrus*, and that for some of the life

history traits considered, this effect was stage-dependent. Overall, however, worms that faced an up-regulated inflammatory response had better performance both in terms of abundance and fecal egg count compared to the control group.

Chronic inflammation of the intestine has become a prevalent disease in many countries (Molodecky et al 2012). In addition to the effort to understand the origin of the disease and the reasons of the raise in disease prevalence that occurred in the last decades, current research focuses on the possible therapeutic role of excretory-secretory (ES) compounds, with immunomodulatory properties, produced by helminthes (Finlay et al. 2014; Heylen et al 2014). Previous findings on animal models showing that gut nematodes ameliorate the symptoms of experimentally induced intestinal inflammation have led to the establishment of clinical trials where helminths have been used to cure inflammatory bowel disease (Helmby et al. 2015). Besides the immediate therapeutic function of nematode ES products, an important yet underestimated question is the possible adaptation of nematodes to the particular immune environment provided by the inflamed intestine. This is particularly relevant because, depending on the stage of the parasite, the inflammatory response might enhance the infectivity of the parasite. For instance, once ingested, *H. polygyrus* L3 larvae penetrate in the intestinal wall where they undergo a couple of molts before emerging back in the intestinal lumen as adult worms. While adult worms have been shown to have an up-regulated expression of *Hp-tgh2* gene, a homolog of the mammalian *TGF-β,* the expression is lower in larvae (McSorley et al. 2010). This possibly suggests a stage-dependent benefit of immune modulation.

We explored the adaptation of *H. polygyrus* to an inflammatory environment using a well-established colitis model induced by the administration of dextran sulphate sodium. DSS alters the intestinal cytokine environment, up-regulating the production of pro-inflammatory cytokines and markers (Chassaing et al. 2014). Here, we assessed the expression of *TGF-β* gene in host spleen and found that DSS administration enhanced its expression. This finding, in addition to the observed decrease in body mass in DSS exposed mice, shows that we successfully altered the intestinal environment worms were exposed to. Interestingly however, the up-regulation of *TGF-β* gene involved the DSS-only treatment, whereas DSS

treated mice that were also infected with *H. polygyrus* had similar *TGF-β* expression than the control group.

Parasite adaptation to the immune environment might depend on the developmental stage that is exposed to the inflammatory response. Based on the finding that the expression of *Hp-tgh2* gene is stage dependent in *H. polygyrus*, we predicted that the inflammatory environment should enhance parasite performance when the larval stage faces the inflammatory challenge. This prediction was also rooted in a previous work that used a DSS model of induced colitis, showing that mice with colitis harbored more larvae and more adults compared to control mice (Donskow-Lysoniewska et al. 2013). In agreement with our prediction and this previous work, we also showed that when larvae encountered an inflamed environment a higher number of adult worms was recovered in the intestine at day 9 p.i. However, the difference vanished at day 12 p.i., suggesting that the difference in worm number is not due to differential mortality but to differential developmental rate.

The fitness consequences of shorter developmental rate are two-fold. On one hand emerging at an earlier age extends the reproductive period, on the other hand shorter developmental time is usually associated with a smaller adult size (Morand and Sorci 1998) and adult size is positively correlated with fecundity (Skorping et al. 1991; Morand and Sorci 1998). Despite a shorter developmental time, worms that were exposed to the inflammatory environment shed more eggs than control worms, even though the per capita fecundity did not differ between groups. This suggests that worms facing the inflammatory environment with a shorter developmental time did not pay the expected penalty in terms of reduced fecundity.

A different picture emerged when adult worms experienced the inflammatory challenge. Obviously, in this case, developmental rate could not have been affected by DSS because the treatment started at day 8 p.i. (e.g., when adults started to emerge in the intestine lumen). Indeed, the number of adult worms recovered in the intestinal lumen did not differ between DSS treated and control mice at day 12 and 16 p.i. However, there was a large difference at day 20 p.i., with 5 times more parasites in DSS treated mice compared to controls. Declining population size of adult worms is a typical finding, due to the progressive expulsion of worms under the joint action of the immune response and peristaltic movements

(Zhao et al. 2008). Our finding that, in an inflamed intestine, adult survival was preserved therefore suggests that the immune system of colitis mice failed to deal with the infection. The mechanisms underlying such finding might involve a strongly shifted immune response towards a Th1 and away from the protective Th2 response (Maizels et al. 2012b). Alternatively, previous work has shown that exposure to an inflammatory environment can change the proteome profile of adult *H. polygyrus*, reducing immune recognition of parasites and improving survival (Donskow-Lysoniewska et al. 2013). However, in this case, the key question is what prevents worms to escape immune recognition when exposed to control intestinal conditions.

We found a similar differential fitness profile between DSS exposed and control worms when looking at the number of eggs shed in the feces. Again, there was no difference early on during the first days post adult emergence, but by day 14 p.i. worms in the DSS group shed substantially more eggs than control worms. In terms of per capita fecundity, however, DSS exposed worms had similar fecundity.

Even though exposing larval or adult stages to the inflammatory environment produced slightly different adjustments in terms of life history traits, overall, whatever the developmental stage, the inflammatory challenge improved parasite performance. Beyond the molecular and immunological mechanisms underlying this improved performance, these results raise several questions. Obviously the first question is related to the capacity of *H. polygyrus* to dampen the host immune response (Maizels et al. 2012a) and to alleviate the symptoms of intestinal inflammatory diseases (Hang et al. 2013). It is very well established now that *H. polygyrus* interferes with the immune response of the host at multiple levels, including the stimulation of Treg cells that dampen both Th1 and Th2 immune response (Grainger et al. 2010). At the molecular level, *H. polygyrus* secretes and excretes a number of compounds with different immune interference properties (McSorley et al. 2013) and, in agreement with previous studies, we found that the expression of a gene that produces an homolog of the mammalian *TGF-β* was up-regulated, at the adult stage, in DSS treated mice. Similarly, *H. polygyrus* has been shown to ameliorate the symptoms of experimentally induced colitis in several independent studies (Elliot et al. 2004; Sutton et al. 2008, Blum et al. 2012, Donskow-Lysoniewska et al. 2012). Even though we did not find such

"therapeutic" role of *H. polygyrus* infection in DSS treated mice, we only focused on changes in body mass as an integrative index of severity of disease, and it is possible that more specific markers of inflammation severity, such as presence of blood in the feces might have produced the expected results. But, if an inflamed intestine provides a more suitable environment for the nematode, why does it dampen it? This key question remains open at the moment, and we can only speculate on the possible explanations. The first possible explanation is that worms facing an inflammatory environment adopt a faster pace of life, with accelerated development, enhanced early fecundity but lowered late fecundity and increased late mortality. Such shifts in life history strategies have been documented in a few species in response to threatening environmental conditions (Agnew et al. 2000). A text book example of such life history shifts involves the snail *Biomphalaria glabrata* exposed to *Schistosoma mansoni* miracidia (Minchella and Loverde 1981; Thornhill et al. 1986). In response to the infection, snails increase their investment into egg production because the parasite has a deleterious effect over time. The underlying assumption here is that these life histories shifts, at best, result in the same fitness than the life histories under control environment. To test this hypothesis we would need to monitor the parasite life history traits over the entire life span of the worms. Another possibility is that costs of facing an inflammatory environment are paid not only during the late phase of the infection but also during the following generation. We have recently shown that larval infectivity does vary as a function of parental traits (Lippens et al. under review). It is therefore possible that worms experiencing an inflammatory environment produce offspring with reduced fecundity or survival prospects.

To conclude, our study shows that *H. polygyrus* has the potential to plastically respond to the immune environments provided by its host, both in terms of life history traits and the expression of a gene involved in the interference with the immune response. While exposure of different parasite stages to the inflammatory environment provided slightly different results in terms of life history traits, overall, exposure to inflamed intestine improved parasite fitness. This raises the question of the possible long term adaptation of *H. polygyrus* to inflammatory conditions and the potential consequences of these adaptations on the capacity of nematodes to cure inflammatory diseases.

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Chapitre 3 : Réponses plastiques et micro-évolutives d'un nématode à l'environnement immunitaire de l'hôte

Title: Plastic and micro-evolutionary responses of a nematode to the host immune environment

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Abstract

Parasitic organisms have to cope with the defences deployed by their hosts and this can be achieved adopting immune evasion strategies or optimal life history traits according to the prevailing pattern of immune-mediated mortality. However, parasites often encounter variable immune environments both within and between hosts, promoting the evolution of plastic strategies instead of fixed responses. Here, we explored the plasticity and micro-evolutionary responses of immunomodulatory mechanisms and life history traits to the immune environment provided by the host, using the parasitic nematode *Heligmosomoides polygyrus.* To test if the parasite responds plastically to the immune environment, we stimulated the systemic inflammatory response of mice and we assessed i) the expression of two genes with immunomodulatory functions (*Hp-Tgh2* and *Hp-CPI*); ii) changes in the number of eggs shed in the faeces. To test if the immune environment induces a micro-evolutionary response in the parasite, we maintained the nematode in mice whose inflammatory response was up- or down-regulated during four generations. We found that *H. polygyrus* plastically responded to a sudden rise of pro-inflammatory cytokines, up-regulating the expression of two genes involved in the process of immune modulation, and enhancing egg output. At the micro-evolutionary level, parasites maintained in hosts experiencing different levels of inflammation did not have differential expression of *Hp-Tgh2* and *Hp-CPI* genes when infecting unmanipulated, control, mice. However, parasites maintained in mice with an up-regulated inflammation shed more eggs compared to the control line. Overall, our study shows that *H. polygyrus* can plastically adjust the expression of immunomodulatory genes and life history traits, and responds to selection exerted by the host immune system.

Introduction

In response to selection pressures exerted by the immune system, parasites have evolved fascinating strategies to evade immunity through physiological and biochemical mechanisms (Schmid-Hempel 2008). Nematodes are among the best examples of parasites that can down-regulate the host immune response for their own benefit (Maizels et al. 2004).

Along the course of a nematode infection, the immune environment provided by the host is likely to change. The vertebrate immune response is a complex process with successive and interacting components (innate and acquired immunity), involving different cellular and molecular effectors (Akira et al. 2006). After its penetration into the host, a nematode faces the innate immune response (de Veer et al. 2007). Then, the recognition of parasite epitopes stimulates the Th2 immune pathway and the activation of the immune regulatory network (Gause 2003). Consequently, the immune response shifts from a Th1 response to a Th2-driven reaction. Thus, from its penetration into the host to its death a parasitic nematode deals with different immune components which modify its immediate environment. Since many nematode species establish long lasting infections, they are also likely to encounter variable immune environments as long as the host cyclically engages in stressful activities (i.e., reproduction, migration) or has to deal with secondary pathogen infections. Parasitic nematodes have also to cope with spatial variation in their environment during their "migration" through different host tissues and organs that provide different immune environments (Read and Skorping 1995). At the intra-host level, therefore, nematodes have to face spatial and temporal variation in immune environments.

Between generations, nematodes also deal with different immune environments. Host populations are characterized by a strong variation in the ability of individuals to mount an immune response (Hayward 2013). This variability, shaped by genetic and ecological factors, promotes the coexistence of susceptible, tolerant and resistant hosts to specific parasites. It also implies that from one generation to the other, parasites are likely to experience different immune environments, depending on whether they will end up infecting a resistant, a susceptible or a tolerant host. Thus, the benefits and costs of immunomodulatory strategies deployed by the parasite are likely to vary across generations.

In addition to directly interfering with the host immune response, parasites might respond to perceived risk of immune-mediate mortality by adjusting the age-specific investment into reproduction. This is very similar to the predictions that models on the evolution of life history traits have provided for free-living organisms (Michod 1979). Increased risk of mortality should produce a rise in current reproduction because saving energy for future reproduction might be worthless (Paterson and Barber 2007).

Adaptation to specific environmental conditions is usually achieved through two main mechanisms: phenotypic plasticity and genetic selection. Theoretical work has shown that living in a variable and unpredictable environment should promote the evolution of phenotypic plasticity (Scheiner 1993). Based on this theoretical prediction and the variability parasites have to face both within and between hosts, one can predict that gut nematodes i) should have evolved mechanisms allowing them to rapidly shift between different environmental types, through plastic responses (Viney and Diaz, 2012), both in terms of immunomodulatory strategies and life history trajectories (Lippens et al. 2015); ii) should adapt their investment into immune evasion effectors according to the experienced level of immune aggression.

Here, we tested whether parasitic nematodes exposed to variable immune environments have plastic immunomodulatory mechanisms and life history traits, and whether there is a micro-evolutionary response after four generations of selection under specific immune environments. To this purpose, we used the association between the gut nematode *Heligmosomoides polygyrus* and its rodent host, *Mus musculus musculus*. Previous work has shown that *H. polygyrus* secretes molecules with immunomodulatory properties (Hewitson et al 2009, Valanparambil et al. 2014). These include excretory-secretory products that interfere with dendritic cell functioning or the expansion of T regulatory lymphocytes (Treg) (McSorley et al. 2013). Here, we focused on the expression level of two genes: *Hp-Tgh2*, a homolog of the anti-inflammatory cytokine transforming growth factor beta (TGF-β) (McSorley et al. 2010) and *Hp-CPI*, a cysteine protease inhibitor, involved in the processes of escaping immune recognition (Schierack et al. 2003).

At the intra-host level, we experimentally modified the immune environment experienced by *H. polygyrus* by activating the inflammatory response of mice with the injection of

Escherichia coli lipopolysaccharide (LPS). At the inter-host level (i.e. between generations of nematodes), we maintained *H. polygyrus* in hosts whose inflammation was up-regulated (mice treated with anti-TGF-β antibodies), down-regulated (mice treated with TGF-β) or left as unmanipulated control. TGF-β is one of the main suppressive cytokines with effects on lymphocyte proliferation and differentiation (Li MO et al. 2006). Previous work has shown that TGF-β and anti-TGF-β antibody injections in mice effectively down- and up-regulate immune effectors in response to infection (Omer and Riley 1998, Li et al. 1999, Doligalska et al. 2006, Beiting et al. 2007 et De'Broski et al. 2008).

We assessed the expression of *Hp-Tgh2* and *Hp-CPI* genes and parasite egg shedding in response to the LPS challenge. Lines of parasites maintained under the three selection regimes were used to infect control mice and their *Hp-Tgh2* and *Hp-CPI* expression and egg shedding were assessed through time.

At the intra-host level, we predicted that the LPS challenge should produce a plastic response in terms of up-regulation of genes involved in immunomodulation. In terms of adjustment of egg shedding, several scenarios can be envisioned. First, the up-regulation of the inflammatory response induces strong immune constraints on the parasites, resulting in a reduction in egg shedding. Second, changes in the immune environment might be perceived as a danger signal by the parasite and produce an adaptive response in terms of increased egg shedding.

At the inter-host level, selection over a stable environment might favour parasite genotypes with differential expression of immunomodulatory genes (depending on the environment experienced). In this case, we should expect that lines selected in TGF-β treated mice should have down-regulated expression of immunoregulatory genes in control mice, and lines selected in mice treated with anti-TGF-β antibodies should have an up-regulated expression. In terms of parasite egg shedding, if experiencing a harsher immune environment reduces parasite survival, we predicted that lines maintained in mice treated with anti-TGF-β antibodies should have the highest egg shedding when infecting control hosts (Paterson and Barber 2007).

Materials & Methods

Intra-host

Eleven-week-old BALB/c jrj female mice (n = 30) were used for the experiment. All animals were purchased from JanvierLabs (Laval, France) and were housed in cages (18.5 x 38 x 22.5cm) containing 5 individuals under standard conditions (temperature: 21°C, light/dark cycle: 12h/12h). Mice had food and water *ad libitum*. *H. polygyrus* larvae were kindly provided by Prof. Rick Maizels (University of Edinburgh) in 2012 and maintained, since then, in our lab in B6CBAF1 female mice. All animal experiments were approved by the Comité d'Ethique de l'Expérimentation Animale Grand Campus Dijon, France (CNREEA n° C2EA – 105, protocol #1212), in accordance with the national guidelines ("Charte nationale portant sur l'éthique de l'expérimentation animale", Ministère de la Recherche et de l'Enseignement Supérieur) on the use of animals for research purposes.

At day 0, we infected mice with 200 L3 larvae by oral gavage. At day 13 post-infection (pi), we measured body mass (0.01 g) and we assessed faecal egg count (FEC, number of parasite eggs per gram of faeces). Briefly, mice were placed in clean individual cages for 4 hours in the morning. Faeces were collected and mice were placed back into their home cage. Faeces were weighted and suspended in a 50% NaCl solution. Then, the solution was placed in a McMaster counting chamber during 15 minutes for flotation before egg counting. We duplicated the measurement for each sample and computed mean values. At day 14 pi, half of the individuals received an intra-peritoneal injection of *E. coli* LPS (LPS, serotype 055:B5, Sigma, St. Louis, MO; $1\mu g.g^{-1}$ of body mass diluted in 100 µl of PBS) and the other half was injected with the same volume of phosphate buffer saline (PBS). At 5, 29 and 53 hours post-injection, we measured body mass and faecal egg count for 5 individuals from each treatment group (at each time point). After faeces sampling, we collected a blood sample by sub-mandibular puncture and euthanized the mice by cervical dislocation. Blood was rapidly centrifuged (4000 rpm, 10 minutes, 4°C) and plasma stored at −80°C. Immediately after mouse death, we collected *H. polygyrus* adults from the intestine. We did not count the number of parasites collected because it takes a relatively long time to have an accurate counting which was not compatible with the proper preservation of RNA for rt-PCR.

Parasites were washed twice in PBS and stored in RNA Later solution (Sigma-Aldrich, St Louis, MO, USA) at -20°C.

Inter-host level

Selection lines

Eight-week-old BALB/c JRj female mice (n=89) were used for the experiment. An initial generation (G0) of *H. polygyrus* L3 larvae was established from parasites maintained in female B6CBAF1 JRj mice. Mouse housing conditions were identical to the previous experiment. At day 0, we infected 15 mice with 200 larvae of *H. polygyrus* (G0). At day 8 and 11 pi (corresponding to the timing of parasite emergence into the intestine), we injected 5 females with 40 µg of mouse anti-TGF-$\beta_{1,2,3}$ antibodies (R&D system, Abington, UK) diluted in 100 µl of PBS, 5 females with 50 ng of recombinant mouse TGF-β_1 (rTGF-β_1) (R&D system, Abington, UK) in 100 µl of PBS, and 5 females with 100 µl of PBS. At day 14 pi, we collected faeces by placing mice in individual cages. Faeces were mixed with distilled water and coal, spread on a Whatmann paper laid on a wet paper towel to keep humidity and put in a Petri dish. Petri dishes were stored for 13 days at ambient temperature, in the dark, and daily humidified. L3 larvae were collected after 13 days and stored overnight in a water solution at 4°C, before infection of a second batch of mice corresponding to the G1 generation of nematodes. We performed four passages following this procedure over the three selection lines.

Gene expression levels and life-history traits

After four generations of selection, 200 larvae from each of the three selection regimes were used to infect unmanipulated female BALB/c mice. Due to stochastic events along the experiment we could infect 5, 4 and 2 mice for PBS, TGF-β and anti-TGF-β lines, respectively. We monitored fecal egg count at days 11, 15, 18, 21, 25, 32, 46, 60 and 74 pi following the protocol detailed above. Faecal egg counts of selected lines were compared to FEC of 10 unmanipulated female BALB/c mice that had been infected with 200 larvae of the ancestral population of worms (G0).

We also investigated the effect of selection regimes on the expression of immunomodulatory genes at 15 days pi. To this purpose, we collected larvae of the G4 for

each selection line and infected 5 unmanipulated female BALB/c mice (per selection line) with 200 larvae. At day 15 pi, mice were euthanized by cervical dislocation and adult worms collected and stored as described above. Gene expression of parasites from the three selection lines was compared to the gene expression of parasites from the ancestral population (5 unmanipulated mice infected with 200 G0 larvae, adult parasites collected at day 15 pi).

Cytokine quantification

We assessed the concentration of 4 cytokines (TNF-α, IL-4, IL-6, and IL-10) in the plasma, with a Luminex technology on Bio-plex200 instrument (Biorad®). We used Milliplex® Map kit (MCYTOMAG-70K, Millipore) on a total of 25 µl of plasma following manufacturer kit protocol. The flow cytometry run based on standard curve was carried out using Bio-Plex software (Biorad). Values below or above standard curves were replaced using the most conservative estimations (i.e., the lower and upper values of the standard curve for each cytokine). We could assess cytokine concentrations for 28 individuals out of 30 due to small plasma volumes for two individuals.

RNA extractions and cDNA bank

Total mRNA was extracted from approximately twenty *H. polygyrus* adults per mouse using TRIzol/ chloroform protocol recommended by the manufacturer (Invitrogen, Carlsbad, CA, USA). RNA was precipitated using isopropanol, washed with 75% ethanol, and re-suspended in 50 µl of RNase free water. We eliminated DNA contamination using DNAse Treatment and Removal Reagents Ambion kit (Thermo Fisher scientific, Waltham, MA USA) following the manufacturer protocol. RNA extractions were electrophoresed on 2% agarose gels and visualised using ethidium bromide staining. Degraded samples of RNA extractions were eliminated. Then, total RNA concentration was estimated using a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). We normalized concentrations to 250 ng/µl with RNase free water and stored at -80°C. cDNAs bank was generated from 2 µl of extracted RNA (500 ng/reaction) in a 20 µl reaction using Improm-II Reverse Transcription System kit (Promega, Madison, WI, USA), following the manufacturer protocol.

Quantitative PCR

Based on sequences available in Genbank (FJ410912.1 for *Hp-Tgh2*, KC181863.1 for *Hp-CPI*) we designed the following specific primers: Hp-Tgh2-F (CGGTGTGTCTGCCTGAAGAT) Hp-Tgh2-R (CGTTGTATTTGTGCGGTGCA); Hp-CPI-F (CTCGCCTCGTCTATCGTCAC), Hp-CPI-R (CCGCCTTCCATGCTTTTTCC). These primers allowed the amplification of a 108 bp and 100 bp cDNA fragments for *Hp-Tgh2* and *Hp-CPI* gene, respectively. We used *Hp beta-actin* as an endogenous control as advocated by McSorley et al. (2010), with primers Actin-F (TGAGCACGGTATCGTCACCAAC) and Actin–R (TTGAAGGTCTCGAACATGATCTG) which allowed the amplification of a 171 bp cDNA fragment.

We quantified *Hp-Tgh2* and *Hp-CPI* expression by performing a real-time PCR (RT-PCR) using an Applied Biosystems StepOne Plus thermocycler (Applied Biosystems). For each sample and gene, three replicates were carried out in a total volume of 20 µl reaction, which included 10 µl SYBR Green Master Mix 2X (Applied Biosystems), 0.5 µM of each primer, 1 µl of cDNA diluted 20 times and RNAse/DNase-free water to complete the total volume. The PCR amplification was as follows: a first denaturation step at 95°C for 10 minutes, then 40 PCR cycles including denaturation and annealing step at 95°C for 15 seconds and elongation at 60° C for 1 minute. To prove the specificity of the assay, melting curves for all reactions were determined. This procedure consisted of incubations for 15 seconds at 95°C, 60 seconds at 60°C and a final slow heating with a rate of 0.3°C per second up to 95°C with continuous fluorescence measurement. A negative control (water) was added on each plate to ensure the absence of any contamination.

Hp-Tgh2 and *HP-CPI* mRNA relative expression levels were estimated for each sample using the method developed by (Pfaffl et al. 2008). We used *Hp beta-actin* as the endogenous reference gene as advocated by (McSorley et al. 2010). We calculated Qn = [(ETar + 1)-CpTar]/[(ERef + 1)-CpRef] with ETar, ERef, CpTar and CpRef being, respectively, the mean efficiencies and the crossing points of the target and endogenous reference genes. The mean PCR efficiencies were estimated using the LinRegPCR software (Ruijter et al. 2009). Crossing point values were estimated for each sample using the second derivative maximum method implemented in StepOne software (Thermo Fisher scientific,

Waltham, MA USA). We estimated, and considered in the analysis, Cp mean corresponding to the average of triplicate RT-PCR reactions for target and reference genes.

Statistical analyses

We analysed the following host and parasite traits: cytokines in the plasma (log-transformed), body mass, FEC (log-transformed), *Hp-Tgh2* and *Hp-CPI* expression levels. We ran ANOVA models that included treatment (PBS *vs*. LPS), time post-injection and their interaction as independent variables. For the analyses of body mass and FEC, we used a generalized linear mixed model with a normal distribution of errors and mouse identity declared as a random variable, to compare pre-injection values and values at 5, 29 and 53 hours post-injection. Degrees of freedom were estimated using the Satterthwaite method.

We analysed the effect of selection regimes on FEC estimated from day 11 to 74 pi using a generalized linear mixed model on log-transformed values. The model included the selection regimes, day post-infection and their interaction as fixed factors and mouse identity as random factor. The significance of fixed effects was determined using type 3 test with Satterthwaite approximation for degrees of freedom.

We used one-way ANOVAs to test the effect of the selection regimes on *Hp-Tgh2* and *Hp-CPI* expression.

Statistical analyses were performed with R and SAS.

Results

Intra-host

Mice injected with LPS had a rapid increase of circulating IL-6, TNF-α, and IL-10 (IL-6: time, $F_{2,22} = 75.11$, $p < 0.0001$, treatment, $F_{1,22} = 41.78$, $p < 0.0001$, time x treatment, $F_{2,22} = 36.39$, $p < 0.0001$; TNF-α: time, $F_{2,22} = 70.01$, $p < 0.0001$, treatment, $F_{1,22} = 55.23$, $p < 0.0001$, time x treatment, $F_{2,22} = 99.02$, $p < 0.0001$; IL-10: time, $F_{2,22} = 61.81$, $p < 0.0001$, treatment, $F_{1,22} = 87.05$, $p < 0.0001$, time x treatment, $F_{2,22} = 95.47$, $p < 0.0001$; Figure 1). On the contrary, circulating IL-4 (a Th2 cytokine) did not increase in LPS treated mice, and even tended to be higher in PBS individuals (time, $F_{2,22} = 0.02$, $p = 0.9841$, treatment, $F_{1,22} = 6.77$, $p = 0.0163$, time x treatment, $F_{2,22} = 1.49$, $p = 0.2482$; Figure 1). While circulating TNF-α, IL-6

and IL-10 were strongly and positively correlated (ranging from r = 0.87 for IL-6 and IL-10 to r = 0.96 for TNF-α and IL-10, all p's < 0.0001, n = 28), IL-4 tended to be negatively correlated with the other cytokines, even though the correlation coefficients were weak and did not reach statistical significance (ranging from r = -0.31 for IL-4 and IL-6 to r = -0.32 for IL-4 and IL-10, all p's > 0.05, n = 28).

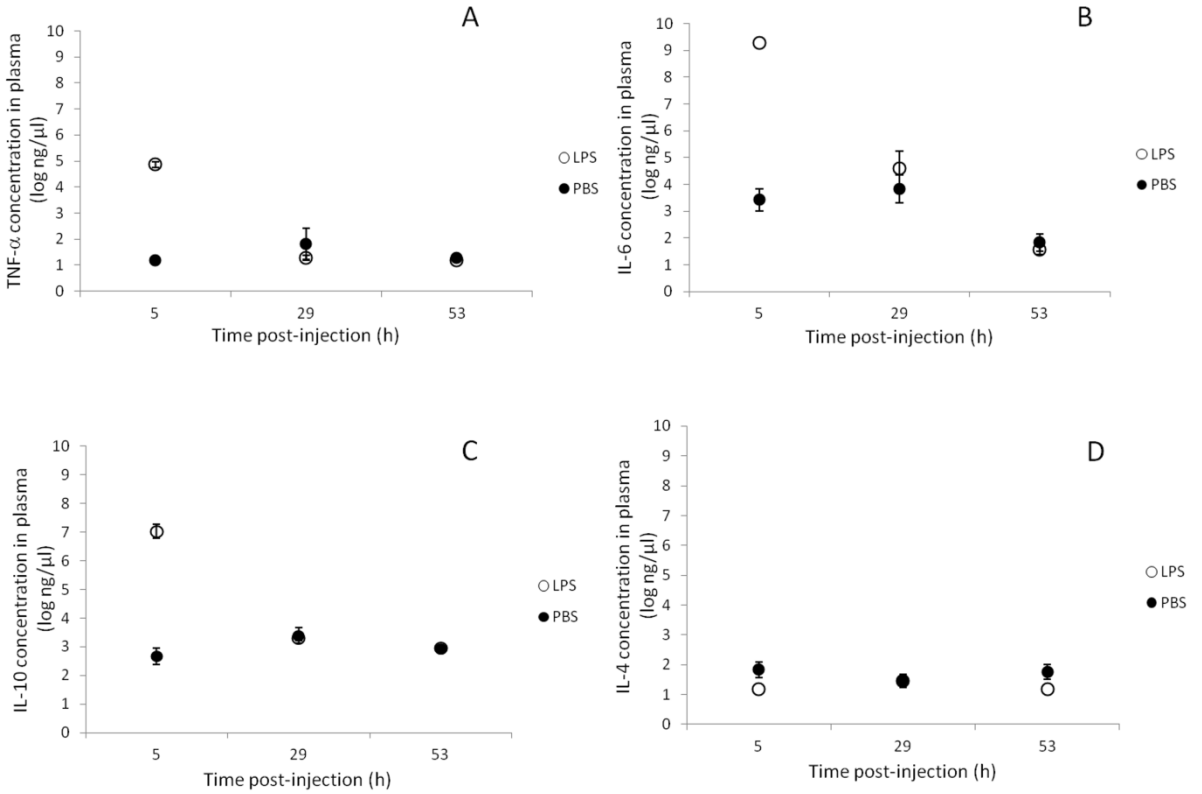

Figure 1**.** Circulating levels (log ng/µl) of TNF-α (A), IL-6 (B), IL-10 (C) and IL-4 (D) at 5, 29 and 53 hours post- injection (red dots = LPS; black dots = PBS). Mean values ± standard errors are reported.

Rapidly after injection, mice treated with LPS lost body mass while body weight of controls remained stable (time: $F_{3,26.2} = 24.11$, $p < 0.0001$; treatment: $F_{1,28.3} = 14.30$, $p = 0.0007$; time x treatment: $F_{3,26.2} = 33.33$, $p < 0.0001$; Figure 2).

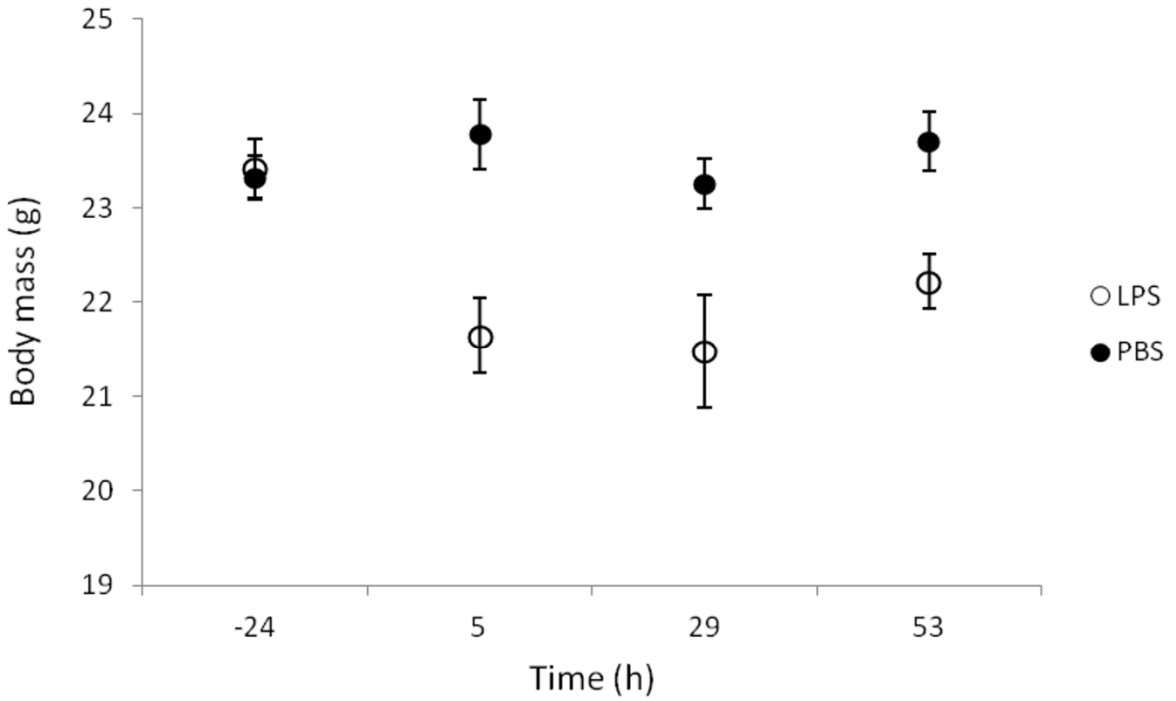

Figure 2. Body mass (g) of mice at 24 hours pre-injection and at 5, 29 and 53 hours post-injection (red dots = LPS; black dots = PBS). Mean values ± standard errors are reported.

We did not have enough *H. polygyrus* mRNA for three mice. Therefore, *Hp-Tgh2* and *Hp-CPI* expression levels were assessed for worms from 27 hosts. Changes across time of the expression of *Hp-Tgh2* tended to differ between treatments (Figure 3), even though the time x treatment interaction did not reach statistical significance ($F_{2,21}$ = 2.84, p = 0.0809). This might be due to low statistical power and visual inspection of figure 3A suggests that the expression of *Hp-Tgh2* might differ between treatments at certain time points. We therefore conducted post-hoc tests for each time point and found that *Hp-Tgh2* expression differed between treatments at 29 hours post-injection ($F_{1,8}$ = 5.28, p = 0.0507), with *H. polygyrus* from mice injected with LPS having a slightly higher *Hp-Tgh2* expression level compared to parasites from control mice (Figure 3). No difference was found at 5 and 53 hours post-injection (all p's > 0.4).

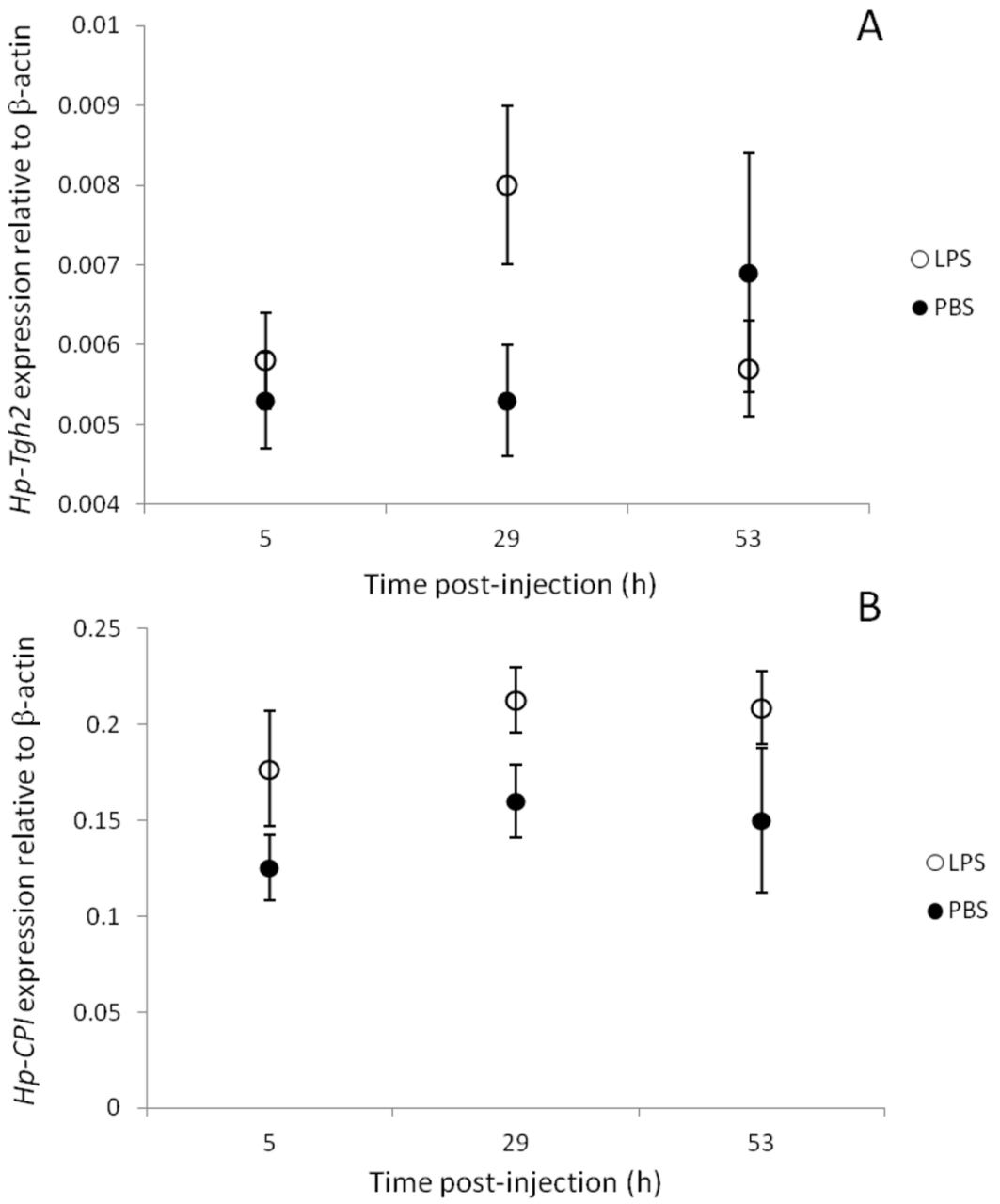

Figure 3. *Hp-Tgh2* (A) *Hp-CPI* (B) expression relative to β-actin in *H. polygyrus* at 5, 29 and 53 hours post-injection (red dots = LPS; black dots = PBS). Mean values ± standard errors are reported.

HP-CPI expression varied between treatments ($F_{1,21}$ = 8.37, p = 0.0087) but not through time post-injection ($F_{2,21}$ = 1.5, p = 0.2458); the time x treatment interaction was not statistically significant neither ($F_{2,21}$ = 0.02, p = 0.9845). *H. polygyrus* from mice injected with LPS had higher *Hp-CPI* expression levels compared to controls from 5 to 53 hours post-injection (Figure 3).

Egg shedding was consistently higher in mice injected with LPS compared to controls, except for the pre-injection values (time: $F_{3,24} = 11.45$, $p < 0.0001$; treatment: $F_{1,24} = 18.36$, $p = 0.0003$; time x treatment: $F_{3,24} = 9.63$, $p = 0.0002$; Figure 4).

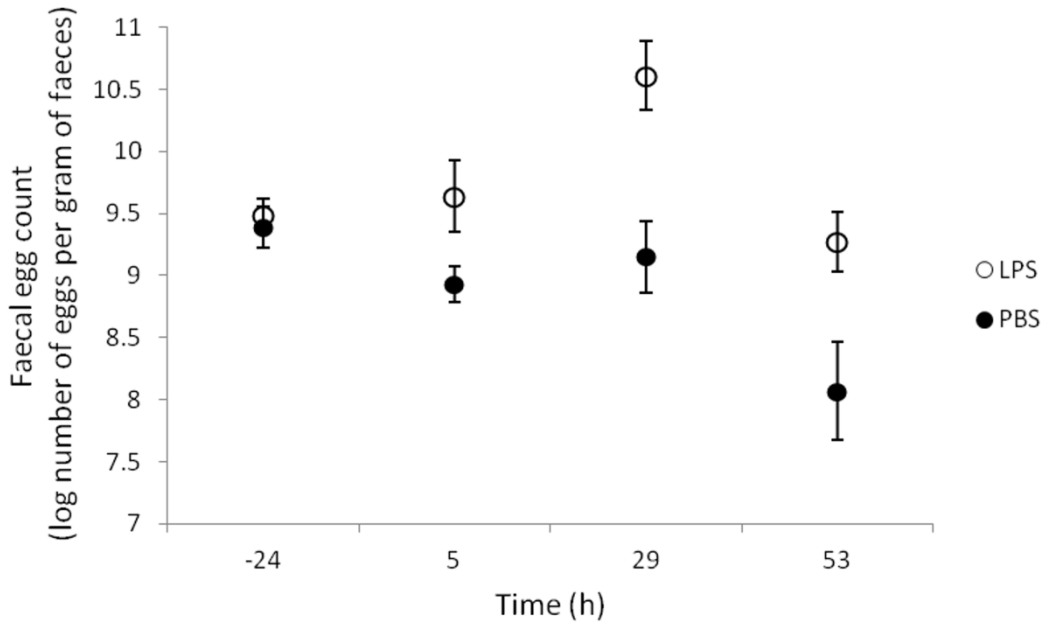

Figure 4. Faecal egg count (log-transformed number of eggs per gram of faeces) at 24 hours pre-injection and at 5, 29 and 53 hours post-injection (red dots = LPS; black dots = PBS). Mean values ± standard errors are reported.

Inter-hosts

Expression of *Hp-Tgh2* and *Hp-CPI* genes did not differ among selection lines (*Hp-Tgh2,* $F_{3,15} = 1.27$, $p = 0.3216$; *Hp-CPI*, $F_{3,15} = 0.18$, $p = 0.9104$, Figure 5).

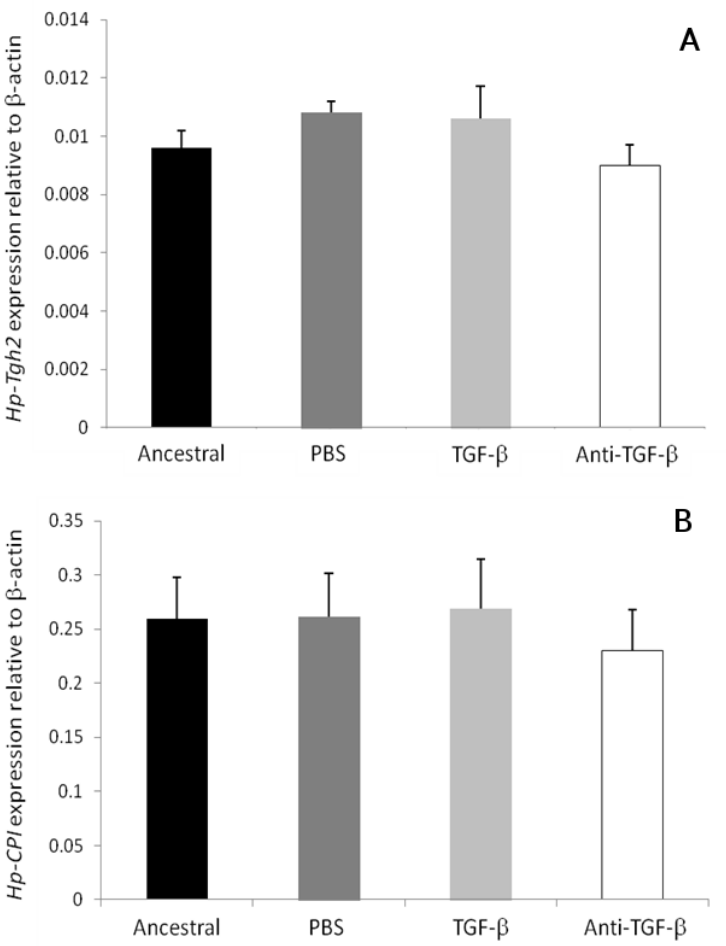

Figure 5. *Hp-Tgh2* (A) and *Hp-CPI* (B) expression relative to β-actin in *H. polygyrus* maintained during four generations in mice treated with TGF-β (green), anti-TGF-β antibodies (red), or PBS (blue) and infecting control, unmanipulated, hosts. Black histograms refer to the values for the ancestral (pre-selection) line. Mean values ± standard errors are reported.

The mixed linear model investigating the effect of selection regimes on FEC showed a strong time x selection interaction (time: $F_{1,164} = 160.46$, $p < 0.0001$; selection regime: $F_{3,30.3} = 1.87$, $p = 0.1551$; time x selection regime: $F_{3,164} = 8.80$, $p < 0.0001$). All selected lines tended to have higher FEC compared to the ancestral line, possibly due to worm adaptation to BALB/c hosts. However, egg shedding remained high in mice infected with parasites that had evolved in anti-TGF-β hosts (Figure 6).

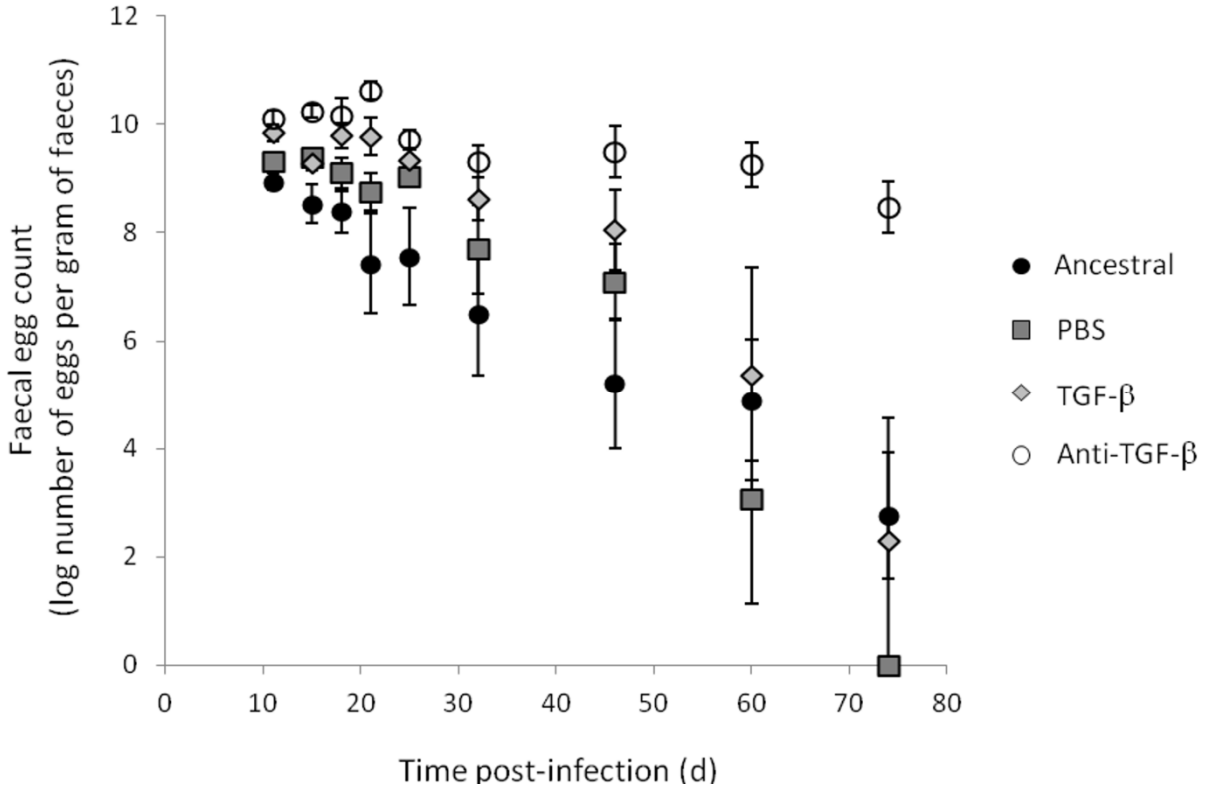

Figure 6. Faecal egg count (log-transformed number of eggs per gram of faeces) of *H. polygyrus* maintained during four generations in mice treated with TGF-β (green), anti-TGF-β antibodies (red), or PBS (blue) and infecting control, unmanipulated, hosts. Black dots refer to the values for the ancestral (pre-selection) line. Faecal egg count was assessed from 11 to 74 days pi. Mean values ± standard errors are reported.

Discussion

The aim of this study was to explore the plasticity of immunomodulatory mechanisms and life history traits of the nematode *H. polygyrus* in response to an inflammatory environment. To this purpose, we stimulated the inflammatory response of *H. polygyrus*-infected mice with a LPS injection. In agreement with our prediction, we found that two immunomodulatory genes were weakly but significantly up-regulated few hours after a change in the inflammatory environment. This was also accompanied by an enhanced parasite egg shedding.

We also conducted a serial passage experiment where *H. polygyrus* was maintained in hosts with an over-expressed, a down-regulated, or an unmanipulated inflammatory response. In line with the idea that the immunomodulatory capacity mostly responds to the current immune environment experienced by the nematode, we did not find any difference in the expression of two immunomodulatory genes among the selected lines when infecting

control hosts. However, parasites maintained in hosts with an up-regulated inflammatory response had higher FEC when infecting unmanipulated hosts, suggesting that the inflammatory environment induced a permanent change in the nematode life history traits.

Intra-host change of immune environment

H. polygyrus responded to a sudden induction of the host inflammatory response with a weak but significant up-regulation of *Hp-Tgh2* and *Hp-CPI* genes. *Hp-Tgh2* was slightly up-regulated only at one time point (29 hours post-injection) whereas *Hp-CPI* was up-regulated during the whole course of the study (5, 29 and 53 hours post-injection). The plasticity of the immunomodulatory mechanisms in response to the immune environment implies that *H. polygyrus* can detect environmental cues perceived as danger for its survival/persistence and deploy the appropriate response to down-regulate host immunity (Babayan et al. 2010).

Hp-Tgh2 produces a TGF-β mimic presenting a conserved activity domain of the TGF-β protein family (McSorley et al. 2010). Previous work showed that the TGF-β mimic stimulates the expansion of Tregs (Grainger et al. 2010). Tregs are crucial for the regulation of inflammation and more generally to dampen the immune response (Taylor et al. 2009). The expression of *Hp-CPI* gene was up-regulated rapidly after the LPS injection (5 hours post-injection) and remained high at 29 and 53 hours post-injection. CPI protein induces cellular hyporeactivity, modulates cytokines production and interferes with antigen-presenting cells (Schierack et al. 2003). In *H. polygyrus*, Sun et al. (2013) showed that *Hp-CPI* alters the differentiation of bone-marrow dendritic cells when stimulated with LPS and suppresses the expression of major histocompatibility II gene, two components of the antigen-presenting cell process. Overall, an up-regulated expression of *Hp-Tgh2* and *Hp-CPI* should provide a better protection to the worm against the host immune response.

In addition to the up-regulation of immunomodulatory genes, we found that the inflammatory environment also induced a rapid change in parasite egg shedding. We used faecal egg count as an overall proxy of parasite performance because we could not count the number of adult worms recovered in the intestine. Even though we collected worms, the need to rapidly preserve them to avoid RNA deterioration, prevented us to accurately count them. Therefore, our assessment of parasite life history traits relies on the number of eggs shed in the faeces. Obviously, high FEC can be due to large population size of nematodes,

reflecting a high infection success, or to highly fecund parasites. Previous work conducted on *H. polygyrus* has shown that FEC positively covaries with the number of adult parasites (Brindley and Dobson 1982, Sitepu et al. 1986). We can discard the possibility that the induced inflammatory response affected developmental time, since the inflammatory stimulation occurred 14 days pi, a few days after parasite emergence in the intestine and the beginning of egg shedding. The short time lapse (i.e., hours) between the inflammatory stimulation and the response in terms of egg shedding also suggests that the increase in FEC is probably not due to differential mortality between more or less fecund worms within hosts but to a plastic adjustment of investment into reproduction (Hashimoto et al. 2010). Whether this represents an adaptive response of the parasite or the mere consequence of changes in environmental constraints is a key question. LPS stimulation strongly polarizes the immune response towards a Th1 profile, and Th1 effectors tend to inhibit the Th2 response that targets intestinal nematodes. Therefore, it could be that activating the Th1 response simply relaxed the immune selection pressure on the worm, finally promoting parasite fitness. In agreement with this hypothesis, we found that circulating levels of IL-4 tended to be lower in LPS mice, even though the difference between LPS and PBS individuals was much smaller than for Th1 cytokines. IL-4 also tended to be negatively correlated with TNF-α and IL-6 even though the correlation coefficients did not significantly differ from zero. Therefore, while we found evidence for an inhibitory effect of LPS stimulation on the Th2 response, the magnitude of the effect was small, potentially suggesting that the increased parasite egg shedding in LPS-treated mice was not merely due to relaxed immune pressures.

LPS is known to produce a series of physiological and behavioural changes (fever, reduced activity, cachexia), and poor condition may simply make host exploitation easier for the parasite (Beldomenico et al. 2008). Alternatively, one could speculate that the cues produced in response to the LPS injection can be seen as signals of poor future survival prospect, either because of direct immune-mediated parasite mortality, or because of increased risk of host mortality. In this case, the adaptive response would be to increase investment into current reproduction even if this is paid later on in terms of future reproduction or survival. In other terms, the inflammatory environment might have modified the optimal solution of the trade-off between current *vs.* future reproduction. In addition to the current *vs.* future reproduction trade-off, the inflammatory environment might also

affect the number vs. quality of offspring trade-off as suggested by (Babayan et al. 2010). Future work should investigate whether eggs produced by worms experiencing an up-regulated inflammatory response have similar fitness prospects (hatching success, infection success, etc.) than eggs produced by control worms.

Inter-host change of immune environment

After four generations of selection in hosts with up- or down-regulated inflammation, *H. polygyrus* did not have different expression of two immunomodulatory genes in control mice. This finding corroborates the idea that the expression of *Hp-Tgh2* and *Hp-CPI* is essentially induced by the current environmental conditions. Inducible responses are supposed to evolve in variable and unpredictable environments. High levels of expression of immunomodulatory genes might actually be costly for the parasite if the environment does not require such high investment in immune interference. First, hosts differ in their degree of immune aggression and a fixed strategy of immune interference might be worthless and incur costs if the production of immunomodulatory molecules consumes metabolic resources. Second, down-regulating, or more generally interfering with host immunity can be costly for the parasite if this exposes the host to opportunistic infections with the associated mortality risk (Cornet and Sorci 2010, Guivier et al. 2016). Therefore, inducible, plastic expression of the immunomodulatory genes could be the best strategy for *H. polygyrus* to maximize its persistence within the host.

Alternative explanations for the absence of response to selection might be that i) environmental conditions were not harsh enough to provide sufficient selection pressures; ii) four generations were not enough to produce a response to selection, if the additive genetic variation is low. Even though we cannot discard these alternative explanations, the finding that parasite life history traits did respond to selection indicates that i) we successfully altered the environmental conditions the different selected lines were exposed to; ii) four generations of selection were enough to produce a micro-evolutionary change. In addition to this, previous work supports the idea that environmental conditions were selective for the parasite and four generations were enough to observe a response to selection. First, Doligalska et al. (2006) injected anti-TGF-β antibodies in BALB/c mice infected with *H. polygyrus* and found that neutralizing TGF-β enhanced inflammation and

depressed parasite performance (both egg shedding and number of adult parasites in the intestine). Grainger et al. (Grainger et al. 2010) also showed that inhibiting TGF signalling reduced *H. polygyrus* burden, even though suppressing directly TGF-β did not affect parasite numbers. Second, serial passages of *H. polygyrus* in Q mice showed that parasites rapidly evolved higher infectivity (the number of adult worms recovered at day 11 and 21 pi) in Q mice but had impaired performance in inbred C_3H mice (Dobson and Owen 1977). In other experiments, *H. polygyrus* were passaged through different strains of naïve and previously infected (immunized) mice. Worms rapidly responded to selection, in terms of infectivity and egg shedding, with a plateau occurring after six generations (Dobson and Tang 1991). Parasites passaged through immune hosts also had better performance compared to parasites from naïve hosts (Su and Dobson 1997). Overall, these previous results are in agreement with those reported here, even though we modified different aspects of the immune environment. Few passages were enough to observe a response to selection and parasites in the most immunocompetent hosts had the best performance when infecting unmanipulated mice.

We found that *H. polygyrus* maintained in hosts with an up-regulated inflammatory response had higher FEC when infecting control, unmanipulated, hosts. This finding is in agreement with the result of our previous experiment where worms exposed to an inflammatory environment plastically responded by enhancing their investment into egg production. Of course, as mentioned above, higher FEC in the anti-TGF-β line could come from either higher investment into egg production or a larger population size due to better infectivity. Even though we cannot determine which parasite life history traits actually responded to selection, we can safely say that parasites evolving in anti-TGF-β mice had a better "performance" compared to the other selection lines when infecting unmanipulated mice. Further work is actually needed to precisely identify the life history trait(s) that responded to selection.

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Chapitre 4 : Réponse micro-évolutive d'un nématode parasite à une inflammation intestinale.

Title: Micro-evolutionary response of a gut nematode to intestinal inflammation

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Abstract

Parasitic helminths interfere with the host immune response of their hosts to establish long-lasting chronic infection. While favorable to the parasite, the capacity to dampen the immune response can also provide a benefit to the host in terms of reduced risk of immune disorders and immunopathology. The immunomodulatory role of nematodes has actually been exploited in clinical trials to treat a number of human inflammatory and immune diseases. However, how parasites adapt to an inflammatory environment remains a poorly explored question. Here, we conducted a serial passage experiment where the gut nematode *Heligmosomoides polygyrus* was maintained during 9 generations in mouse hosts that had a drug-induced intestinal inflammation or in control hosts. The life history traits of parasites from the selected lines were assessed in hosts that were either exposed to the inflammatory drug or kept as controls, in a fully factorial design. This allowed us to explore, conjointly, the effect of plastic and microevolutionary response to the inflammatory environment. In addition to the nematode life history traits, we also assessed the severity of the intestinal inflammation. We found that *Heligmosomoides polygyrus* adapted to the inflammatory environment through both plastic and microevolutionary responses. In particular, per capita fecundity was globally enhanced in worms that experienced the inflammatory environment and that were selected in the inflammatory environment. Interestingly, we also found that worms selected in the inflammatory environment were better able, after 9 generations of selection, to alleviate the drug-induced inflammatory symptoms. This latter result further highlights the potential therapeutic role of gut nematodes to treat inflammatory diseases.

Introduction

A common feature shared by virtually all parasitic organisms is that they have to cope with the defenses mounted by their hosts. Vertebrates have a complex and sophisticated anti-pathogen defense, the immune system. Parasites have evolved an astonishingly diversity of mechanisms of immune evasion, that range from hiding or becoming invisible to the immune system to directly suppressing the immune response (Schmid-Hempel 2008). Helminths are often put forward as being one of the best examples of parasites with immunosuppressive effects (Maizels et al. 2004). Compared to micro-parasites, helminths are large, complex, metazoan that offer many antigenic sites for the recognition by immune cells. Their size often induces traumatic lesions during their penetration and migration within the host body which activates and stimulates the host immune response (Allen and Wynn 2011). Finally, they have relatively long life cycles and can persist for years within their definitive hosts (Gems 2000), which implies a long lasting interaction with the host immune system. For all these reasons, the persistence of helminths in their definitive hosts requires a finely-tuned regulation of the host immune response (Maizels et al. 2016).

Helminthiases are still widespread infections of humans in tropical countries, while in Europe and North America, infection with intestinal worms has almost disappeared (WHO 2009, Lustigman et al. 2012). Interestingly, the temporal trend of decline in the prevalence of helminthiasis in wealthy countries has been paralleled by a sudden increase in the incidence of immune and inflammatory diseases such as inflammatory bowel disease, multiple sclerosis or allergies (Bach 2002). It is tempting therefore to speculate on the possible role that helminths played as regulators of the human immune response during our evolutionary history and that their eradication has disrupted this fine-tuned equilibrium giving rise to more severe immune disorders (Sorci et al. 2016). Even though most evidence is based on epidemiological associations between incidence of immune diseases and prevalence of infection (Fleming and Cook 2006), some experimental work supports the view that helminths are key to prevent the risk of immune disorders (see Finlay et al. 2014 for a review). At the molecular level, the mechanisms underlying the protective effects have been fairly well established at least in a few model systems involving rodent hosts (Finlay et al. 2014, Heylen et al. 2014; McSorley et al. 2013; Bashi et al. 2015). Obviously, the capacity of helminths to protect and to alleviate the symptoms of immune diseases paves

the way for a possible therapeutic role of such organisms. Recently, clinical trials have been conducted with living parasites to treat patients suffering from a number of immune diseases and even though the results are not conclusive at 100%, administering immunomodulating helminths can indeed contribute to ameliorate the disease symptoms (Wammers et al. 2014; Fleming and Weinstock 2015; Evans and Mitre 2015; Maizels 2016).

Beyond the effects of helminths on the host immune system, an associated question is the potential of parasites to adapt to the immune environment provided by the host, within a generation and among generations. Even though helminths have relatively long life cycles compared to viruses or bacteria, they still have much shorter generation time than their hosts which gives them an advantage in terms of adaptive potential with respect to the immune system of their hosts. An interesting question is therefore how parasites do respond when exposed to an up-regulated immune response. Serial passage is a powerful tool used to address this type of experimental evolution (Ebert 1998). Lineages of parasites are maintained over generations in hosts expressing an up-regulated immune phenotype whereas control lines are maintained in control hosts. After a few generations of serial passages, the phenotype of the selected parasites is compared between lines and between the ancestral and the derived lines. Such an experimental evolution approach might be particularly relevant not only to address the fundamental question of the adaptation potential of parasites but also to predict the possible evolutionary trajectory of the therapeutic capacity of these organisms.

We investigated the micro-evolutionary response and the adaptation to the intestinal inflammatory response using the nematode *Heligmosomoides polygyrus* as a model system. *Heligmosomoides polygyrus* is a gut nematode that is commonly used to study the molecular dialog between the parasite and the host immune system (e.g., Urban et al. 1991; Shea-Donohue et al. 2001, Ince et al. 2009 ; Hang et al. 2010, Reynolds et al. 2012). This species is a natural parasite of rodents with a direct life cycle, the host getting infected when ingesting the infectious larvae. Upon infection, larvae penetrate into the intestinal wall and after a few days go back into the intestinal lumen as adults and start releasing eggs that are shed in the external environment with the host feces.

H. polygyrus is an ideal model to study the micro-evolutionary response to the immune environment because it has been shown to interfere with the host immune response in several ways (Grainger et al. 2010; McSorley et al. 2013). The nematode produces excretory/secretory molecules that provide protection towards the host immune response, inhibiting the process of antigen presentation (Manoury et al. 2001; Sun et al. 2013) or activating and stimulating the population of Treg lymphocytes that dampen both the Th1 and Th2 responses (Grainger et al. 2010; McSorley et al. 2013). Because of this capacity to dampen the host immune response, *H. polygyrus* has been shown to protect mice from the inflammatory symptoms of induced colitis (Elliott et al. 2004; Sutton et al 2008, Hang et al. 2010; Blum et al 2012, Donskow-Lysoniewska et al 2012).

Previous work has also shown that *H. polygyrus* do respond to the selection exerted by the host immune response over a small number of generations. Su and Dobson (1997) showed that serial passages of *H. polygyrus* in immunocompetent mice (mice that were previously exposed to the nematode) induced changes in major parasite life history traits (fecundity and infectivity) after a few generations of selection. These results show that lab populations of *H. polygyrus* still have standing genetic variation that allows a rapid response to selection exerted by the host immune response and adaptation to the host environment. More recently, our group conducted a serial passage experiment where *H. polygyrus* was maintained in hosts injected with TGF-β (one of the main anti-inflammatory cytokines), an anti-TGF-β antibody (that therefore neutralizes TGF-β), and in control hosts (Guivier et al. under review). In agreement with previous results, we found that *H. polygyrus* rapidly shifted its life history traits (egg shedding) according to the selection regime. Su and Dobson (1997) and our own work, however, used a systemic approach, and did not explore the specific effect of intestinal inflammation on the adaptive potential of the parasite and its capacity to resolve and alleviate the inflammatory symptoms.

Here, we specifically addressed this question. We conducted a serial passage experiment where *H. polygyrus* was maintained in hosts with induced colitis [due to the ingestion of dextran sulfate sodium (DSS)] or in control hosts. After 9 generations of selection, worms from each selection line were used to infect mice with inflamed intestine or control hosts in

a fully factorial design. This allowed us to disentangle the effect of the current immune environment from the micro-evolutionary response to inflammation.

Materials and Methods

Ethic statements

All animal experiments were approved by the Comité d'Ethique de l'Expérimentation Animale Grand Campus Dijon (CNREEA n° C2EA – 105) and by the "Ministère de la Recherche et de l'Enseignement Supérieur" (project N°01867.01) according to the national guidelines ("Charte nationale portant sur l'éthique de l'expérimentation animale", Ministère de la Recherche et de l'Enseignement Supérieur) on the use of animals for research purposes.

Mice and selection protocol for parasite lines

BALB/c female mice were purchased from JanvierLABS (JRj) and housed (five individuals per cage 18.5 x 38 x 22.5cm enriched with shelters) at the Université de Bourgogne. They were maintained under constant temperature (24°C) and photoperiod (12L:12D), and received food pellets and filtered tap water ad libitum.

Mice used for the serial passages were infected with 150 L3 larvae of *H. polygyrus* in 0.1 ml of water, by oral gavage using a feeding needle on an insulin syringe of 1 ml. The original population of parasites was maintained in B6CBAF1 female mice and was kindly provided by Rick Maizels (Center for Immunity, Infection, and Evolution, Edinburgh, Scotland). Five mice were used for each selection regime per generation. The two selection regimes were: exposure to an inflammatory environment or to a control environment. To this purpose, mice were given either a dextran sulfate Sodium (DSS) solution in drinking water during 4.5 days (starting two days prior to the *H. polygyrus* infection) or regular drinking water. DSS is a complex branched glucan which induces colitis (Chassaing et al. 2014). We used a 1% DSS solution from generation 0 to generation 3 and then moved to a stronger selection regime from generation 4 to generation 9 (3.5%).

In addition to the serial passages, we used 20 mice to assess the life history traits of the original population of *H. polygyurs* (generation 0, G_0), and 40 mice to assess the life history traits of the selected lines after 9 generations (G_9). To this purpose, at G_0, 10 mice were exposed to DSS (1% during 4.5 days) and 10 mice were left as control. Two days after the

start of the DSS treatment, each mouse was infected with 150 L3 larvae from our original population. We monitored parasite fecal egg count at day 9, 12, 16, 19, 23 and 26 post-infection, as follows. Mice were transferred at 9 am in individual cages with a grid on a humidified towel paper at the bottom to prevent feces desiccation. Mice were left four hours in these cages and then put back in their shared cages. Feces produced during this four-hour period were collected and 350 mg were smashed and suspended in 2.5 ml of water. Thereafter, 5 ml of salted water (75% of saturation) were added to allow eggs to float. After agitation, a fraction of this suspension was transferred into a McMaster chamber for egg count. We performed two counts per sample and used the mean values. Fecal egg count is expressed as number of eggs per mg of feces. We used these daily counts to compute the total egg production as the sum of each fecal egg count throughout the study.

At day 13 post-infection half of the mice were killed by cervical dislocation. The abdomen was immediately opened and we counted the number of adult worms (and the number of female worms) in the intestinal lumen. The second half of mice was euthanized at day 27 p.i., and we used the same procedure to count adult worms in these mice. Counting female worms allowed us to compute per capita fecundity at day 12 and 26 p.i.

After 9 generations of selection, parasites from each selection line (DSS and control) were used to infect 10 mice that were exposed to DSS (3% during 4.5 days starting two days prior to the infection) or left as control. Therefore, overall, we had four groups of mice (n = 10 mice per group) where the selection regime and the current environment were crossed in a fully factorial design. Parasite life history traits (fecal egg count, number of surviving parasites at day 13 and 26 p.i., and per capita fecundity) were monitored as described above for the G_0.

Inflammatory symptoms induced by the DSS treatment were assessed using an integrative measure (changes in body mass) and more specific indices (diarrhea, bleeding). Host body mass was measured every two days. Diarrhea, rectal bleeding and perianal inflammation were assessed based on the visual inspection of the perianal region. For each of these indices, we gave a score of 1 if the perianal region was dirty with feces residues or blood, or if the region was fur-loss and reddened. For each mouse, the three scores were

summed up to give a global index that varied between 0 (no inflammatory symptoms) to 3 (maximal inflammation).

Statistical analysis

The number of adult worms collected in the intestinal lumen at day 13 and 27 p.i. was analyzed using a generalized linear model with a Poisson distribution of errors. At G_0, the model included time p.i., the immune environment (DSS vs. control) and the interaction between the two. At G_9, the model included time p.i., the immune environment, the selection line and the interactions between the three variables.

Overall fecal egg count was analyzed using a one-way [with immune environment (G_0)] and two-way ANOVA [with immune environment, selection line and the interaction between the two (G_9)].

Per capita fecundity at days 12 and 26 p.i. was analyzed using a linear model with normal distribution of errors. At G_0, the model included time p.i., the immune environment and the two-way interaction. At G_9, the model included time p.i., the immune environment, the selection line and the two and three-way interactions.

Changes in host body mass at G_9 were analyzed using a linear mixed model that included mouse identity as a random factor (to take into account the repeated nature of the data) and time p.i., squared time p.i., the immune environment, the selected line and the two- and three-way interactions as fixed factors.

Changes in the score of intestinal inflammation at G_9 were analyzed using a linear mixed model that included mouse identity as a random factor (to take into account the repeated nature of the data) and time p.i., the selected line and the two-way interaction as fixed factors.

The comparison between the original population (G_0) and the selected lines (G_9) was restricted to the control environment, to get rid of the difference in the DSS dose that was used at G_0 (1%) and at G_9 (3%), using one-way ANOVAs.

We always started with the full model (that included all the possible interactions) and we dropped the non-significant terms step-by-step, to select the final model.

All statistical analyses were performed with R software V3.0.3.

Results

Parasite abundance

At G_0, the number of adult parasites recovered in the intestinal lumen at day 13 and day 27 post-infection did not differ between immune environments (DSS vs. control) ($F_{1,16}$ = 0.0075, p = 0.9319; fig. 1A). Parasite number decreased with time p.i. ($F_{1,16}$ = 8.2271, p = 0.0112; fig. 1A) but the interaction between time p.i. and the immune environment was not statistically significant ($F_{1,16}$ = 0.0222, p = 0.8835).

At G_9, only time p.i. significantly explained variation in number of parasites, being lower at day 27 than at day 13 p.i., whatever the selection line or the environment (fig. 1C and D, table 1).

When comparing the original population to the line that evolved in control hosts we found that the number of adult parasites at day 13 p.i. was significantly lower in the evolved line ($F_{1,8}$ = 6.3803, p = 0.0355; fig. 1B), suggesting on overall decrease in infectivity. However, worms maintained in DSS-treated hosts tended to have similar adult population size to the original population at day 13 p.i. ($F_{1,8}$ = 4.544, p = 0.0656; fig. 1B), indicating that exposure to the inflammatory environment contributed to maintain a similar performance to the original population. At day 27 p.i., the two selected lines had similar adult population size to the original population (control, $F_{1,8}$ = 0.2516, p = 0.6294; DSS, $F_{1,7}$ = 0.0455, p = 0.8372; fig. 1B).

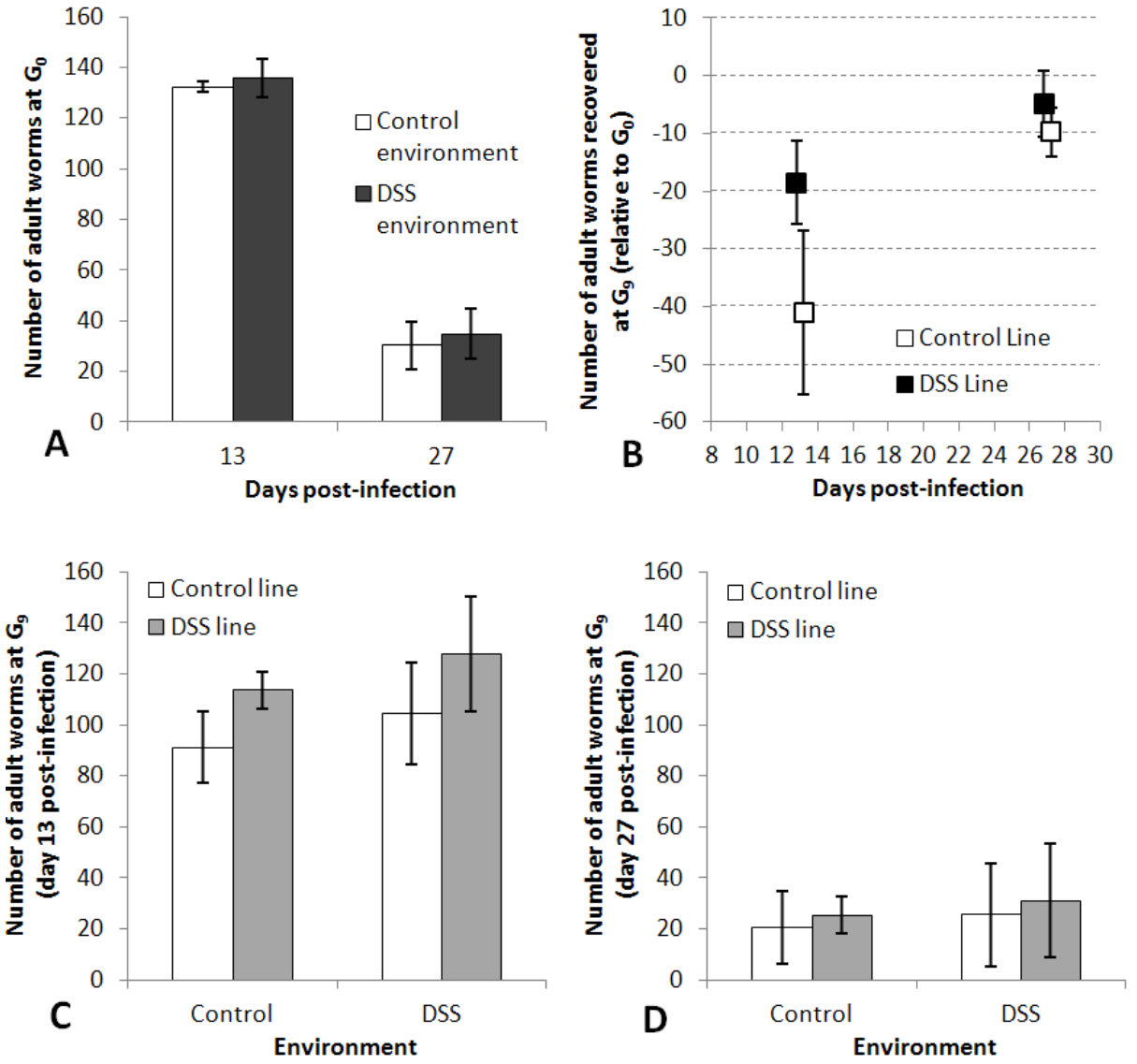

Figure 1. Number of adult worms in the intestinal lumen at G_0 for the two immune environments (A); number of adult worms in the intestinal lumen at G_9 (day 13 p.i.) as a function of the immune environment and the selection line (B); number of adult worms in the intestinal lumen at G_9 (day 27 p.i.) as a function of the immune environment and the selection line (C); difference in number of adult worms in the intestinal lumen between G_9 and G_0 for the two selected lines in the control environment. For each panel, means $\pm$ SE are reported.

Table 1. Generalized linear model of number of adult worms recovered at day 13 and 27 p.i. for G_9. A) full model; B) selected model.

Sources of variation			
A) full model	F	df	p
Time p.i.	28.778	1,30	<0.001
Environment	0.005	1,30	0.9463
Line	0.146	1,30	0.7049
Time p.i.*Environment	0.035	1,30	0.852
Time p.i.*Line	0.000	1,30	0.9882
Environment*Line	0.000	1,30	0.9834
Time p.i.*Environment*Line	0.000	1,30	0.9993
B) selected model	F	df	p
Time p.i.	98.197	1,36	<0.001
Environment	1.336	1,34	0.2557
Line	2.987	1,34	0.093

Cumulative fecal egg count

We analyzed the overall egg shedding over the study period. At G_0, overall fecal egg count was not different between immune environments ($F_{1,8}$ = 2.2277, p = 0.1739; fig. 2A).

At G_9, parasites facing an inflammatory environment tended to have a higher overall egg shedding whatever the selection line (table 2; fig. 2B).

Between G_0 and G_9, there was no difference in the overall egg shedding whatever the selection line (control, $F_{1,8}$ = 0.7761, p = 0.4040; DSS, $F_{1,8}$ = 2.659, p = 0.1416).

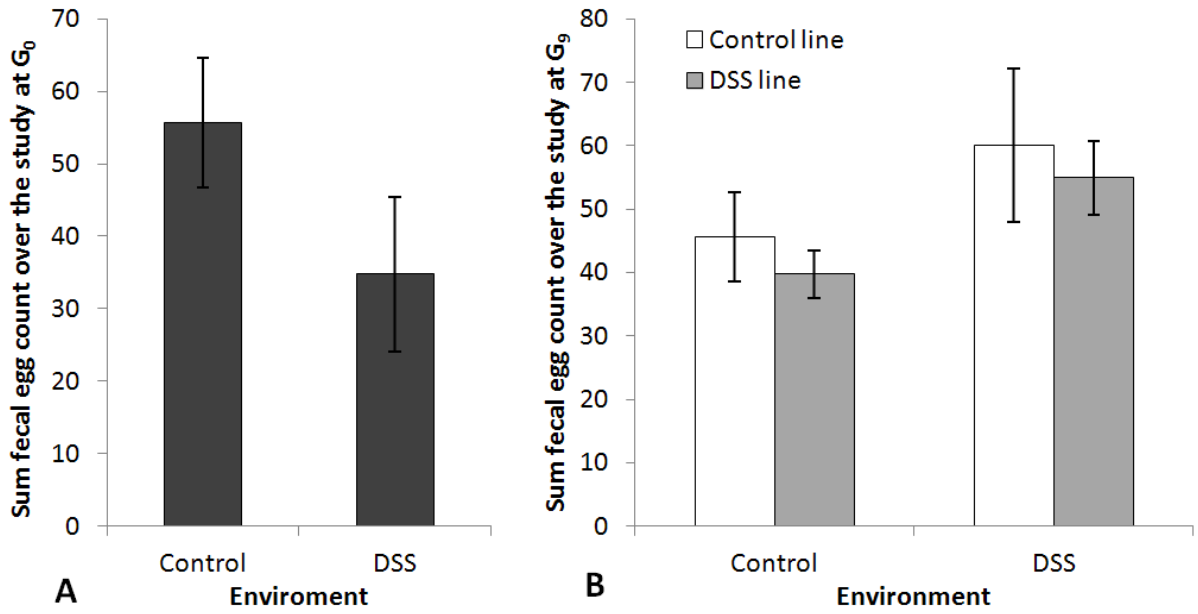

Figure 2. Total fecal egg count at G_0 for the two immune environments (A); total fecal egg count at G_9 for the two immune environments and the two selection lines (B).

Table 2. Two-way ANOVA on the total fecal egg count at G_9.

Sources of variation			
A) full model	F	df	p
Line	0.218	1,16	0.6471
Environment	1.909	1,16	0.1860
Line x environment	0.002	1,16	0.9622
B) selected model	F	df	p
Line	0.533	1,17	0.4755
Environment	3.860	1,17	0.0660

Per capita fecundity

At G_0, per capita fecundity was globally similar for the two immune environments over time, except for worms in the control environment at day 26 p.i., that had a much higher per capita fecundity compared to the other groups (fig. 3A). This resulted in a significant interaction between time p.i. and immune environment (table 3).

At G_9, the statistical analysis showed that per capita fecundity dependent on the three-way interaction between time p.i., selection line and immune environment (table 4). To make the interpretation of the results easier, we decided to run two supplementary models, one for data collected at day 12 p.i. and one at day 26 p.i. (fig. 3C and D).

At day 12 p.i., per capita fecundity was affected by the immune environment, per capita fecundity being higher in worms exposed to DSS, whatever the selection line (fig. 3C,

table 5). At day 26 p.i., worms selected in DSS-treated mice and exposed to the inflammatory environment had higher per capita fecundity compared to the other groups (fig. 3D), resulting in a statistically significant interaction between selection line and immune environment (table 6).

The comparisons across generations in the control environment showed that while at day 12 there was no difference in per capita fecundity between the original and the selected lines (control, $F_{1,8} = 0.2286$, $p = 0.6454$; DSS, $F_{1,8} = 0.0636$, $p = 0.8073$), at day 26 per capita fecundity was higher in the original populations compared to the two evolved lines (control, $F_{1,8} = 8.3139$, $p = 0.0204$; DSS, $F_{1,7} = 15.428$, $p = 0.0057$; fig. 3B).

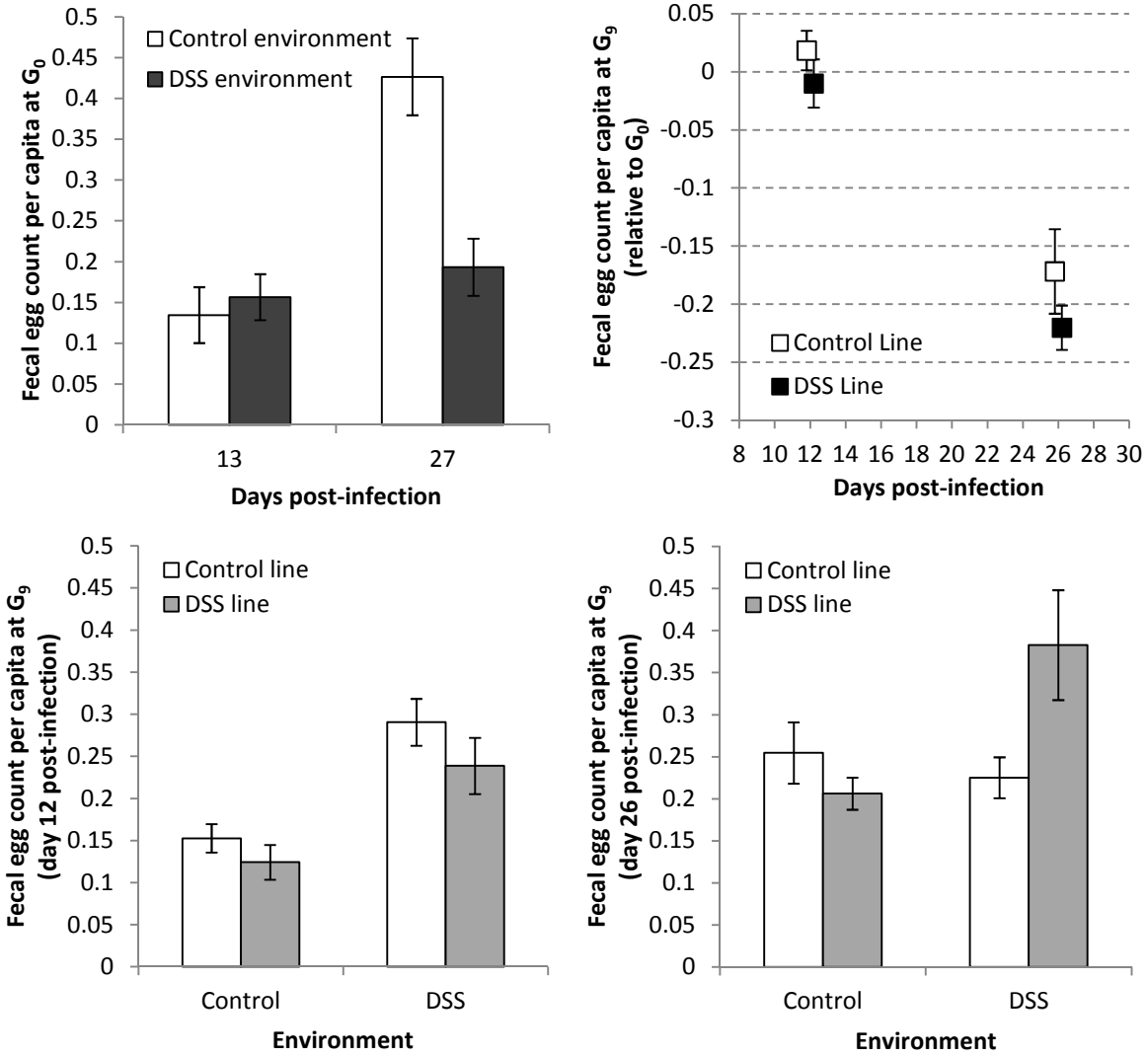

Figure 3. Per capita fecundity at G_0 for the two immune environments (A); per capita fecundity at G_9 (day 12 p.i.) as a function of the immune environment and the selection line (C); per capita fecundity at G_9 (day 26 p.i.) as a function of the immune environment and the selection line (D); difference in per capita fecundity between G_9 and G_0 for the two selected lines in the control environment (B). For each panel, means $\pm$ SE are reported.

Table 3. Two-way ANOVA on per capita fecundity at G_0.

Sources of variation	F	df	p
Time p.i.	0.438	1,15	0.5182
Environment	4.610	1,15	0.0485
Time p.i.*Environment	11.250	1,15	0.0043

Table 4. ANOVA on per capita fecundity at G_9.

Sources of variation	F	df	p
Time p.i.	8.849	1,30	0.0057
Environment	0.375	1,30	0.5447
Line	5.344	1,30	0.0278
Time p.i.*Environment	0.775	1,30	0.3856
Time p.i.*Line	8.819	1,30	0.0058
Environment*Line	2.420	1,30	0.1303
Time p.i.*Environment*Line	5.294	1,30	0.0285

Table 5. Two-way ANOVA on per capita fecundity at G_9 for day 12 p.i.

Sources of variation			
A) full model	F	df	p
Environment	10.047	1,16	0.0059
Line	2.051	1,16	0.1713
Environment*Line	0.209	1,16	0.6535
B) selected model	F	df	p
Environment	25.594	1,17	<0.001
Line	2.579	1,17	0.1267

Table 6. Two-way ANOVA on per capita fecundity at G_9 for day 26 p.i.

Sources of variation	F	df	p
Environment	7.867	1,14	0.014
Line	6.273	1,14	0.0252
Environment*Line	5.358	1,14	0.0363

Severity of colitis symptoms

Variation in body mass was expressed as the percent change with respect to the initial values (when the DSS treatment started, two days prior to the infection).

At G_0, 10 days after the start of the DSS treatment, mice had gained around 10% of their initial body mass whatever the group (figure 4 and table 7).

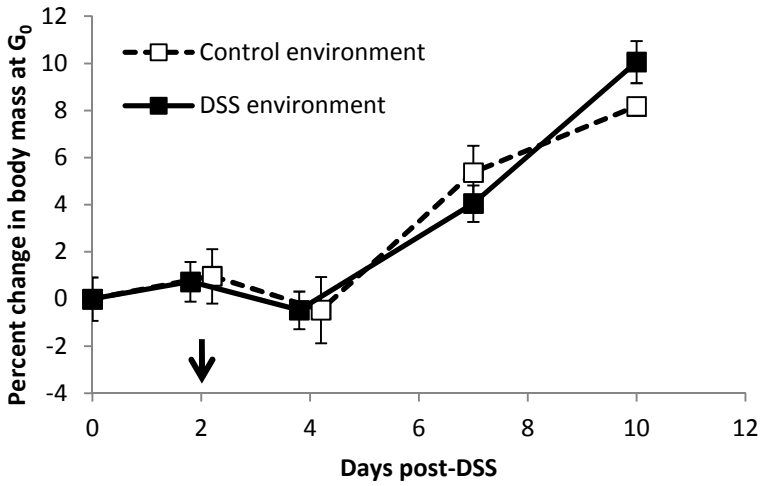

Figure 4. Percent changes in body mass from the start of the DSS treatment at G_0 for the two immune environments: (A) Control environment (B) DSS environment. The arrow indicates the day of infection with *Heligmosomoides polygyrus*. Means ± SE are reported.

Table 7. Linear mixed model on changes in body mass at G_0. A) full model; B) selected model.

Sources of variation			
A) full model	Ward F	df	p
Time post-DSS	87.903	1,78	<0.001
Environment	0.055	1,23.76	0.8171
Time post-DSS*Environment	0.660	1,78	0.4192
B) selected model	Ward F	df	p
Time post-DSS	155.601	1,79	<0.001
Environment	0.004	1,18	0.9484

At G_9, the statistical model showed that changes in body mass was marginally affected by the three-way interaction between time, immune environment and selected line ($F_{1,391.59}$ = 3.5587, p = 0.0599; fig 5). Visual inspection of figure 5, shows that while for three out of the four groups there was a gain of body mass with time (on average +9% at day 11 post-DSS treatment), mice exposed to the inflammatory treatment and infected with worms selected in a control environment lost body mass at day 7 and 9 post-DSS (-3% of the initial body mass). For a comparison, at this time point, the other three groups had already gained 3% of their initial body mass. To make the interpretation of the three-way interaction easier, we run two supplementary models splitting the data according to the inflammatory environment mice were exposed to. In the control environment, mice increased their body mass whatever the line of worms they were infected with (table 8). On the contrary, in the

DSS environment, we found a statistically significant interaction between time and selection line (table 9).

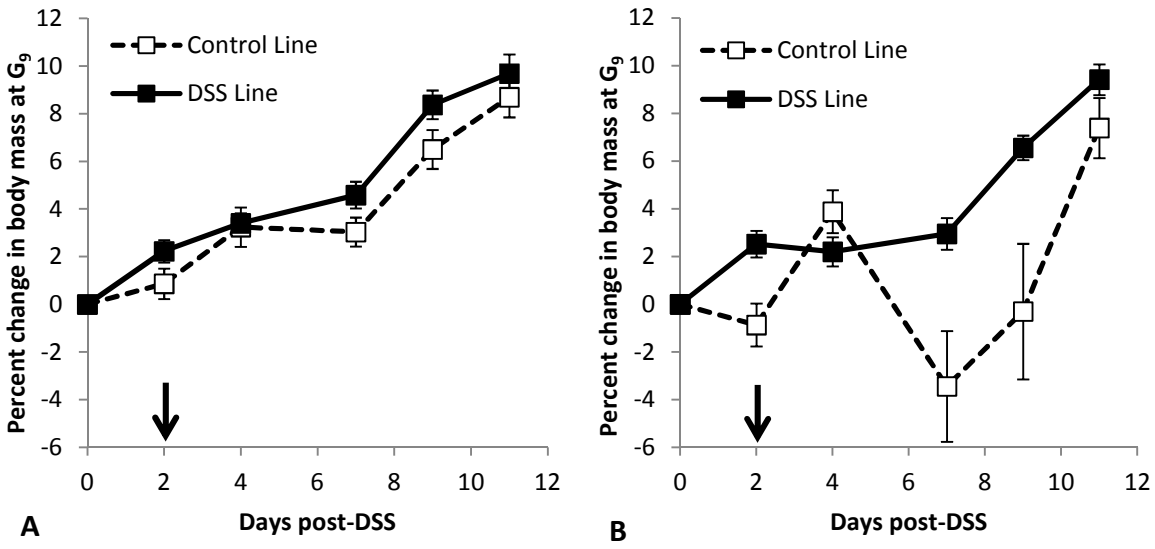

Figure 5. Percent changes in body mass from the start of the DSS treatment at G_0 for the two immune environments: (A) Control environment (B) DSS environment. The arrow indicates the day of infection with *Heligmosomoides polygyrus*. Means ± SE are reported.

Table 8. Linear mixed model for changes in body mass at G9 in the control environment. A) full model; B) selected model.

Sources of variation			
A) full model	Ward F	df	p
Time post-DSS	31.495	1,196.00	< 0.0001
Squared Time post-DSS	2.143	1,196.00	0.1448
Line	0.888	1,48.90	0.3506
Time post-DSS*Line	1.825	1,196.00	0.1783
Squared Time post-DSS*Line	0.665	1,196.00	0.4157
B) selected model	Ward F	df	p
Time post-DSS	43.158	1,198.00	< 0.0001
Squared Time post-DSS	8.288	1,198.00	0.0044
Line	2.181	1,38.00	0.1480

Table 9. Linear mixed model for changes in body mass at G9 in the inflammatory environment. A) full model; B) selected model.

Sources of variation			
A) full model	Ward F	df	P
Time post-DSS	0.965	1,194.01	0.3270
Squared Time post-DSS	3.531	1,194.01	0.0617
Line	1.346	1,53.54	0.2511
Time post-DSS*Line	4.870	1,194.01	0.0285
Squared Time post-DSS*Line	1.502	1,194.15	0.2219
B) selected model	Ward F	df	P
Time p.i.	0.062	1,195.11	0.8042
Squared Time p.i.	14.875	1,195.15	0.0002
Line	1.085	1,52.52	0.3024
Time p.i.*Line	7.425	1,195.61	0.0070

In addition to changes in body mass, we also assessed the severity of colitis using a synthetic index of diarrhea, rectal bleeding and perianal inflammation.

At G_0, we did not find any signs of diarrhea or rectal bleeding, based on visual examination of the perianal area, during the course of the DSS treatment.

At G_9, the first diarrheal, bleeding and perianal inflammation signs, were visible at day 7 post-DSS. We found that mice infected with worms passaged in control (non-inflamed) hosts consistently suffered from more severe inflammation at day 7, 9 and 11 post-DSS (fig. 6 - table 10). Mice infected with worms kept in DSS treated hosts fully recovered from the inflammatory symptoms by day 11 post-treatment.

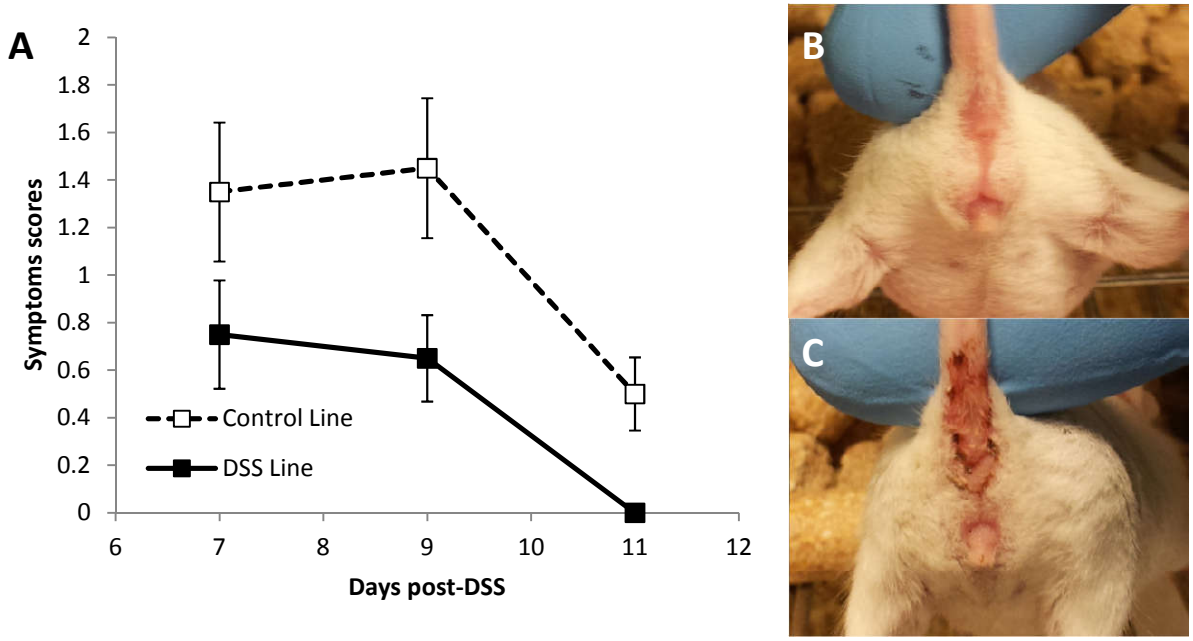

Figure 6. A) Synthetic score of inflammation severity of DSS treated mice at G_9. Means ± SE are reported. B) and C) Pictures of two mice with different inflammatory symptoms.

Table 10. Linear mixed model on the score of inflammation severity at G_9. A) full model; B) selected model.

Sources of variation			
A) full model	Ward F	df	p
Time post-DSS	11.810	1,78.00	< 0.0001
Line	1.831	1,105.91	0.1789
Time post-DSS*Line	0.105	1,78.00	0.7468
B) selected model	Ward F	df	p
Time post-DSS	27.1828	1,79.00	< 0.0001
Line	6.1516	1,38.00	0.0177

Discussion

While recent work has shown that intestinal nematodes can alleviate the symptoms of inflammatory diseases due to their immunomodulatory properties (Finlay et al. 2014; Maizels 2016), the adaptation and the micro-evolutionary response of helminths to an inflammatory environment remain poorly explored. Here, using a serial passage experiment where the nematode *Heligmosomoides polygyrus* was maintained during 9 generations either in mice experiencing an intestinal inflammation or in control hosts, we show that exposure to an inflammatory environment produced shifts in the parasite life history traits, in terms of per capita fecundity, and increased the therapeutic capacity to alleviate the colitis symptoms.

Heligmosomoides polygyrus has been a widely used model species to investigate the molecular dialog between invading parasites and the host immune response. Excretory/secretory molecules in the proteome of *H. polygyrus* have been shown to have multiple immunomodulatory effects (McSorley et al. 2013) and to be produced differently at different stages of the parasites (McSorley et al. 2010). These immunomodulatory effects are actually shared by many parasitic helminths which rely on these strategies of immune interference for their own persistence within the host (Maizels et al. 2004).

These findings provide a mechanistic basis to the epidemiological observations that prevalence of infection with helminths is usually negatively correlated with the occurrence of several immune and inflammatory diseases (Bach 2002). For instance, prevalence of multiple sclerosis per country is tightly associated with the prevalence of infection with the whipworm *Trichuris trichura* (Fleming and Cook 2006). Epidemiological studies are by nature based on correlative evidence and one cannot firmly establish a causal relationship between helminths and incidence of immune disorders based on such evidence. Nevertheless, experimental work has further confirmed the hypothesis that helminths can indeed play a protective role towards immune diseases. Work conducted on animal models has for instance shown that Non-obese diabetic (NOD) mice infected with different species of helminths (*S. mansoni, T. spiralis, H. polygurs*) are protected from the risk of developing diabetes (Cooke et al. 1999; Liu et al. 2009; Saunders et al. 2007). Evidence of the protective effect of helminths also comes from studies where humans were either treated with anthelminthic drugs (and the risk to contract an immune disease monitored after the clearance of the infection) either directly exposed to the infection. For instance, a placebo-controlled study where Gabonese children were treated with the anthelminthic drugs praziquatel and mebendazole showed an increased rate of allergic reaction to a skin test in dewormed children (van den Biggelaar et al. 2004). It should be noted nevertheless, that not all studies where anthelminthic drugs have been used report an increased risk to contract immune diseases (Wammers et al. 2014). Direct infection of humans refers to the different clinical trials that have been implemented to test the safety and efficacy of helminthic therapy to treat immune diseases. More than 20 trials have been conducted or are under progress (Fleming and Weinstock 2015). Again, even though not all trails have reported conclusive effects of helminth infection on remission from immune diseases, in some cases

infection does alleviate disease symptoms. For instance, in 29 patients with Crohn's disease, given 2500 *Trichuris suis* ova every 3 weeks for 12 weeks, 79% reported an improvement in disease symptoms (Summers et al. 2005a). In a placebo-controlled study, 43% of patients with ulcerative colitis given *T. suis* ova reported alleviated disease symptoms, vs 17% of patients in the placebo group (Summers et al. 2005b).

Our results show that the nematode capacity to alleviate inflammatory symptoms is a trait that can evolve if parasites are repeatedly exposed to an inflammatory environment. This, further, highlights the potential therapeutic role of immunomodulatory nematodes to treat syndromes associated with intestinal inflammation.

As any living organism, parasites can adapt to the environmental conditions they experience by adopting plastic responses or micro-evolutionary shifts. Our experiment allowed us to tease apart these two responses, since selected lines of worm in inflamed or control hosts were used to infect mice that were either exposed to the inflammatory drug or experienced control conditions, in a fully factorial design. We assessed three major parasite life history traits, the number of adult worms recovered in the intestinal lumen at day 13 and 27 p.i., the cumulative egg shedding over the course of the experiment and the per capita fecundity. We found that both phenotypic responses to the environment and micro-evolutionary shifts did shape the life history traits of *H. polygyrus*. Overall, exposure to an inflammatory environment for a given generation and selection in an inflammatory environment across generations improved fitness-linked traits. The cumulative egg shedding tended to be higher in worms that were exposed to the DSS treatment (even though the statistical support was weak), and the per capita fecundity was higher in worms exposed to the DSS when selected in DSS mice at day 26 p.i. In addition to the overall positive effect of the inflammatory environment on fitness-linked traits, this last finding also suggests a "local adaptation" effect where DSS-selected worms had higher per capita fecundity only when exposed to the inflammatory environment.

The overall positive effect of inflammatory environment on parasite life history traits corroborates previous work conducted on *H. polygyrus*. Donskow-Łysoniewska et al. (2013) treated mice with DSS and infected then with *H. polygyrus*. They found that being exposed to DSS improved parasite infectivity (number of L4 and adult worms recovered in the

intestine) and in vitro egg production. In a previous work we exposed different stages *H. polygyrus* (either larvae either adults) to a DSS-induced inflammatory environment and we also found that whatever the parasite stage that was exposed to the inflammatory environment, infectivity, adult survival and egg output were improved in DSS-exposed worms (Lippens et al. under review). In addition to the positive effect of intestinal inflammation, also a systemic inflammation, induced by a LPS injection produced an increased egg shedding immediately after the inflammatory challenge (Guivier et al. under review).

We did not explicitly investigate the mechanisms underlying this improved performance of DSS-exposed and/or selected worms, but we can speculate about some possible explanations. DSS-induced inflammation is accompanied by a raise of several pro-inflammatory cytokines (Chassaing et al. 2014) and it might be that parasites perceive an inflamed intestinal environment as a danger signal and adopt a faster pace of life (Ricklefs and Wikelski 2002). This latter hypothesis would imply that long term exposure to an inflammatory environment does impair late life-history traits (longevity and/or late fecundity), and that a shift towards improved early life history traits produces similar lifetime reproductive success. Testing this hypothesis would need to monitor parasite performance over longer period of time compared to the course of the experiment reported here. However, previous work has shown that late fecal egg count (at 3 and 7 months p.i.) was significantly lower in *H. polygyrus* infecting mice that had experienced a LPS challenge at day 28 p.i. compared to control mice, corroborating the idea that the inflammatory challenge might have deleterious effect on late-expressed fitness components (Guivier et al. 2016). Whether this is enough to fully compensate the early benefits remains however, an open question.

We induced an inflammatory syndrome at the intestinal level to explore the microevolutionary responses of *H. polygyrus*. Previous work used the same parasite species to explore the microevolutionary response when parasites were exposed to naïve or previously immunized hosts. Su and Dobson (1997) passaged *H. polygyrus* in naïve or previously twice infected Q mice for more than 30 generations. The two lines of parasites were then tested in naïve Q mice. The results showed that parasites passaged in previously

infected mice had better egg output, and better survival compared to the naïve line. This suggests that exposure to the immune response of previously immunized hosts, induced permanent changes in parasite life history traits.

After 9 generations of selection, we found that number of adult worms in the intestine and per capita fecundity were lower compared to the initial population. This reduced performance of evolved lines after 9 passages might be due to the depletion of genetic variation due to repeated bottlenecks and/or evolution of increased immunogenicity over time. Similar results were obtained by previous work, where *H. polygyrus* maintained during 10 generations in Q mice, increased their infectivity (+6%) in homologous infection, suggesting an initial parasite adaptation to the host. This initial adaptation was however followed by an increased expulsion of parasite at day 28 p.i., generation after generation, due to stronger immune responses to structurally homogenous antigens from the selected lines (Dobson and Owens 1977). Overall, the seminal work conducted by Dobson and coworkers a few decades ago suggested that while *H. polygyrus* rapidly responds to the selection exerted by the immune response of the host, maintaining the selection regime over many generations resulted in a plateau with no further changes in parasite phenotypes (Dobson and Tang 1991). This limit to selection was actually reached after 6 generations of selection. Since we assessed the selected lines after 9 generations of selection, it is possible that we were already behind the selection limit.

To conclude, we showed that exposure to an inflammatory intestinal environment, induces both phenotypic and microevolutionary shifts in the life history trajectories of the gastrointestinal nematode *Heligmosomoides polygyrus*, towards faster pace of life and improved capacity to alleviate the inflammatory symptoms. Whether these microevolutionary shifts are also associated with costs and the underlying molecular mechanisms remain to be explored.

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Chapitre 5 : Est-ce qu'une infection à un jeune âge accélère le vieillissement : un test expérimental chez des souris infectées par *Plasmodium*

Title: Does early infection accelerate aging: an experimental test with *Plasmodium* infected mice

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Abstract

While the antagonistic pleiotropy hypothesis has provided a fertile ground of investigation to explain the evolution of aging, the study of the molecular/physiological functions underlying the early benefits/late costs paradigm remains elusive. Here we experimentally tested the hypothesis that while early activation of the inflammatory response confers benefits in terms of protection towards an infectious threat, it also incurs late costs in terms of reduced reproductive output at old age and life span. We used the association between the protozoan *Plasmodium yoelii* and its rodent host. Mice were infected with *P. yoelii* at the age of 7 weeks. Half of the infected mice were also treated with an anti-IL-10 antibody to further up-regulate the inflammatory response. Control groups were injected with heat killed *P. yoelii* (vaccine), with the anti-IL-10 antibody or left unmanipulated. We found that the stimulation of the inflammatory response provided an early benefit in terms of reduced parasitemia during the acute phase of the infection and reduced anemia. After the acute phase, mice were treated with an antimalarial drug to clear the infection. Overall reproductive success, assessed during three consecutive reproductive bouts when mice were 487 - 613 days old, was low showing that mice were in a senescing state. Although we found that previously infected mice had lower reproductive success than vaccinated and anti-IL-10 treated mice, unmanipulated mice also had low reproductive success. At the moment, when 52% of the cohort died, Plasmodium-infected mice have about a two-fold higher survival compared to the control groups. This is contreary to the antagonistic pleiotropy hypothesis. Although not all data are available yet, the current results do not support the idea that early exposure to infectious diseases accelerates aging.

Introduction

Aging is associated with a progressive decline of physiological performance eventually leading to reduced reproductive output and survival (Monaghan et al. 2008). How and why organisms' age have been two major questions for biologists. Understanding the molecular and physiological mechanisms underlying the age-associated decline in performance is key for the treatment of age-associated diseases (Fontana et al. 2010). This is particularly relevant in the context of the current shift towards older age classes that occurs in many countries. Understanding the evolutionary forces that explain the decline in Darwinian fitness with age has a more fundamental interest. Why do organisms age has been a puzzling question for evolutionary biologists for decades (Kirkwood and Austad 2000). Indeed, why should natural selection promote a decline in fitness? The solution to this apparent paradox was provided by William Hamilton a few decades ago by showing that the strength of selection weakens with age and therefore selection is mostly blind to what happens in the late, post-reproductive life (Hamilton 1966). The assumption that selection weakens with age is shared by most of the evolutionary theories of aging, including the antagonistic pleiotropy hypothesis. Williams (1957) suggested that genes impairing fitness at old ages will be selected for if they have fitness enhancing roles at young ages. Given that the intensity of selection is stronger during early life, the benefits of such pleotropic genes will outweigh any late occurring cost. The antagonistic pleiotropy hypothesis has attracted considerable attention and many studies have indeed shown that individuals with better performance at early age do age faster and earlier than individuals with poor early performance [see Lemaitre et al. (2015) for a recent review]. The mechanisms underlying such antagonistic pleiotropy remain however poorly known.

The inflammatory response is an excellent candidate function with potentially antagonistic properties across ages. Inflammation is an essential component of antimicrobial defense and response to trauma. Animal models with knocked out genes of the inflammatory response have very poor survival prospects when facing an infectious threat (see for instance Kurtz et al. 2003). Inflammation is however, by nature, a non-specific defense that can also damage host tissues and many age-associated diseased have an inflammatory origin (including cancer and cardio-vascular diseases) (Sorci and Faivre 2009, Reverri et al. 2014, Wu et al. 2014). Chronic inflammation is actually a disease status of old

age that has been coined “inflammaging” (Vasto et al. 2007, Candore 2010). Therefore, while the inflammatory response certainly confers a benefit in terms of immune protection, it can also generate life threatening disease at old age.

In line with this idea, a few years ago Finch and Crimmins (2004) analyzed the age specific mortality rate of human cohorts in Sweden. They showed that high infant mortality (presumably due to infectious diseases in times with rudimental medical care) was associated with high old age mortality in the same cohorts. They named it the “cohort morbidity phenotype” and suggested that this reflects the late negative consequences of inflammatory processes occurring at early ages. This study stimulated further work. For instance, Barbi and Vaupel (2005) reanalyzed the same data (in addition to data from other sources) and suggested that period effects are actually more important to explain the changes in old age mortality rate compared to the cohort effects. Using completely different data from cohorts born in the 17^{th} and 18^{th} centuries in Canada, Gagnon and Mazan (2009) reported a negative association between early and late mortality within cohorts. Evidence based on historical human data is correlative by nature and prone to the potential confounding effect of several factors. As such demographic analyses of human cohorts have provided mixed, highly debated results (Bengtsson and Lindstrom 2003, Catalano and Bruckner 2006; Bengtsson and Broström 2009, Gagnon and Mazan 2009, Myrskylä 2010).

Experimental approaches are therefore needed to properly test the antagonistic pleiotropy hypothesis. Such experimental evidence is however almost lacking. To the best of our knowledge, a single study explored the effect of immune activation on aging. Pursall and Rolff (2011) exposed *Tenebrio molitor* larvae to a series of immune challenges and found that insects exposed to the immune insults aged earlier than the control groups. The immune response of insects however differs with many respects from the vertebrate immune response and using inert antigens to elicit the immune response does not allow testing the benefits of immune activation. A proper test of the antagonistic pleiotropy hypothesis requires exploring not only the late costs but also the early benefits.

We used here the association between the protozoan *Plasmodium yoelii* and its rodent host to test this hypothesis. We infected mice at a young age (7 week old). Upon infection, *Plasmodium yoelii* induces an inflammatory response that is followed by an antibody based

response (Couper et al. 2008, Chen et al. 2010, Bakir et al. 2011). To further stimulate the inflammatory response we treated half of the infected mice with an anti-IL-10 antibody. IL-10 is one of the principal anti-inflammatory cytokines that contributes to the regulation of the immune response and the resolution of inflammation. Blocking IL-10, either at the phenotypic level (with antibodies) or at the genetic level (in knocked out models) produces on overproduction of pro-inflammatory effectors and a reduction in parasite density (improved resistance) (Redpath et al. 2014). In addition to the non-infected control group, we also had two supplementary controls, one injected with the anti-IL-10 antibody and left free from the parasite and one injected with dead *P. yoelii*. These vaccinated animals should produce an antibody response against the parasite antigens, while having a minimal inflammatory response. We monitored animals during the acute phase of the infection and then treated them with an antimalarial drug to cure the infection. Such malaria free animals were then monitored during their entire lifespan. In addition to the longevity, we also assessed reproductive success of old animals by letting them to breed during three consecutive reproductive bouts. We therefore had two components of organismal fitness measured at old ages, reproductive success and survival.

If the inflammatory response elicited by the infection with *P. yoelii* has antagonistic pleiotropic functions, we expect that i) anti-IL-10 treated mice should better resist to the infection than mice only infected with *P. yoelii*; ii) *P. yoelii* infected mice (and particularly so those also treated with anti-IL-10 antibodies) should have lower reproductive output at old age and reduced longevity compared to the different control groups.

Materials and methods

Ethics statement

All animal experiments were approved by the Comité d'Ethique de l'Expérimentation Animale Grand Campus Dijon, France (CNREEA n C2EA – 105) and by the ''Ministère de la Recherche et de l'Enseignement Supérieur" (project N01867.01) in accordance with the national guidelines (''Charte nationale portant sur l'éthique de l'expérimentation animale", Ministère de la Recherche et de l'Enseignement Supérieur) on the use of animals for research purposes.

Animals

Sixty BALB/c mice were purchased from JanvierLABS (JRj, France) and housed (six individuals per cage - 18.5 x 38 x 22.5 cm - enriched with shelters) at the Université de Bourgogne, France. Mice were maintained under a constant temperature (24°C) and a photoperiod of 12:12 (L:D). They were fed with standard mouse pellets ad libitum and had ad libitum access to filtered tap water.

When they were 7 week old, they were allocated to one out of five different groups (n = 12 mice per group). One group of mice was infected with 10^5 *Plasmodium yoelii* 17XNL by intraperitoneal injection of 100 µl of citrate saline diluted blood. One group of mice was infected with the same dose of *P. yoelii* and also received a treatment with an anti-IL-10 antibody (intraperitoneal injection of 20 µg of anti-IL-10 antibodies). Mice were treated four times, at day -1, 1, 3, and 5 post *Plasmodium* infection. One group of mice received the intraperitoneal injection with 10^6 heat dead *P. yoelii*. One group of non-infected mice was treated with the anti-IL-10 antibody at the same dose during four non-consecutive days. The last group was left as a non-manipulated control. When infected mice were injected with the parasite, the other groups received an intraperitoneal injection of the same volume of PBS. When anti-IL-10 mice were injected with the antibody, the other groups were injected with an equivalent volume of PBS (50 µl).

Plasmodium yoelii infection

Cryopreserved blood with *P. yoelii* 17XNL was diluted with citrate saline and 100 µl of this diluted blood was injected to 5 mice (100 µl per mouse). At day 12 post-infection, 2 µl of blood from were collected from the tip of the tail to estimate red blood cell counts and a drop of blood (also from the tip of the tail) was used for smears to count the number of parasites. Smears were fixed with methanol, stained with Giemsa and the number of parasites per 10,000 red blood cells counted under an optical microscope. Red blood cell counts and number of parasites per 10,000 red blood cells were then used to compute the amount of blood and the dilution (with citrate saline) required to infect 24 mice with a dose of 10^5 parasites in a volume of 100 µl of diluted blood. Blood required to infect the 24 mice was obtained by puncture of the facial vein.

Anti-malaria treatment

At day 39 post-infection, all mice were treated with an anti-malarial drug in order to clear the infection (see Staszewski et al. 2012). Pyrimethamine (Vetranal^{TM} - Sigma Aldrich) was diluted in DMSO at a concentration of 7mg/ml. This solution was further diluted 100 times in filtered tap water. The pH was adjusted between 3.5 and 5 by adding HCl. Drinking bottles of all cages were then filled with the Pyrimethamine solution. Bottles were wrapped in aluminium sheets for light protection. The treatment lasted during 5 consecutive days.

Assessing parasitemia

Five µL of blood were collected from the tail tip, diluted in 200 µL of citrate saline (8.5g NaCl; 15g citrate sodium in 1L of distilled water) and centrifuged at 17000g for 3 min at 4°C. The supernatant was then removed and the pellet frozen at -80°C.

Blood smears were done at days 3, 5, 7, 10, 12, 14, 17, 20, 24, 27, 31 (during the acute phase of the infection), and at days 55, 420 and 525 post-infection to make sure that the treatment was effective in clearing the infection.

Parasitemia was also assessed by real-time PCR on StepOne Real-Time PCR System of Life Technologies at days 3, 5, 7, 10, 12, 14, 17, 20, 24, 27, 31 (during the acute phase of the infection) and at days 55, 420 and 525 post-infection to make sure that the treatment was effective in clearing the infection.

DNA extraction was performed with the MAGMAX DNA MULTI-SAMPLE KIT (Ambion by Life Technologies) following the protocol provided by the manufacturer with 3µL of blood diluted in 75µL of eluent.

The amplified gene was *P. yoelii* merozoite surface protein 4/5 gene (PyMSP4/5) using an Applied Biosystems StepOne Plus thermocycler (Applied Biosystems by Life Technologies). A probe was used for fluorescence signal. For each sample and gene, three replicates were carried out in a total volume of 20 µl reaction, which included 12.5 µl TaqMan® Universal PCR Master Mix (Applied Biosystems), 0.75 µL of each primer (10µM), 2 µl of extracted DNA, 5µL of probe (10µM) and 3.5µL of RNAse/DNase-free water to complete the total volume. The PCR amplification was as follows: a first step at 50°C for 2 minutes to activate the probe followed by a denaturation step at 95°C for 10 minutes, then 40 PCR cycles including

denaturation and annealing step at 95°C for 15 seconds and elongation at 60° C for 1 minute. To prove the specificity of the assay, melting curves for all reactions were determined. This procedure consisted of incubations for 15 seconds at 95°C, 60 seconds at 60°C and a final slow heating with a rate of 0.3°C per second up to 95°C with continuous fluorescence measurement. A negative control (water) was added on each plate to ensure the absence of any contamination.

A real-time PCR standard was made using blood from one individual at the peak of the acute phase (day 12 post-infection). Blood smears were used to count the number of parasites per 10,000 red blood cells. Blood smears were made in the morning when parasites are in the ring stage. We also assessed the number of red blood cells per µl of blood, and used these two numbers to compute the number of parasites per µl of blood. This standard blood was serially diluted to get a range of parasite doses and use them as standards in the real-time PCR.

Crossing point values (Cp) were estimated for each sample using the second derivative maximum method implemented in StepOne software (Thermo Fisher scientific, Waltham, MA USA). Mean Cp values of three replicates were used.

Numbers of parasites per µl of blood were calculated by linear interpolation using the regression between the $\log_{10}$ number of parasites per µl in the standard and the associated Cp. The detection threshold was between 5 and 50 parasites per µl of blood.

PyMSP4/5 primers used were 5'-ATCCATCTACTAGTTTGAATCAAAATGA-3' for the forward primer and 5'-CCATTTAGTTTATCTGATTTGTTTGTATTT-3' for the reverse primer (94pb). The probe sequence was 5'-6FAM-TCATAATTCAAACCCAGATGC-MGB-3'.

Body mass

Mice were weighted at days 0, 3, 5, 7, 10, 12, 14, 17, 20, 24, 27, 31 during the acute phase of the infection and at days 39, 45, 55, 80, 216, 236, 420, 437, 565, 635, 664 and 703, 740, 746, 754.

Haematology

Two µl of blood were collected from the tail tip, diluted in 18µl of PBS and stored on ice before measurement. Numbers of red and white blood cells were counted using a SCIL Vet

abc Plus+ at days 0, 5, 7, 10, 12, 14, 17, 20, 24, 27, 31 during the acute phase of infection with *P. yoelii* and at days 80, 216, 565, 635 and 746.

Reproductive success

When mice were 487 day old, they were moved in individual cages with a male (we used a different male for each female). Males were left with females during 10 days and then removed. Cages were provided with nesting material. We monitored reproductive success as litter size at birth. When this first reproductive bout was completed, males were put back in the female cage (we kept the same female-male pair) and the procedure repeated as for the first bout. Overall, three reproductive bouts were completed. Females were 613 days old when they were put back in their shared cages.

Mortality

Mice were monitored every other day to check their health status. Mice that showed rapidly declining health status (rapid loss of body mass) or irreversible pathologies (tumoral masses) were euthanized by cervical dislocation.

Statistical analysis

We used linear mixed models to test the effect of experimental treatments on parasitemia, red blood cell counts, white blood cell counts, and body mass over the course of the acute infection. Models included time post-infection (and squared time when appropriate) and experimental group as fixed factors (plus interactions) and mouse identity as a random factor. Interactions were dropped when non-significant.

Late costs of early infection were assessed using body mass, reproductive success and mortality as dependent variables. Body mass measured over age 600 days was analysed using a linear mixed model with age, squared age and experimental group as fixed factors and mouse identity as random factor. Reproductive success was assessed as cumulative litter size over three reproductive bouts and analysed using a generalized linear model with a Poisson distribution or errors. Differences between experimental groups in mortality that occurred up to the age of 811 days were assessed using a χ^2 test.

Results

Plasmodium yoelii activates the inflammatory response

We conducted a pilot experiment to test if exposure to *P. yoelii* activates the inflammatory response. BALB/c female mice were infected at the age of 7 weeks with 10^5 *P. yoelii* and blood collected at day 3 and 6 p.i. Control groups were mice injected with heat killed *P. yoelii* and unmanipulated individuals. Infected mice had substantially higher titres of cytokines in the blood compared to both control and vaccinated individuals, especially at day 3 p.i. (IFN-γ: time, $F_{1,8} = 26.58$, $p = 0.0009$; treatment, $F_{2,8} = 109.82$, $p < 0.0001$; time x treatment, $F_{2,8} = 20.25$, $p = 0.0007$; IL-6: time, $F_{1,8} = 14.83$, $p = 0.0049$; treatment, $F_{2,8} = 26.59$, $p = 0.0003$; time x treatment, $F_{2,8} = 7.97$, $p = 0.0125$; IL-10: time, $F_{1,8} = 0.07$, $p = 0.8006$; treatment, $F_{2,8} = 6.47$, $p = 0.0213$; time x treatment, $F_{2,8} = 7.16$, $p = 0.0165$; TNF-α: time, $F_{1,10} = 0.00$, $p = 0.9985$; treatment, $F_{2,10} = 44.49$, $p < 0.001$; time x treatment, NS; Fig. 1).

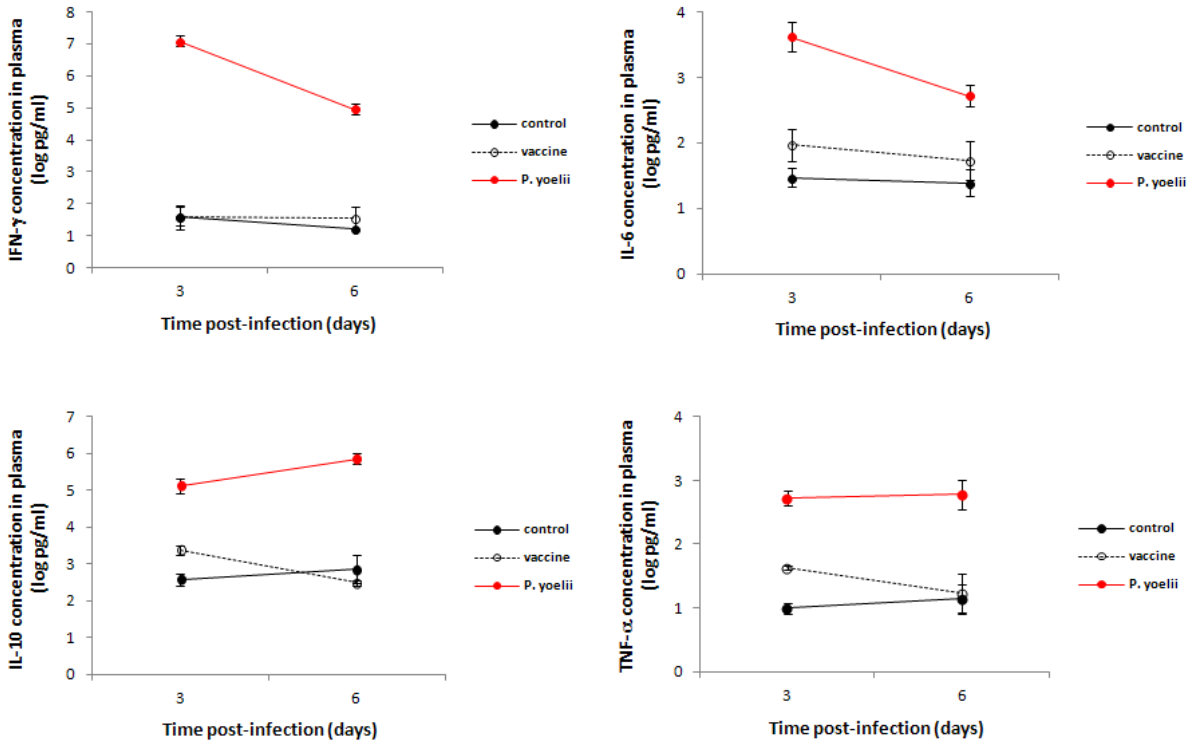

Figure 1. Cytokine (IFN-g, IL-6, IL-10, TNF-a) concentration (log-transformed) in plasma of mice infected with 10^5 *Plasmodium yoelii*, vaccinated with 10^6 heat killed *Plasmodium yoelii*, or left as control, at day 3 and 6 post-infection. Means $\pm$ SE are reported.

Plasmodium infection stimulates leukocytes proliferation

We counted white blood cells during the course of the study and found that *Plasmodium* infected mice had a sharp rise in leucocyte concentration at day 10 and 12 post-infection compared to the other groups (Fig. 2, Table 1).

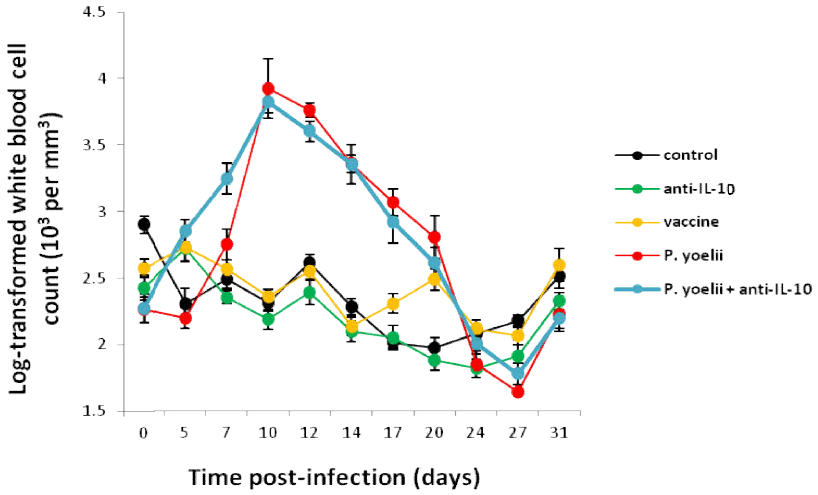

Figure 2. Log-transformed number of white blood cells (10^3 per mm^3 of blood) in the different experimental groups during the course of the acute infection. Means ± SE are reported.

Table 1. Linear mixed model exploring the effect of time and experimental groups on changes in white blood cell count (log-transformed).

Sources of variation	F	df	P
Time p.i.	5.94	1,575	0.0147
Squared time p.i.	35.88	1,574	<0.0001
Treatment	4.90	4,513	0.0007
Time p.i. x Treatment	49.68	4,575	<0.0001
Squared time p.i. x Treatment	63.23	4,574	<0.0001

A more robust inflammatory response reduces parasite burden

Parasitemia peaked at day 12 post-infection for both groups of infected mice (*P. yoelii* and *P. yoelii* + anti-IL-10; Fig. 3A). A linear mixed model showed that, in addition to time and squared time, the two experimental groups had different parasitemia (treatment: $F_{1,222}$ = 9.15, p = 0.0028; time: $F_{1,222}$ = 17.08, p < 0.0001; squared time: $F_{1,222}$ = 34.19, p < 0.0001; time x treatment and squared time x treatment, NS). However, this model poorly fitted the data and using different distributions of errors or data transformation did not improve the fit. We therefore also used a non-parametric test on overall parasitemia per individual (the sum of parasitemia over the course of the study). Two individuals were removed from this

analysis because they died during the acute phase of the infection (one in the *P. yoelii* group and one in the *P. yoelii* + anti-IL-10 group). We therefore only used animals with complete parasitemia values up to day 31 post-infection. This overall parasitemia differed between groups, being significantly lower in the *P. yoelii* + anti-IL-10 group (Wilcoxon two-sample exact test, p = 0.0041, Fig. 3B). We also run day by day tests to investigate whether there was any difference between the two groups at early and late stages of the acute infection. Groups differed at day 5 and 7 post-infection (during the raising phase of parasitemia), with mice treated with the anti-IL-10 antibody harbouring less parasites (day 5: Wilcoxon two-sample exact test, p = 0.0423; day 7: Wilcoxon two-sample exact test, p = 0.0014). This latter difference remained significant after Bonferroni correction (corrected α = 0.0045).

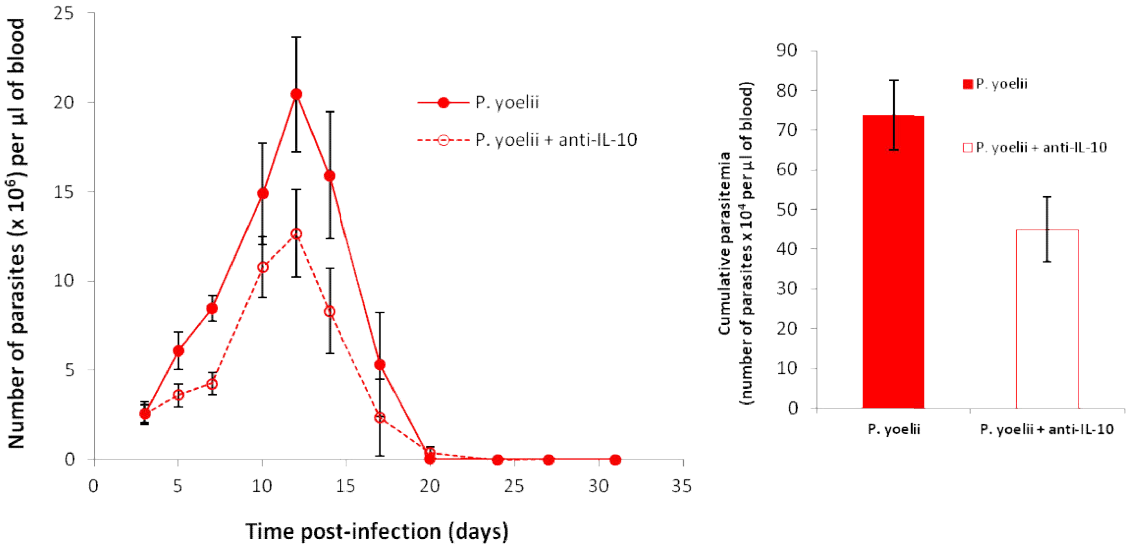

Figure 3. A) Parasitemia in *P. yoelii* and *P. yoelii* + anti-IL-10 treated mice over the course of the acute phase of the infection. Means ± SE are reported. B) Mean overall parasitemia (sum of parasitemia across time) for the two experimental groups. Means ± SE are reported.

A more robust inflammatory response reduces early cost of infection

We used changes in red blood cells as a proxy of cost of infection (as mentioned above, only two mice died during the acute phase of the infection, one in the *P. yoelii* group and one in the *P. yoelii* + anti-IL-10 group). Infected mice showed a drop in red blood cell counts with minimum values reached at day 12 and 14 post-infection (Fig. 4, Table 2).

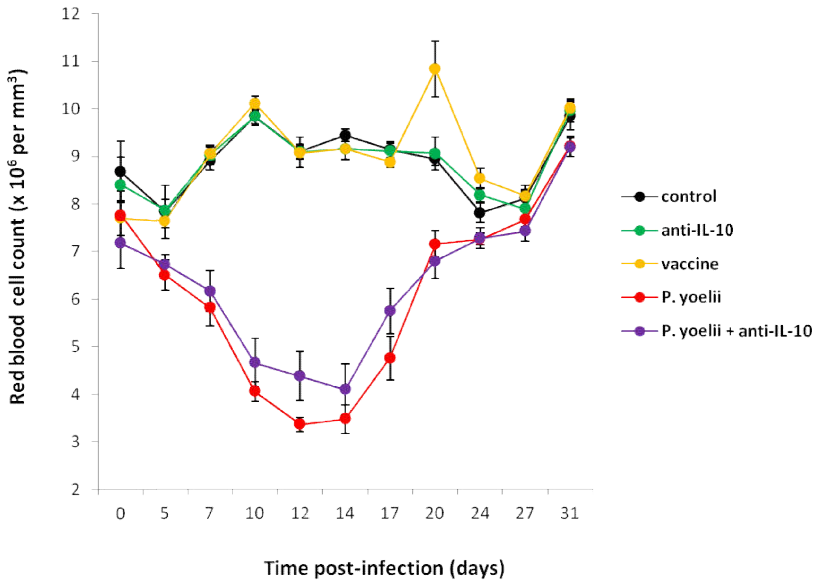

Figure 4. Changes in red blood cell count (x 10^6 per mm^3 of blood) during the acute phase of infection with *P. yoelii* for the different experimental groups. Means ± SE are reported.

Table 2. Linear mixed model exploring the effect of time and experimental groups on changes in red blood cell count.

Sources of variation	F	df	P
Time p.i.	35.04	1,573	<0.0001
Squared time p.i.	71.18	1,571	<0.0001
Treatment	3.00	4,496	0.0183
Time p.i. : Treatment	42.63	4,573	<0.0001
Squared time p.i. : Treatment	55.86	4,571	<0.0001

A comparison of the two infected groups showed that treating mice with the anti-IL-10 antibody reduced the cost of the infection in terms of anaemia (Table 3).

Table 3. Linear mixed model exploring the effect of time and experimental groups on changes in red blood cell count. Only the two infected groups (P. yoelii and P. yoelii + anti-IL-10) were considered here.

Sources of variation	F	df	P
Time p.i.	166.03	1,221	<0.0001
Squared time p.i.	249.69	1,219	<0.0001
Treatment	0.37	1,120	0.5437
Time p.i. : Treatment	3.95	1,221	0.0480
Squared time p.i. : Treatment	4.71	1,219	0.0310

Infection with *Plasmodium* can also produce a loss of body mass. However, we found that infected mice tended to gain more mass during the acute phase of the infection compared to the other groups (Fig. 5, Table 4)

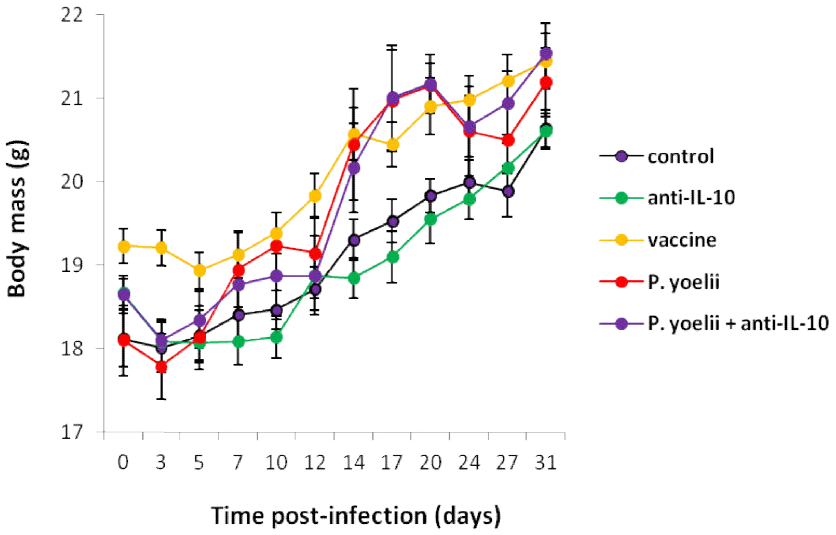

Figure 5. Changes in body mass (g) during the acute phase of infection with *P. yoelii* for the different experimental groups. Means ± SE are reported.

Table 4. Linear mixed model exploring the effect of time and experimental groups on changes in body mass.

Sources of variation	F	df	P
Time p.i.	1403.30	1,636	<0.0001
Treatment	1.81	4,60.8	0.1381
Time p.i. : Treatment	8.30	4,636	<0.0001

Changes in body mass over the course of the acute infection did not differ between the two infected groups (Table 5).

Table 5. Linear mixed model exploring the effect of time and experimental groups on changes in body mass. Only the two infected groups (P. yoelii and P. yoelii + anti-IL-10) were considered here.

Sources of variation	F	df	P
Time p.i.	441.81	1,244	<0.0001
Treatment	0.08	1,24.1	0.7739
Time p.i. : Treatment	0.29	1,244	0.5886

Early infection and late costs

We used several proxies of performance at late ages to test the hypothesis that early infection accelerates aging. At the moment, when mice are 811 days old, 52% of the initial cohort has died. The results on the late costs are therefore likely to change as long as the cohort completely extinguishes.

Body mass

Visual inspection of changes in body mass showed that mice gained mass up to the age of 600 days (Fig. 6). We therefore compared the slopes relating body mass to age from day 600 onwards. While time and squared time were statistically significant, we did not find any effect of experimental groups, suggesting that the slope of age-associate decline in body mass was similar for all groups (Fig. 7, Table 6).

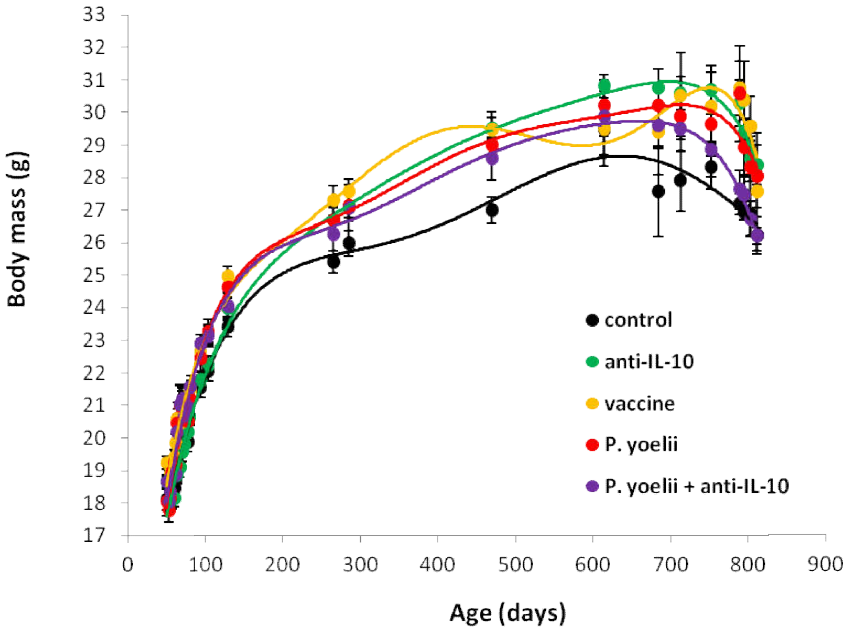

Figure 6. Changes in body mass (g) during the whole study period for the different experimental groups. Lines represent a polynomial fit. Means ± SE are reported.

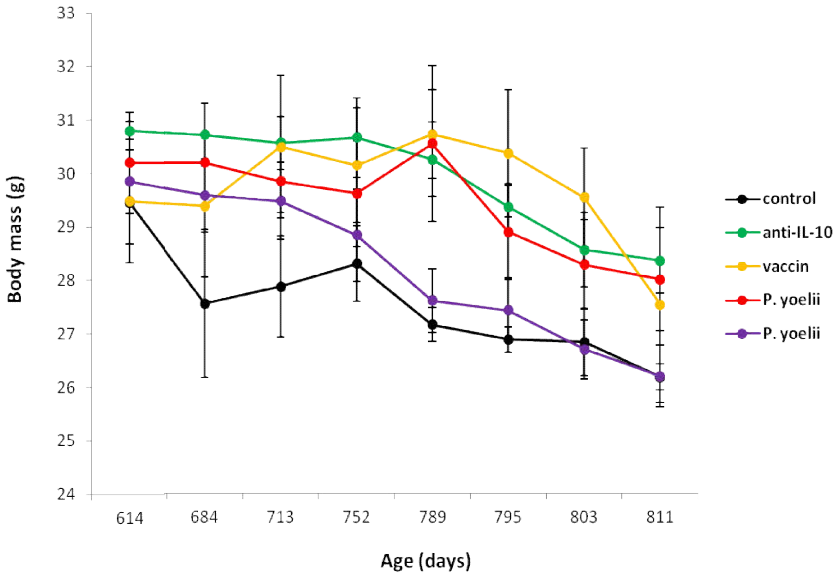

Figure 7. Changes in body mass (g) after the age of 600 days for the different experimental groups. Means ± SE are reported.

Table 6. Linear mixed model exploring the effect of time and experimental groups on changes in body mass after age 600 days.

Sources of variation	F	df	P
Age	9.63	1,229	0.0022
Squared age	7.20	1,228	0.0078
Treatment	1.16	4,230	0.3303
Age : Treatment	1.13	4,229	0.3409
Squared age : Treatment	1.07	4,229	0.3721

Reproductive success

The overall reproductive output over three bouts was low, the average litter size being 0.87 pups ± 0.23 (n = 52). There was no difference in litter size between groups (generalized linear model with a Poisson distribution of errors, χ^2 = 7.77, p = 0.1003, n = 52, Fig. 8.). However, when putting together the two groups of *Plasmodium*-infected mice and the three groups of control mice, the reproductive success of infected mice was lower than the control (χ^2 = 4.41, p = 0.0358, n = 52).

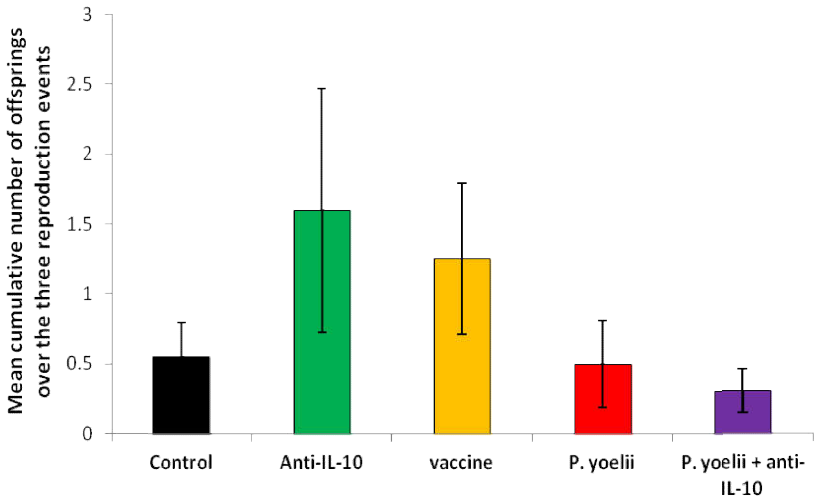

Figure 8. Cumulative reproductive success (litter size at birth) during three consecutive bouts for the different experimental groups. Means ± SE are reported.

Mortality

When 811 days old, an overal 52% of the cohort had died. Mortality ranged from 67% for the vaccine group to 27% for the *P. yoelii* group. However, mortality did not differ among experimental groups ($\chi^2_4 = 5.99$, p = 0.1994, Fig. 9). When putting infected mice in one group and non-infected mice in another groups, mortality differed between groups (32% vs. 65%, $\chi^2_1 = 5.79$, p = 0.0162).

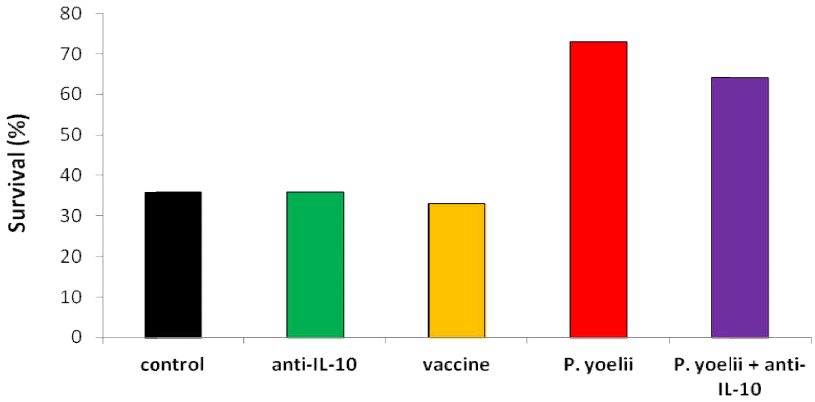

Figure 9. Proportion of surviving mice at the age of 811 days for the different experimental groups.

Discussion

The antagonistic pleiotropy hypothesis has been one of the most fertile grounds of study to explain the evolution of aging. However, the vast majority of these studies have focused on the age-dependent expression of fitness-association traits while neglecting the molecular and/or physiological functions underlying this antagonistic expression (Lemaitre et al. 2015).

In its original version, the antagonistic pleiotropy hypothesis postulates that genes associated to declining performance at old ages can be nevertheless selected for provided they confer a benefit at young ages (Williams 1957). Therefore, the trade-off between early and late fitness-associated traits emerges because a given function is beneficial early on and becomes deleterious later on. There have been a few suggestions for such functions.

Androgens, for instance, are important determinants of reproductive success because they play a role in sexual maturation, the development of primary and secondary sexual traits, dominance and sexual behaviors. Androgens are however also associated with increased incidence of some cancers in males (Boddy et al. 2015). This anecdotal evidence, however, has not been backed up by any experimental work.

The immune response, and in particular the inflammatory response, is another excellent candidate function for such antagonistic effects through ages. The inflammatory response is among the first lines of defense against damage induced by infection or trauma. As any component of the immune response, inflammation requires first the recognition of impairment of organismal integrity (either due to infection or trauma). This recognition step is followed by an amplification phase where inflammatory mediators recruit immune cells to the site of infection/trauma. The final step is characterized by a series of end signals aiming at stopping the response and promoting tissue repair. The inflammatory response is rapidly induced (within minutes/hours following the insult) and non-specific, in the sense that inflammatory effectors do not specifically target the invading (infectious) organisms. As such, acute and chronic inflammation can generate substantial self-damage to the host. Septic shock is probably the most severe damage, often lethal, associated with a runaway inflammatory response.

In addition to these immediate costs, inflammation is more often associated with delayed pathologies of variable severities. Chronic inflammation induces fibrosis with associated organ failure, cardiovascular diseases, senile dementia, arthritis, inflammatory bowel disease, and many cancers have an inflammatory origin. It is therefore straightforward to speculate that the inflammatory response could be a function conferring benefits in terms of protection towards infection and trauma and costs in terms of inflammatory diseases.

Finch and Crimmins (2004) analysed historical demographic data of human cohorts in Sweden and found that infant mortality was correlated with old age mortality within cohorts. They interpreted this as evidence that cohorts that were exposed to heavy infectious threats at young ages (as shown by high infant mortality) paid a subsequent cost in terms of old age mortality. While appealing, results based on historical human age-dependent mortality rates fail to establish a causal relationship between infection/inflammation and late mortality. Here we provide the first experimental test of the antagonistic pleiotropy properties of the inflammatory response.

We infected young mice with *Plasmodium yoelii*. We also treated half of the infected mice with an antibody that neutralizes the anti-inflammatory cytokine IL-10, further boosting the inflammatory response against the pathogen. Our aim was two-fold: i) assess the early benefits of the inflammatory response during the acute phase of the *Plasmodium* infection; ii) investigate the costs paid at late ages.

We found that the pathogen does activate the inflammatory response and that mice with the most potent inflammatory response had lower parasitemia. They also paid a lower cost of infection in terms of loss of red blood cells. These results indicate therefore that activation of the inflammatory response in response to an early infectious threat confers benefits.

Although, at the moment, we do not have the final results on the late costs, current evidence provides mixed support to the antagonistic pleiotropy hypothesis. Currently, only 52% of the initial cohort of mice has died (at the age of 811 days), and it is therefore possible that mortality acceleration has not occurred yet. However, it is striking to note that at the moment, contrary to the prediction, survival is actually much better in infected mice compared to the different control groups. Survival to the age of 811 days ranged from 33% to 36% for the control mice, whereas it was 67% and 73% for the two infected groups. In addition to this striking difference, we also found that age-dependent decline in body mass (an integrative trait that is usually used to identify senescing individuals) did not differ between experimental groups. Finally, reproductive success assessed during three consecutive reproductive bouts when mice were between 487 and 613 day old, did not differ between experimental groups. Reproductive success was very low for the whole

sample of mice, showing that they were already in a status of reproductive senescence. Although it is true that the two infected groups had lower reproductive success compared to two control groups (the vaccinated and the antibody treated mice), they had similar reproductive success to the unmanipulated control.

Overall, at the current stage of cohort mortality, we can say that our study only provides support to the first prediction of the hypothesis, that is activation of the inflammatory response does confer an early protection towards infection-induced damage. On the contrary, we do not have any clear evidence supporting the view that this also generates late costs. Decline in body mass at old age and reproductive success was similar for the different groups and more surprisingly, mice that were infected with *Plasmodium* at young age seem to have better longevity compared to the control groups. This latter result obviously requires being confirmed when the entire cohort of individuals will be extinguished. Nevertheless, we can already speculate on the possible reasons that might lead to this finding.

Although, Finch and Crimmins (2004) suggested that early exposure to inflammatory insult might induce an over-expression of inflammatory markers at old ages, other studies have found that early exposure to infectious diseases diminishes the level of inflammation at adulthood. For instance, McDade et al. (2010) used a longitudinal survey of a human population in the Philippines and found a positive correlation between the number of episodes of intestinal infections during infancy and the level of C-reactive protein at adulthood. This finding is in agreement with the so called hygiene hypothesis postulating that exposure to microbial antigens is an essential component of the regulation of the immune response. Epidemiological and experimental evidence has indeed shown that reduced exposure to antigenic stimulation increases the risk to contract auto-immune diseases at later ages (Rook 2009, Sorci et al. 2016).

Koop and Medzhitov (2009) also suggested that infection might be seen as an environmental stressor that shut down investment into growth and reproduction to improve maintenance and repair. According to them, infected individuals might enter an antiaging state exactly as caloric reduced animals do. This hypothesis therefore predicts a negative correlation between infection and aging.

Finally, early exposure to pathogens that activate the pro-inflammatory Th1 immune response might indirectly promote protection against cancerous cell proliferation (Cramer and Ave 2011), and as such improve life span.

To conclude, we provide an experimental test of the hypothesis that inflammation has antagonistic pleiotropic action promoting early performance at the expenses of late fitness. In agreement with the hypothesis, we found that inflammation reduced *Plasmodium* burden and the early cost of infection. However, even though the cohort still has about 50% of surviving mice, we do not have any clear evidence for accelerated senescence in infected mice. On the contrary, up to now, infected mice survived better than non-infected controls. This result needs to be confirmed in the upcoming weeks/months.

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Chapitre 6 : Microbes, parasites et maladies immunitaires

Title: Microbes, parasites and immune diseases

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Abstract

Western societies have witnessed a sharp increase in the incidence of many immune diseases (e.g., inflammatory bowel disease, type-1 diabetes, allergies) during the last decades. Concomitantly, the same countries have gone through major human-driven environmental changes that have resulted in improved hygiene, sanitation and medical treatments. These environmental changes have produced a mismatch between the environment our immune system has evolved with and our current environment. It has therefore been suggested that the current 'epidemics' of immune disorders might be due to the lack of proper 'stimulation and/or regulation' of the immune system by microorganisms and metazoan parasites that our immune system had learnt to tolerate. Although this idea has attracted considerable attention, it is important to fully acknowledge the evolutionary forces that might lead microorganisms and parasites to induce immunopathology or protect the host from immune disorders. In this article we will review why parasites can indeed confer a protection towards over-zealous immune functioning for their own interest. We will also discuss how taking evolutionary thinking into account can guide our decision taking rule and improve our way to deal with diseases that arise from a dysregulated immune response.

Introduction

Improved hygiene and sanitation, the discovery of effective drugs and vaccines have profoundly changed human exposure to microorganisms and parasitic agents, even though this has been mostly true for wealthy countries. While improved medical conditions have undoubtedly contributed to the spectacular raise in human longevity that we have witnessed in the last decades, it is striking to note that many debilitating diseases whose etiology is due to a dysregulated immune functioning have increased in frequency during the same laps of time (Bach 2002). It is therefore tempting to draw a parallel between the reduced exposure to infectious agents and the raise of immune disorders (Will-Karp et al. 2001; Yazdanbakhsh et al. 2002). However, while epidemiological and experimental results support the view that some parasite species do alleviate the risk of immunopathology, others appear to exacerbate it (Kamradt et al. 2005). The heterogeneity in the outcome of the interaction between infectious agents and the host immune response might reside in the evolutionary interests of the parasites (Sorci et al. 2012). When parasites rely on a down-regulated immune response for their own persistence with the host (in other terms for their own Darwinian fitness) we do expect them to exert a protective role against immune disorders. On the contrary other parasites adopt different strategy for their own survival (such as hiding from or escaping the immune response) and in this case it is hard to envisage a protective role. Sometimes, pathogens can even benefit from an up-regulated immune response in terms of access to privileged sites, or accelerated spread within the host; in this case, pathogens should even promote immunopathology. In addition to this, long-term coevolution between the immune system and parasitic organisms might have promoted the evolution of tolerance strategies where the host better cope with the pathogen by reducing the cost of the infection instead of eradicating it (Schneider and Ayres 2008). Even though tolerance to the infection and immunological tolerance are not synonymous terms, in many cases tolerant hosts pay a reduced cost of infection because they substantially reduce the cost induced by an over-zealous immune response (Ayres and Schneider 2012 ; Vale et al. 2014). This very broad view clearly illustrates the need to implement the evolutionary thinking into the study of the immunological and epidemiological determinants of immune diseases.

The aim of the article is to provide such an integrative view. We will first briefly introduce the immunological mechanisms that promote or prevent immunological damage. We will then discuss the epidemiological and experimental evidence in support or against the view that the raise in incidence of immune disorders is due to a change in the environmental conditions our immune system is experiencing nowadays. We will finally put forward a few possible ideas on how evolutionary thinking, beyond our fundamental understanding of the evolution of immune diseases, might feed the policy and contribute to improve our decision taking rule.

The immune system: a two-edged sword

Despite its crucial role to protect organisms from parasites and malignant cells, and to repair wound and injuries, immunity may also inflict severe damage when it fails to distinguish between self and non-self (9). An erroneous recognition of self-antigens by T and B lymphocytes can have devastating effects on host homeostasis, depending on the organ that is targeted by the mis-oriented immune response. A classical example of such mis-targeted immune response is type-1 diabetes where T-cells and antibodies attack pancreatic β-cells (Sell and Max 2001). In addition to mis-directed response, immune disorders can also occur when the immune system, while focusing on the right target (the invading pathogen) produces an over-zealous, poorly controlled, response. Over-expressed inflammatory responses occurring, for instance, during a septic shock are one of the most striking examples of how a dysregulated cytokine storm can generate overwhelming harm to the vital functions of the host.

Given the potential costs of mis-directed or dysregulated immune response, it is not surprising that hosts have evolved regulatory filters that reduce the likelihood of immune damage. These filters involve both central and peripheral mechanisms. For instance, most auto-reactive Tlymphocytes are deleted in the thymus, or are anergized (i.e., silenced) in peripheral organs if they escaped the deletion in the thymus. Similarly, following a wound, injured cells produce danger signals that attract immune cells into the area. These immune cells further produce signaling molecules that constitute a sort of “switch on” signal that polarizes the immune response towards a Th1/Th17 or a Th2 profile and promote the clearance of the invading pathogen and the healing of the wound. However, to avoid an

overzealous response the production of “switch on” signals is accompanied by the production of “switch off” signals that contribute to the resolution of inflammation and circumvent its negative effects (Kim et al. 2007). Regulatory T-cells (Treg) are a particularly important class of lymphocytes that secrete anti-inflammatory cytokines such as interleukin (IL)-10 or transforming growth factor (TGF)-β with strong inhibitory effects on pro-inflammatory cytokines. It is therefore straightforward to see that ensuring host homeostasis requires a finely-tuned balance between effectors that promote the immune response and regulatory feed-backs that prevent the immune response to get out of control. However, the necessity to have regulatory mechanisms to avoid immune damage opens the door for a possible exploitation by pathogens and parasites (Belkaid 2007). The evolutionary success of pathogens and parasites resides on their capacity to survive and multiply within a host and transmit to other hosts. Ineluctably, this requires escaping the host immune response (Finlay and McFadden 2006; Schmid-Hempel 2008). Therefore, while immune regulation is essential to avoid immunopathology, it can be used by parasites as a Trojan horse to promote the within-host establishment.

Pathogenic microorganisms and metazoan parasites exhibit an astonishing diversity of mechanisms of interference with the host immune system, and not all of them imply a downregulated immune response. For instance, *Plasmodium* and *Trypanosoma* escape immunity by providing a moving target to immune effectors, or in other terms by expressing highly polymorphic surface proteins that vary in time during the course of the infection, only one gene being expressed at time. Consequently, different surface proteins are expressed over the course of the infection and the expression of a novel surface protein leaves the immunity behind, providing an escape route to the parasite (Niang et al. 2009 ; Horn 2014). Interestingly, the same parasite species may rely on several escape mechanisms since the rodent malaria (*Plasmodium yoelii*) has also been shown to directly down-regulate the host immune response by inducing the differentiation of Th0 lymphocytes into Treg with immunosuppressive properties (Hisaeda et al. 2004).

Helminths are probably the masters of immune regulation among parasitic organisms (McSorley and Maizels 2012; McSorley et al. 2013 ; Finlay et al. 2014). Contrary to many microparasites that rapidly multiply within the host, macroparasites usually require long

developmental time to mature into the adult stage and adults can reproduce and shed eggs for weeks, months or even years for some human diseases (Carme and Laigret 1979). This suggests that parasitic worms have to cope with the host immune system for very long period of time.

Infection with helminthes usually polarizes the immune system towards a Th2-response (Finlay et al. 2014). A Th2 response is indeed required to expel the worms in most cases. Interestingly, effectors of the Th2 response are key for the wound healing process and it has been recently suggested that the Th2 response may represent a tolerance mechanism to helminths by contributing to the repair of wound induced by migrating worms (Gause et al. 2013). Polarization of the immune response towards a Th2 profile has a number of consequences for the host since Th2 cytokines tend to inhibit the production of Th1, proinflammatory, effectors. Therefore, by polarizing the immune response towards a Th2 type of response, helminths down-regulate the more harmful Th1-type of response. In addition to this, and perhaps more importantly, helminths induce the differentiation and expansion of Treg which further down-regulate over-zealous inflammatory responses (both Th1 and Th2). Several worm species can induce the production of the anti-inflammatory TGF-β or directly produce a TGF-β mimic (McSorley and Maizels 2012 ; McSorley et al. 2013 ; Finlay et al. 2014). They also stimulate alternatively activated macrophages (Finlay et al. 2014). Finally, helminth secretion and excretion products interfere with dendritic cells by inducing their apoptosis and inducing tolerogenic properties (Nono et al. 2012).

Evidence that induction of Treg is an essential component of parasite fitness has been provided in several experiments. For instance, experimental infection of mice with the nematode Litomosoides sigmodontis, a rodent model of human filariasis, induces the differentiation and proliferation of Treg. Once infection has established, experimental neutralization of Treg results in parasite mortality and reduced parasite burden by comparison with control mice (Taylor et al. 2007). This study clearly shows that interference with the immune system improves parasite fitness.

Paradoxically, an up-regulation of the immune response may in some cases benefit to the parasite. This is the case when leucocytes are themselves targeted by the pathogen to

spread within the host. For example, Ebola virus, which infects monocytes and macrophages, stimulates the attraction and recruitment of even more macrophages which provide further potential targets for viral replication and dissemination (Zampieri et al. 2007).

Benefits of an up-regulated inflammatory response can also arise when pathogens use the host immune response as a weapon against competitors. Salmonella enterica serovar Typhimurium triggers an inflammatory response that confers a selective advantage during competition with commensal bacteria. Avirulent strains of the bacterium lack this capacity to trigger inflammation and fail to establish unless the inflammatory response is experimentally induced (Stecher et al. 2007). The above consideration shows that single species of parasites may trigger or suppress immune disorders, depending on their own evolutionary interest; and the picture may become very complex when considering the communities of parasites and commensals exploiting a host.

Epidemiology of immune diseases

While it is well established now that many immune diseases have increased in frequency during the last 60 years in Europe and North America, the underlying causes are still debated (Rook 2008 ; Brown et al. 2013). Epidemiological studies have highlighted a number of environmental factors that are either positively or negatively associated with the likelihood to be affected from any of these immune disorders.

One of the most popular hypotheses that has been put forward a few decades ago, postulated that the improved hygiene and sanitation conditions of households in western countries have changed the exposure risk to infectious diseases producing a dysregulation of the immune system (Strachan 1989). This hypothesis that was first named “the hygiene hypothesis” has gone through a series of refinements (“old friends” (Rook 2010) or the “biodiversity” hypothesis (Hanski et al. 2012)). These refined hypotheses postulate that the observed raise in autoimmune and inflammatory disorders is not simply due to improved hygiene but rather to a mismatch between the current environment our immune system is exposed to and the one under which it has evolved for hundreds of thousands of years (Rook 2010) and have received support on the basis of the association between a number of traits describing the life style and the risk to develop allergic and autoimmune diseases. For instance, Strachan (Strachan 1989) reported that the household size is negatively correlated

with the incidence of hay fever, possibly because in families with several siblings the probability of transmission of microbial diseases (childhood diseases) among sibs increases with the number of children per family. Subsequent work has, however, shown that the benefits of having older siblings (in terms of reduced risk of immune disorders) might be due to the colonization rate of commensal bacteria (Penders et al. 2013). Similarly, children living in farms have been shown to have much reduced risk to suffer from allergies and atopy compared to children living in urban settings (Von Mutius 2010), and these observations have recently been backed up by gene expression studies showing that the expression of regulatory cytokines (such as IL-10 and TGF-β) was up-regulated in children living in farms (Frei et al. 2014). The impact of environmental changes on the incidence of atopy and allergic diseases can be astonishingly rapid, as suggested by a recent study. Poland acceded to the European Union in 2004. Becoming a member of the European Union resulted in a number of changes in the agricultural policies adopted by farmers. Sozanska et al. (2013) compared the incidence of atopy and asthma between 2003 and 2012 in people living in villages and urban settings. They found that the prevalence of atopy increased from around 8 to 18% in rural settings while it remained constant in towns (around 20%). The increased prevalence of atopy in rural areas correlated with major changes in exposure to animals and animal products (contact with cows decreased from 24% to 4%, and the percent of villagers who drank unpasteurized milk decreased from 35% to 9%). Therefore, a nine year period was long enough to witness major changes in the risk of immune disorders following environmental exposure to cows and cow products.

At a larger scale, the incidence of immune diseases (i.e., multiple sclerosis and type-1 diabetes) follows a clear North to South cline, with higher disease prevalence in the northern European countries compared to South Europe and in North America compared to South America (Bach 2002). While these geographic trends might be explained by genetic differences between populations, comparisons of disease incidence among migrating and resident populations consistently point towards a major role of environmental conditions, since immigrants tend to rapidly acquire the risk profile of resident populations (Ko et al. 2014). For instance, immigrants from Pakistan to the UK tend to acquire the some risk to develop type-1 diabetes as local residents which is 11-fold higher than the incidence of the disease in Pakistan (Bach 2002).

Although these epidemiological studies strongly suggest a major role for the local environment on the etiology of immune disorders, they incompletely inform us on the nature of these environmental factors. In support to epidemiological studies, a large body of literature shows that in animal models, exposure to certain microorganisms and parasitic metazoan does indeed reduce the risk of autoimmunity or ameliorates disease symptoms.

Coevolution with "old friends"

For hundreds of thousands of years, humans have been exposed to a diversity of microorganisms whose effect ranges from lethal, highly exploitative pathogens, to beneficial symbionts. In front of this diverse set of foreigner organisms, the immune system does not behave in the same way. Actually, in many instances hosts might benefit from tolerating the infection instead of adopting an aggressive strategy towards the invader. Tolerance is clearly a strategy that confers benefits to the parasite (it can persist within the host in front of a permissive immune response) and for the host (it reduces the cost of an over-reactive immune response, which in many cases outweighs the direct cost of parasite exploitation (Graham et al. 2005)).

Gut microbiota and helminths have attracted considerable attention in the last years as potential organisms with tolerogenic effects (Rook 2008; Brown et al. 2013). Parasitic helminths usually induce long-lasting relatively benign infections, unless the immune system does not over-react against them (Alves et al. 2006; Korten et al. 2011). A classical example of devastating consequences of helminthic infections involves the filarial nematode Wuchereria bancrofti that induces lymphedema and, in its most severe form, elephantiasis. Lymphedema is associated with enhanced Th1/Th17 responses and reduced Treg population (Babu et al. 2009).

Similarly, our gastrointestinal tract is the natural habitat for an extremely complex community of commensal organisms that include bacteria, viruses, protozoa, helminths. One of the daunting tasks of the immune system is to tease things apart, not only in terms of distinguishing between self and non-self, but also between commensal and potentially pathogenic organisms. Gut bacteria are regularly sensed by the immune system but they do not elicit an improper immune response, unless the immune system over-reacts to them. Inflammatory bowel disease is a debilitating syndrome that has increased in prevalence

during the last decades which is believed to be due to a dysregulated inflammatory response against gut bacterial stimuli (Braus and Elliott 2009).

It is therefore possible that the current "epidemics" of immune disorders does not arise as a mere consequence of improved hygiene, but rather as a consequence of the disruption of a longlasting coevolutionary history between our immune system and these "old friends" (Rook 2010).

There is a wealthy of experimental evidence supporting this view. Disruption of the gut microbiota during infancy due to an over-use of antibiotics has been shown to induce a number of pathologies during adulthood, including a higher risk of several inflammatory diseases (Stensballe et al. 2013; Shaw et al. 2010). More generally, reduced contact with natural environments and biodiversity, which characterize our modern lifestyle in highly urbanized settings, can also disrupt the composition of the microbiota (Hanski et al. 2012; Rook 2013). For instance, the biodiversity around the house of Finnish teen-agers suffering from atopy has been shown to be lower compared to healthy individuals, and this goes in parallel with reduced diversity of gammaproteobacteria on the skin (Hanski et al. 2012) (figure 1).

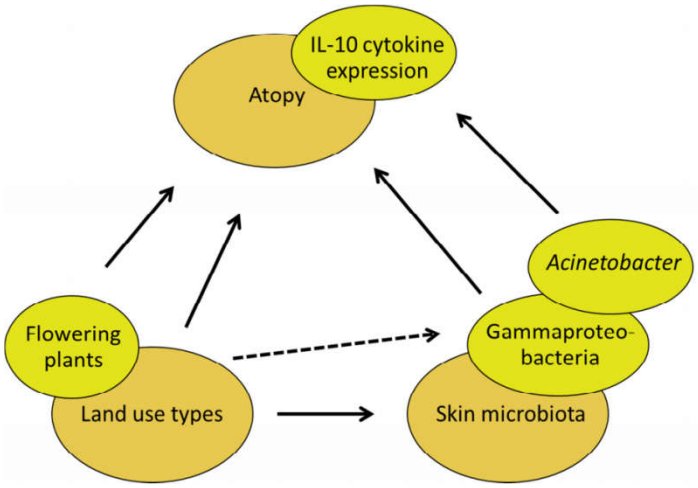

Figure 1. Association pattern between environmental biodiversity, skin microbiota and atopy in Finnish teenagers. Land use types around the houses of the studied subjects affect the generic diversity of skin microbiota and more marginally the generic diversity of gammaproteo-bacteria (dashed line). Skin microbiota correlates with the occurrence of atopy and IL-10 expression. Land use types and the diversity of flowering plants in the yard of the studied subjects also correlate with the occurrence of atopy. Redrawn with permission from Hanki et al. (2012).

Implementing evolutionary thinking as a way to improve our policy and practice

Even though there is a general consensus to say that understanding why we are still susceptible to diseases requires taking into account evolutionary theory, it is less clear whether we could or should implement evolutionary thinking into our decision taking rules as to improve therapeutic practices. Actually, in many cases, our policy already follows such "evolutionary" guidelines. Many of our medical treatments do not aim at killing the pathogens but rather at reducing the symptoms. This has a parallel to the tolerance strategy many hosts adopt to face a parasitic attack. Commonly used non-steroidal anti-inflammatory drugs, for example ibuprofen or aspirin, do boost tolerance. They limit the symptoms of infection, such as inflammation and the associated pain, without directly affecting pathogen burden. Conversely, the classical antibiotic treatment against bacterial infection only aims the eradication of the pathogen. Interestingly, evolutionary thinking also tells us that each of these strategies has its drawbacks. On one hand, tolerating the infection reduces the symptoms but does not stop parasite transmission. When we take an aspirin we certainly feel better but we keep spreading the virus. On the other hand, strategies that aim at killing the pathogen strongly select for mechanisms that allow the pathogen to escape them, antibiotic resistance being one of the most dramatic examples of this. In addition to the emergence of antibiotic resistance, undue or excessive use of antibiotics, especially during infancy (or in utero), has been shown to have profound effects on the community of gut bacteria with potentially negative consequences on the risk of several inflammatory diseases at adulthood (Stensballe et al. 2013 ; Shaw et al. 2010 ; Willing et al. 2011).

Taking into account the coevolutionary history we have with our parasites and commensals has also promoted the idea that we might use the tolerogenic properties of helminths and bacteria, as therapeutic agents (figure 2). Patients suffering from multiple sclerosis fortuitously infected by intestinal helminths entered a phase of remission compared to non-infected patients and the course of the disease resumed when the patients were treated with anti-helminthic drugs (Correale and Farez 2007; Correale and Farez 2011). Similar results have been obtained in many studies involving animal models (Heylen et al. 2014; Maizels et al. 2014). Mice experimentally induced to develop a form of inflammatory bowel disease (ulcerative colitis) have much attenuated symptoms (reduced

diarrhoea, reduced colon inflammation) when infected with the nematode *Heligmosomoides polygyrus* (Sutton et al. 2008; Donskow-Lysoniewska et al. 2012).

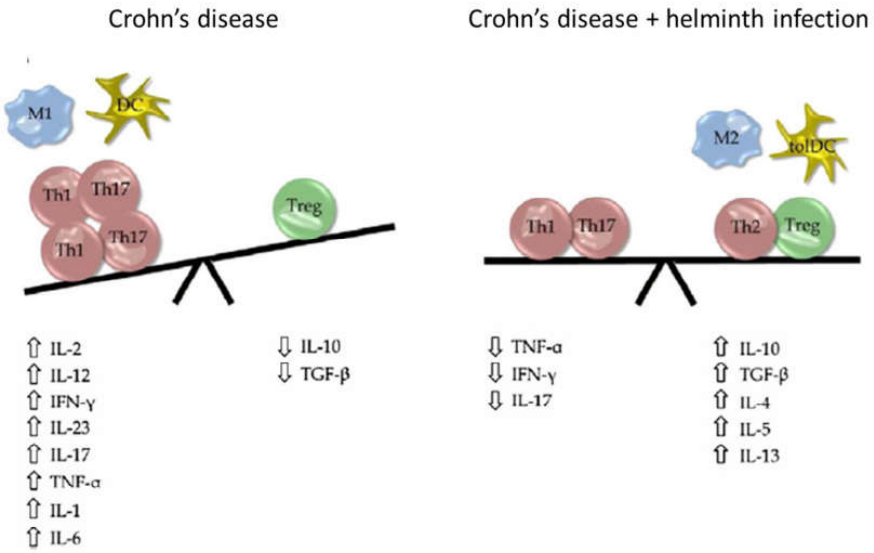

Figure 2. Crohn's disease is a form of inflammatory bowel disease that is characterized by the shift towards a pro-inflammatory status driven by Th1/Th17 cells and cytokines, and M1 macrophages and dendritic cells (DC). Helminth therapy is thought to restore intestinal integrity by expanding Treg, tolerogenic DCs, alternatively activated macrophages (M2) and Th2 cells and cytokines. Redrawn with permission from Heylen et al. (2014).

Following these promising results, several clinical trials have been started with a few helminth species to cure immune diseases, such as inflammatory bowel diseases and multiple sclerosis (Weinstock and Elliott 2013; Fleming 2013). However, there are a number of limitations due to the potential undesirable effects of using living organisms. Current efforts are devoted to isolate the effectors that promote the tolerogenic effect of helminths and use them instead of living organisms (Robinson et al. 2013) (figure 3).

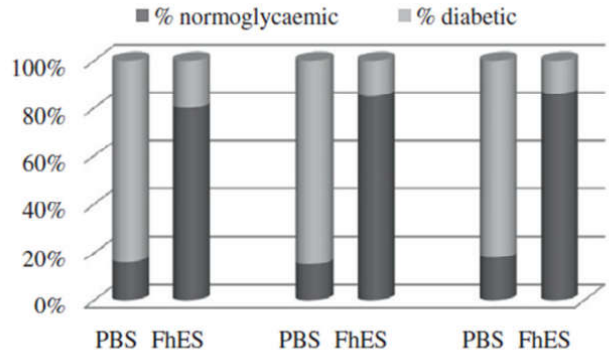

Figure 3. Non-obese diabetic (NOD) mice develop spontaneous hyperglycemia and type-1 diabetes. However, when treated with the excretory/secretory products of the trematode parasite Fasciola hepatica (FhES), only 15% of the individuals were hyperglycemic compared to 82% for control (PBS) mice. Reproduced with permission from Robinson et al. (2013).

The tolerogenic properties of helminths also have very interesting potential for the treatment of organ transplanted patients. Solid-organ transplantation is accompanied by long-lasting immunosuppressive drug treatment to prevent rejection. However, immunosuppressive treatment has a number of very undesirable side-effects, including the risk of contracting opportunistic diseases or developing cancer. An alternative strategy to immunosuppression might be improved immunological tolerance (silencing the immune response towards the allograft while preserving general immunocompetence). Johnston et al. (Johnston et al. 2014) reviewed the current evidence for a role of helminths on allograft survival and found that several worm species do indeed improve the survival of the allograft in the absence of immunosuppressive drug treatments.

Faecal transplant or bacteriotherapy is another promising avenue to fight a number of immune disorders that might be rooted into a disrupted microbiota (Borody and Khoruts 2012; Smits et al. 2013; Udayappan et al. 2014). Faecal transplants have been successfully tested in randomized controlled trials to treat type-2 diabetes and Clostridium difficile intestinal infection, and in case series studies against irritable bowel syndrome, inflammatory bowel disease and multiple sclerosis (Smits et al. 2013).

Beyond the exciting development of novel therapies for diseases that have proved difficult to cure, evolutionary thinking might provide a useful guide to adopt a life style that might minimize the risk of becoming sick. The spectacular raise in immune disorders is certainly due to the environmental changes that our modern, post-industrialized, societies

are facing. However, it is becoming increasingly clear that improved hygiene per se is not the causal agent but rather the reduced exposure to certain commensals and tolerogenic parasites. In line with this view, children in day care centres that have implemented programmes of improved hygiene (i.e., using an alcoholbased hand rub) do not have increased risks to develop immune disorders (Dunder et al. 2007). It seems in turn that our immune system has to be educated to learn how to distinguish between harmful and benign tolerogenic microorganisms. Failure to promote early education of the immune system with the proper environmental stimuli can thus produce the undesirable immune disorders observed nowadays (Djuardi et al 2011; Ege et al. 2011).

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Chapitre 7 : Les bénéfices de la protection immunitaire face aux coûts de l'immunopathologie : une synthèse basée sur les modèles de cytokines KO

Title: Benefits of immune protection versus immunopathology costs: a synthesis from cytokine *KO* models

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Keywords: Costs, benefits, immune response, immunopathology, cytokines, KO

In preparation.

Abstract

An overreacting or misdirected immune response can produce severe damage to host tissues and organs, and in several infectious diseases, the most severe symptoms are often due to immunopathology rather than a direct effect of pathogen multiplication. Given the fitness consequences of immunopathology, why natural selection has not succeeded to eradicate immune damage? One hypothesis for the maintenance of immunopathology posits that the benefits of immune protection towards infectious agents outweigh any, possibly delayed, cost in terms of immune damage. Here, we used a large dataset covering different infectious diseases to explore this question. We used data on cytokine knocked-out (*KO*) models [and the corresponding wild-type (*WT*) controls] to test the association between parasite density and survival. We used differences in parasite density and survival between *WT* and *KO*, which gives an internal control for variation in pathogen species, host strain, parasite dose, inoculation route, etc. Based on the previously reported finding that disease severity and parasite density are not always linked, we predicted that if cost of infection (here measured with mortality rate) is essentially due to immunopathology, there should not be a tight association between parasite density and survival. On the contrary, if mortality is essentially due to pathogen proliferation we expect a tight association between difference in parasite density and survival. The results provide strong support to this latter hypothesis. In 85% of *WT* – *KO* comparisons, an increase in parasite density was associated with an increase in mortality, and a decrease in parasite density was associated with a decrease in mortality. The type of *KO* cytokine affected this pattern, with pro-inflammatory *KO* being associated with higher parasite burden and higher mortality and anti-inflammatory *KO* being associated with lower parasite density and lower mortality. Overall, these findings are in agreement with the idea that immunopathology costs persist because immune protection incurs immediate benefits in terms of pathogen clearance/control.

Introduction

By definition pathogens and parasites exploit resources provides by an unrelated organism, called host (Combes 2001). Parasitic exploitation diverts host resources from host functions and as such is considered costly for the host. Indeed, infection is usually associated with impaired host fitness, either in terms of increased mortality or decreased reproductive output (Schmid-Hempel 2008). Such infection-associated costs define parasite virulence (Read 1994). The evolution of virulence has been a puzzling question for evolutionary biologists for decades. Indeed, why should a parasite that relies on host survival for its own survival kill its host? A solution to this paradox was provided a few decades ago when it was suggested that parasite fitness does not depend on parasite survival but on its transmission to other susceptible hosts (Anderson and May 1982, Ewald 1983). The model, coined the trade-off model of virulence, assumes that parasite transmission increases with parasite multiplication within the host, and that host mortality increases with parasite multiplication (Alizon et al. 2009). Therefore, while parasite multiplication (host exploitation) improves transmission rate it also incurs host death. Parasite fitness is therefore maximized at intermediate level of multiplication (exploitation) (see for instance de Roode et al. 2008; Alizon and Michalakis 2016). This model establishes a direct link between cost of infection and parasite multiplication. However, the severity of disease is often unrelated to parasite abundance or density (see Graham et al. 2005 for a review). In this case, the severity of the disease symptoms is rather associated with an overreacting immune response. Immune costs incurred following an infection are usually called immunopathology (Graham et al. 2005).

While theoretical work has shown that taking into account immune costs does not invalidate the trade-off model of the evolution of virulence (Day et al. 2007; Best et al. 2012), immunopathology raises the question of the evolutionary maintenance of such costs (Graham et al. 2005). Maintenance of immunopathology is even more puzzling given that infection-associated immune costs are not universal. For instance, tolerance, as opposed to resistance, is among the strategies hosts can rely on to deal with an infection while limiting its costs (including the immune ones) (Medzhitov et al. 2012).

The most obvious explanation for the persistence of immunopathology relies on the ratio between the benefits of immune protection and the associated immunopathological costs. Immunopathology might be an unavoidable consequence of immune activation and even though it potentially causes damage to the host, a lack of immune activation upon infection would produce even more dramatic fitness consequences for the host due to pathogen proliferation. In addition to this, while failure to keep pathogen proliferation under control incurs immediate costs, several immunopathological diseases (but not all) have late-life onset, and early benefits should outweigh late costs.

While sound, this hypothesis has proved difficult to test over a large spectrum of host-pathogen systems. Here we took advantage of published information on cytokine-knocked out mouse models to test the idea that benefits of immune protection outweigh the immunopathology costs.

Cytokines are signaling molecules produced by different immune cells playing a central role in the regulation (up- and down) and the polarization (Th1/Th2/Th17) of the immune response (Opal and de Palo 2000, Scapigliati et al. 2006, Bachereau et al. 2012). Cytokines exhibit interesting properties such as synergy, redundancy, or inhibitory effects (Paludan 1998, Elenkov et al. 2005). Based on the immune cells that produce them and on their action on the immune response, cytokines have been grouped and classified as having pro- or anti-inflammatory properties or skewing the immune response towards a Th1, Th2, or Th17 type. It is worth noting however, that not all cytokines clearly fall into one of these categories, and their anti- or pro-inflammatory role can also be context-dependent (Cavaillon 2001).

As signaling molecules, cytokines play a central role in the process of resistance to infectious diseases, and as such they have received a lot of attention to identify how they promote clearance of infection (Guidotti and Chisari 2000, Cooper et al. 2011, Beltra and Decaluwe 2016). One useful tool to study the effect of cytokine on pathogen resistance has been to produce mouse strains where the gene that codes for a given cytokine has been inactivated or disrupted. Traits of interest of these knock out (*KO*) mouse models are then compared to those of the corresponding wild type (*WT*) strains, in response to an infectious threat.

We compiled a database comprising 19 *KO* cytokines (or cytokine receptors) from 86 published articles. This gave 126 *WT – KO* comparisons for differences in survival and parasite density, involving 21% of viruses, 52% bacteria, 6% fungi and 22% protozoa. Based on the observation that disease severity may not be correlated with parasite density (Gazzinelli et al. 1996; Kullberg et al. 1998; Long et al. 2006, 2008a,b; Long and Graham 2011), we predicted that if cost of infection is due to immunopathology there should be no clear association between differences in survival and differences in parasite density. On the contrary, if cost of infection is essentially due to pathogen proliferation, given that silencing pro-inflammatory cytokines depresses the anti-microbial response, we should expect a strong association between differences in survival and differences in parasite density.

Materials & Methods

Data acquisition

We searched the literature indexed in the web of science, using a list of key words (including 'KO', 'cytokine', 'infection', 'survival', 'mortality'). For each article, we checked that there was a cytokine *KO* model (either the protein or the receptor), an infectious model (a viral, bacterial, fungal, or protozoan infection), a measure of pathogen density in both *KO* and *WT* hosts, the assessment of mortality in *KO* and *WT* hosts. Some articles also reported the production of cytokines in *KO* and *WT* hosts in response to the infection (not the cytokine that was knocked out).

Data on parasite density, mortality and when available cytokine production, were extracted from published figures using the freely available softwares ImageJ and webplotdigitizer (http://arohatgi.info/WebPlotDigitizer/app/). For survival data, we computed the areas below the survival curves for *WT* and *KO* individuals. For parasite density, when means were reported at different time points, these values were summed up to have a cumulative parasite density. Similarly, when parasite density was reported for several organs, we summed up the means to obtain an overall parasite density value per group (*WT* and *KO*). The full dataset is available as an online supplementary material.

Statistical analyses

For each trait (survival, parasite density and cytokine production), we computed the percent difference between *WT* and *KO* as ($\frac{WT-KO}{WT}$).

Even though computing the percent difference gets somehow rid of the difference in scale between measurements, we decided to avoid using linear models to analyze the data. Instead, we preferred using a semi-quantitative approach. The percent differences between *WT* and *KO* have signs indicating whether *WT* had more parasites than *KO* or suffered more mortality than *KO*. These signs are obviously totally independent from the scale used in the original data. The percent differences in survival and parasite density were plotted one against the other. This gives a four quadrants plot. Each of these quadrants refers to a particular pattern. Moving in a clockwise direction, quadrant 1 refers to the case where *WT* have better survival and lower parasite density than *KO*, quadrant 2 refers to the case where *WT* have better survival and higher parasite density than *KO*, quadrant 3 refers to the case where *WT* have lower survival and higher parasite density than *KO*, and quadrant 4 refers to the case where *WT* have lower survival and lower parasite density than *KO*. Figure 1 reports a schematic illustration of the four quadrants and their meaning. We then tested the distribution of the observed differences across the different quadrants using a χ^2 test. If survival and parasite density are uncoupled, we should observe a random distribution of points across the different quadrants, if parasite density and survival are negatively associated we should find that the majority of points lie in the diagonal across quadrants 1 and 3.

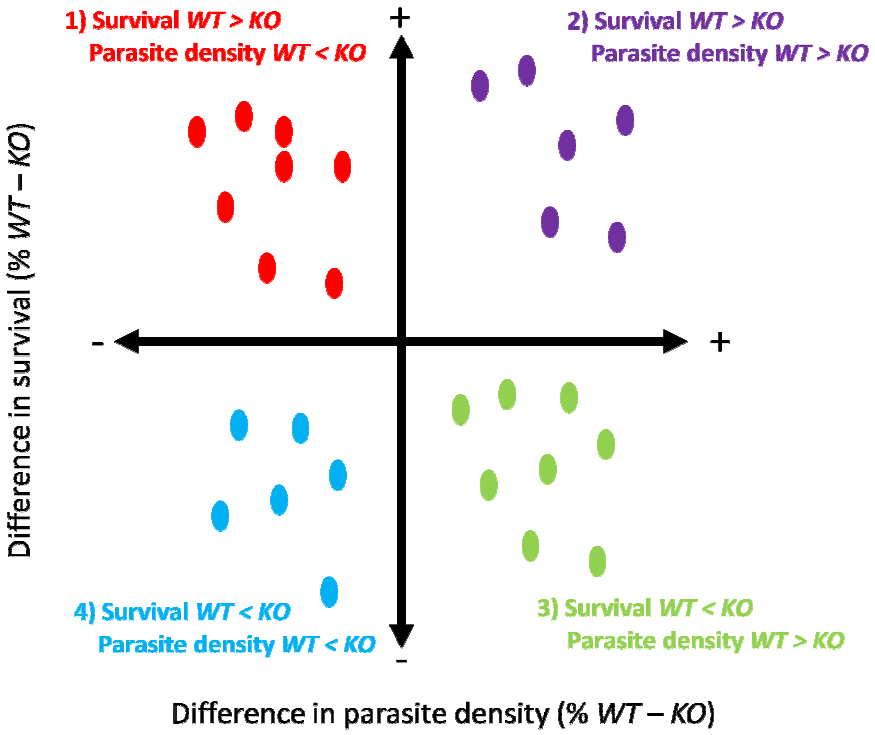

Figure 1. Schematic illustration of the method used to assess the association between differences in parasite density and differences in survival in *WT* and *KO* mice. Plotting the percent differences between *WT* and *KO* defines a four quadrant space, with each quadrant indicating a particular combination of differences between the two variables (parasite density and survival). In the absence of association between parasite density and survival, the distribution of data points should be random across the four quadrants which represents the null hypothesis (H_0).

Results

In the vast majority of cases, suppressing pro-inflammatory cytokines increased parasite density, since 83% of the percent *WT* - *KO* differences were negative ($\chi^2_1 = 48.00$, $p < 0.0001$, $n = 108$, fig. 2A). Sixty one per cent of the percent *WT* - *KO* differences in parasite density were positive when anti-inflammatory cytokines were suppressed. However, this was not significantly different from the random distribution, possibly because we only had 18 comparisons involving anti-inflammatory cytokines ($\chi^2_1 = 0.89$, $p = 0.346$, $n = 18$, fig. 2B). The difference in distribution of signs between pro- and anti-inflammatory cytokines was, however, highly significant ($\chi^2_1 = 17.20$, $p < 0.0001$, $n = 126$).

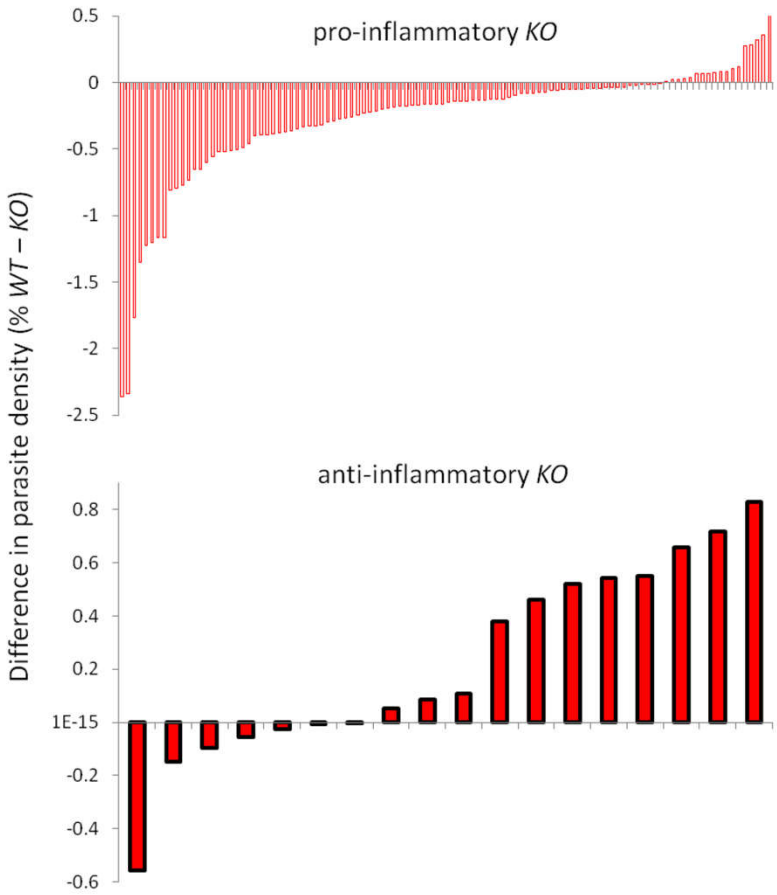

Figure 2. Percent difference in parasite density between *WT* and *KO* mice. A) genes coding for pro-inflammatory cytokines were inactivated; B) genes coding for anti-inflammatory cytokines were inactivated.

We found that the distribution of points across the four quadrants was not random ($\chi^2_3 = 137.05$, $p < 0.0001$, $n = 126$, fig. 3A). In agreement with the hypothesis that survival is tightly negatively associated with parasite density, we found that 85% of the data points laid in the quadrants along the diagonal (quadrants 1 and 3). This pattern was even stronger when computing mean values per cytokine, since 95% of mean values were in quadrants 1 and 3 ($\chi^2_1 = 15.21$, $p < 0.0001$, $n = 19$, fig. 3B).

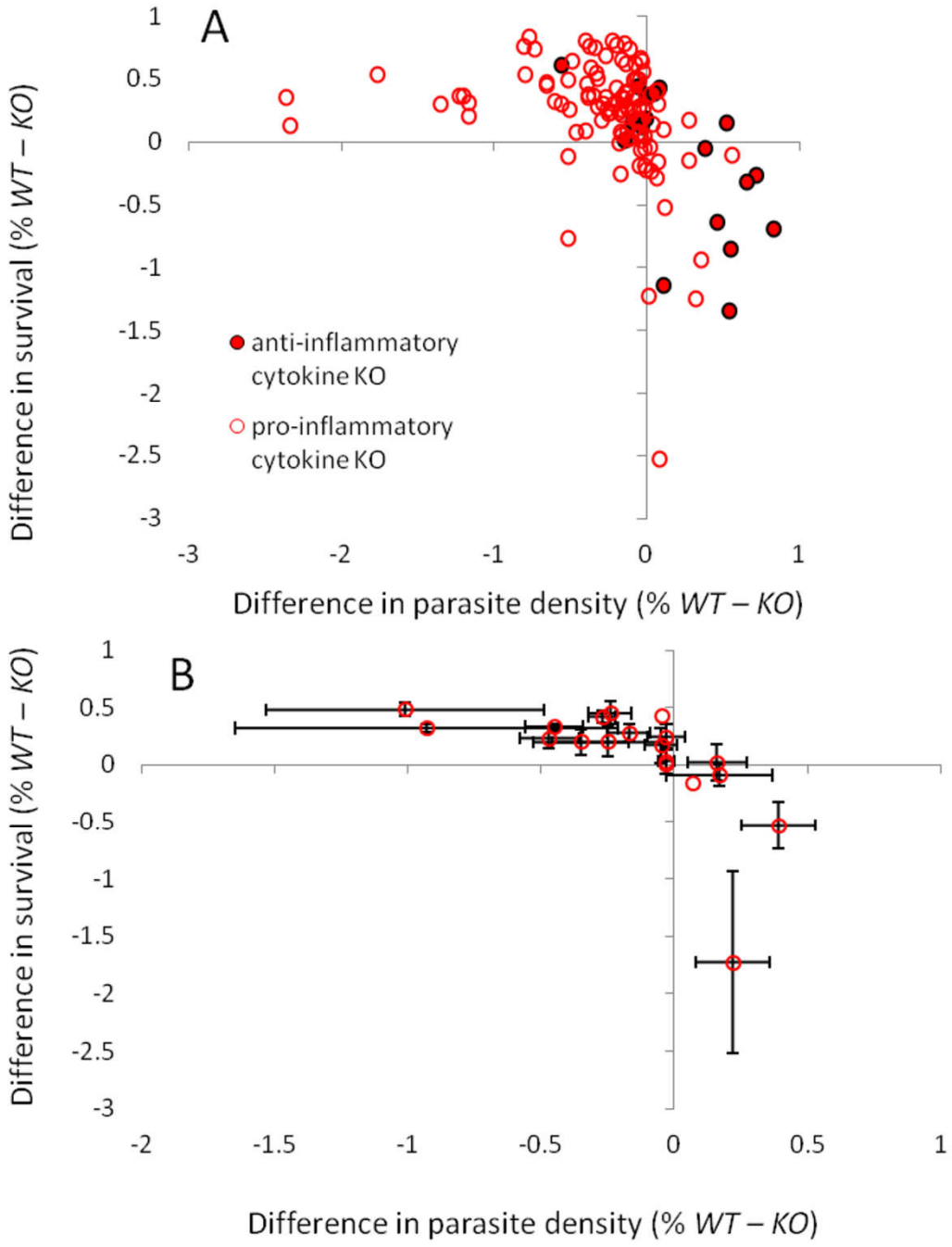

Figure 3. Association between difference in survival and parasite density in *WT* and *KO*. A) Empty and full dots refer to pro-inflammatory and anti-inflammatory *KO* cytokines, respectively. B) Dots refer to the mean values per *KO* cytokine (± SE).

The distribution across the four quadrants differed between pro- and anti-inflammatory cytokines (χ^2_3 = 18.41, p = 0.0004, n = 126, fig. 3A). While 75% of points involving pro-inflammatory cytokines laid in quadrant 1 vs. 39% for anti-inflammatory cytokines, only 10% of pro-inflammatory cytokines were in quadrant 3 vs. 44% for anti-inflammatory cytokines

(fig. 3A). This reflects the general trend that silencing pro-inflammatory cytokines enhanced parasite density and mortality in *KO* individuals.

The distribution of data points across the four quadrants was remarkably consistent when comparing cytokine vs. receptor *KO* models, or over the three major pathogen taxonomic groups (viruses, bacteria and protozoa) (see online supplementary material, figures S1 and S2).

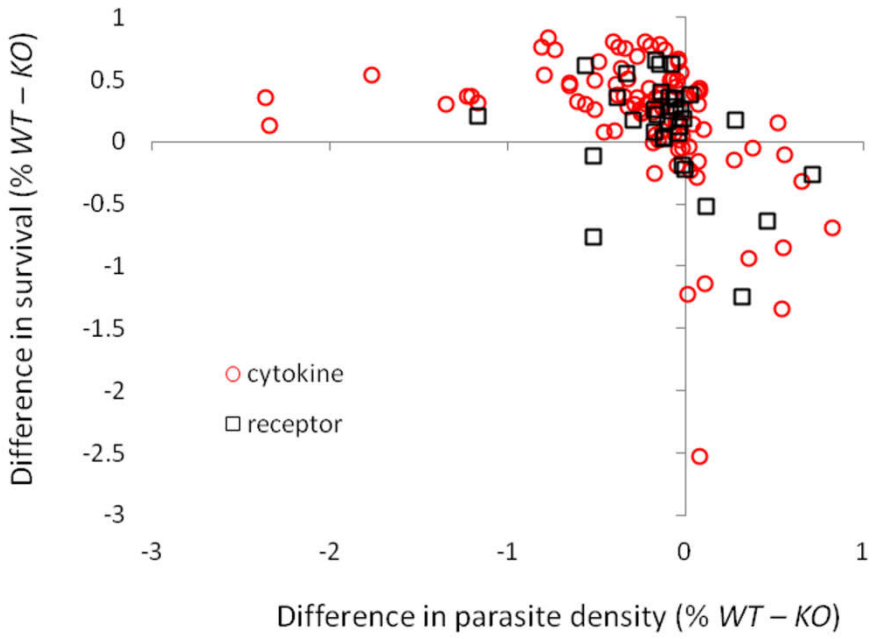

Figure S1. Association between differential survival and parasite density in *WT* and *KO*. Empty dots and squares refer to models where either the cytokine or the receptor gene was knocked out, respectively.

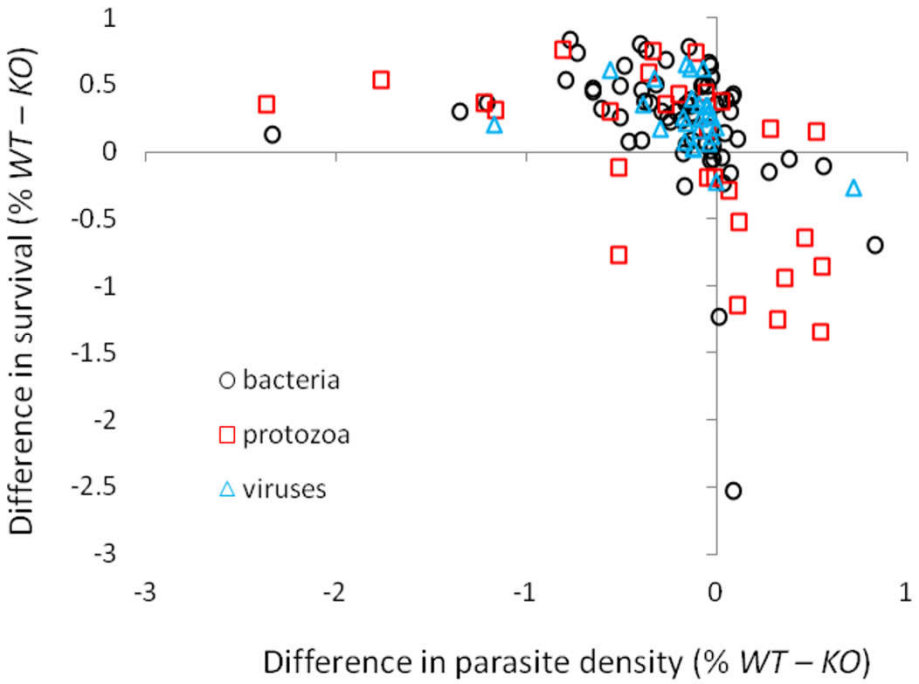

Figure S2. Association between differential survival and parasite density in *WT* and *KO*. Empty dots, squares and triangles refer to infectious models involving bacteria, protozoa or viruses, respectively

We also explored the possible compensatory effects induced by knocking out pro-inflammatory cytokines. Here, two possible predictions can be made. First, failure in producing a given pro-inflammatory cytokine might induce the over-expression of other cytokines with redundant functions. Alternatively, knocking out a gene of a pro-inflammatory cytokine might have pleiotropic effects on other signaling molecules. We did not find support for any of these hypothesis since in about half of the cases (55%), silencing a pro-inflammatory cytokines induced the up-regulation of other pro-inflammatory cytokines (TNF-α, IFN-γ, IL-1β, IL-17) and in half of the case it produced a down-regulation (45%; $\chi^2_1 = 1.17$, p = 0.278, n = 103). Silencing an anti-inflammatory almost always produced an up-regulation of pro-inflammatory cytokines (TNF-α, IFN-γ, IL-1β, IL-17) (in 92% of cases, $\chi^2_1 = 9.31$, p = 0.0023, n = 13).

Discussion

Using a compiled data base from published literature we show that, by far, changes in parasite density are the most important determinant of cost of infection, assessed as host mortality, in cytokine *KO* models. This finding shows that immediate benefits of infection

clearance outweigh the possibly delayed immunopathology costs. This strongly suggests that immunopathology costs persist in face of natural selection because immune protection confers immediate benefits in terms of survival.

Understanding the evolution of infectious diseases requires the study of the molecular dialog between pathogens and hosts but obviously also the study of the fitness costs and benefits for the partners. Traditionally, evolutionary biologists see parasite virulence (host mortality or impaired health) as the unavoidable consequence of within-host multiplication, and within-host multiplication as a trait that increases transmission rate (Alizon and Michalakis 2016). This view therefore establishes a direct link between parasite density and host mortality risk (or health impairment). On the contrary, biomedical science has, since long, stressed the idea that mounting an immune response against an infectious organism can produce devastating collateral effects possibly more severe than the direct damage due to resource exploitation by the pathogen (Lambrecht and Hammad 2012, Sharma and Thomas 2014, Newton et al; 2016, Shin et al. 2016). Text book examples of such infection-induced immunopathology costs involve cerebral malaria, or respiratory distress during influenza (Peiris et al. 2010, Schmidt et al. 2011, Damjanovic et al. 2012). Since the seminal paper published by Graham and coworkers in 2005, evolutionary biologists have started to take into consideration how immunopathology might affect the evolution of parasite virulence and host defenses (Sadd et al. 2006, Belloni et al. 2010, Cressler et al. 2015). While theoretical work has overall shown that the general conclusions of the trade-off model of parasite virulence hold even when host death is due to immune costs (Day et al. 2007, Best et al. 2012), we still have little insights on the evolutionary forces that maintain such immune mediate costs. Pioneering work conducted on rodent malaria has provided a detailed assessment of the relative role of immunopathology and parasite density as drivers of disease severity. Using a combination of experimental infections with different clones of the protozoan *Plasmodium chabaudii* and the blockade of pro-inflammatory (TNF-α) or anti-inflammatory (IL-10) effectors in infected mice, Long and coworkers were able to drive parasite virulence (infection-induced mortality) up and down (depending on the immune treatment used), independently of parasite density (Long et al. 2008a,b). These examples show that immune activation contributes to the cost of infection and that this cost can be decoupled from parasite density.

Although the idea that immunopathology is the unavoidable consequence of immune protection makes perfectly sense, to our knowledge no quantitative study on a broad range of infectious organisms has been produced to support or invalidate this idea. Here we show that inactivating pro-inflammatory cytokine genes exacerbates parasite density and increases mortality rate over a large spectrum of disease models. This produces a tight association between parasite density and mortality, in agreement with the hypothesis that uncontrolled pathogen proliferation induces immediate and potentially devastating costs for the host. Inflammatory diseases are often associated with up-regulation of pro-inflammatory cytokines and failure to shut-down the production of inflammatory effectors (Wallace et al. 2014). Therefore, while immunopathology might potentially be removed by down-regulating pro-inflammatory cytokines (Gottschalk et al. 2016, Liu et al. 2016), the associated overwhelming pathogen proliferation makes such a choice evolutionarily senseless.

Although the strength of our study is that it involves different immune effectors and pathogens, this also raises a series of challenges. Obviously the first challenge is that it is difficult to compare studies that used different pathogenic organisms that have been inoculated to different host strains, at different doses, using different inoculation mode, monitored over different periods of time, etc. We got rid of all these confounding effects by using *WT* individuals as the internal control. Even if studies differ in many aspects, within studies, *KO* and *WT* animals were always exposed to the same inoculum size, monitored over the same period and so on. Therefore, using the difference between *WT* and *KO* gives an objective estimate of the relative effect of knocking out a given cytokine on host mortality and parasite density. To be even more conservative, we decide to refrain from using linear models to explore the relationship between parasite density and mortality, because of the possible effect of different scales used to assess parasite density across infectious organisms. The method that we adopted, based on the distribution of the signed differences, does not allow us to quantitatively estimate the fraction of variance in host mortality due to parasite density; nevertheless, it unambiguously shows that variation in parasite density (up or down, depending on the type of cytokine knocked out) is associated to variation in host mortality. It should also be made clear that the computation of differences in parasite density and host mortality between *WT* and *KO* does not take into account whether the original study

reported a statistically significant difference or not. Actually, in several studies parasite densities or survival were indeed not significantly different between *WT* and *KO*. However, if the signed differences were mostly due to random variation around a zero mean, we should have found a homogenous distribution of the data points across the four quadrants.

A potential problem for any study based on published results is publication bias in support of a given hypothesis. However, the original literature that we used to compile our database does not deal with a particular hypothesis that could have been supported or invalidated. In addition to this, we investigated whether there was a temporal trend in the data by looking at the variation in the differences between *WT* and *KO* among years and we did not find any consistent trend (see online supplementary material, figures S3). Finally, the reported association is so strong that the publication bias should be very substantial to mask the observed pattern, which seems rather unlikely.

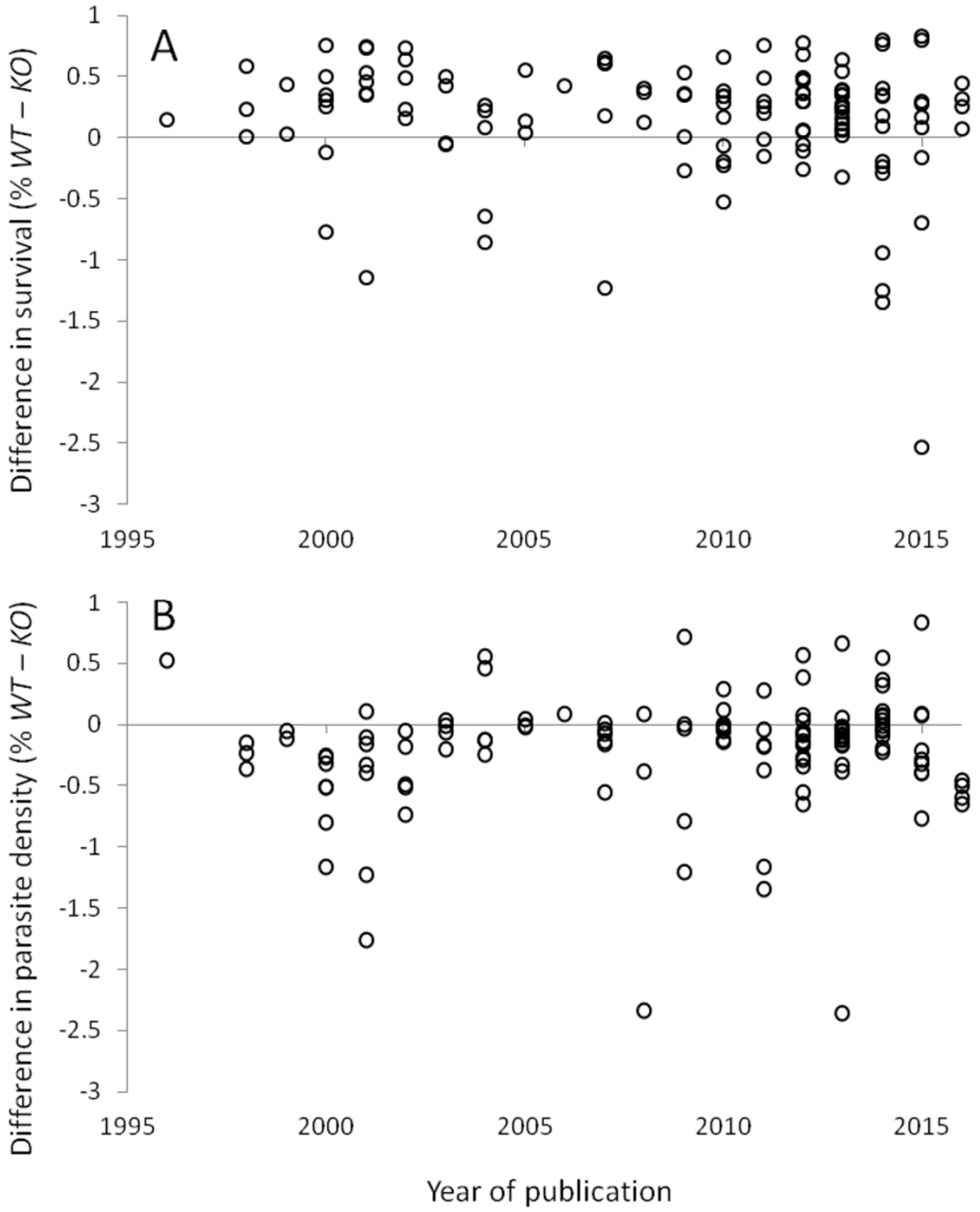

Figure S3. Variation of percent difference in survival (A) and parasite density (B) between *WT* and *KO* as a function of year of publication (n = 126).

While our finding suggests that host mortality is overall tightly associated with variation in parasite density, we do not discard the role of immunopathology as an important component of infection cost. In particular, to stick with the definition of parasite virulence (impairment of host fitness), we focused on studies dealing with pathogens that do induce host mortality (almost no studies in the context of *KO* infected models have investigated reproductive output) and therefore are amenable for the study of the immediate costs of

infection. Several pathogen species are not immediately lethal and instead have more diffuse and long term costs. Further work should include such infectious models to have an even more comprehensive view on the benefits of immune protection vs. the costs of immunopathology.

Cytokine signaling is characterized by properties such as redundancy, synergy, pleiotropy, inhibition (Cavaillon 2001, Elenkov et al. 2005). Silencing genes coding for a given pro-inflammatory cytokine might therefore induce a compensatory effect involving other cytokines with redundant properties. The finding that mice with *KO* pro-inflammatory cytokines overall suffered from increased parasite density and mortality suggests that, if any, the compensatory effect is far from being perfect. To go further in this direction, we investigated whether *KO* individuals had an up-regulated expression of some of the main pro-inflammatory cytokines (TNF-α, IFN-γ, IL-1β, IL-17). We found no consistent pattern since in half of the cases *KO* individuals had down-regulated expression of pro-inflammatory cytokines, and the other half an up-regulated expression. Further work should investigate whether the type of pathogen or the knocked out cytokine might explain this variability. On the contrary, knocking out anti-inflammatory consistently produced an up-regulation of pro-inflammatory cytokines.

To conclude, we provide evidence based on a diverse set of infectious organisms that even though suppressing pro-inflammatory signaling reduces infection-associated immunopathology, pathogen proliferation incurs an immediate major fitness cost in terms of mortality risk. The balance between immediate cost of parasite proliferation and delayed cost of immunopathology therefore provides an evolutionary explanation for the persistence of immunopathology.

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Discussion générale, conclusion et perspectives

Rappel de la problématique et questionnements soulevés

La course aux armements entre les parasites et leurs hôtes a doté chacun des camps de caractéristiques de défenses et d'évitements acquises au cours de leur coévolution. Néanmoins, malgré l'évidente nécessité du système immunitaire dans la lutte contre les agents pathogènes, ce dernier arbore des coûts aussi bien énergétiques qu'immunopathologiques, mais aussi sur le long terme avec l'apparition de maladie inflammatoires avec l'âge, montrant que l'immunité résulte d'un ensemble de compromis évolutifs. Mais dans quelles mesures les bénéfices de l'immunité surpassent ses coûts et expliquent le maintien de cet ensemble de troubles immunitaires observés de nos jours ?

De l'autre côté du front, les parasites comme les helminthes montrent des capacités d'évasion de l'immunité très poussées qui leur permettent de faire face à l'immunité de leur hôte dans la durée et d'être souvent favorables à leur hôte lors d'affections inflammatoires chroniques. Cependant, l'environnement inflammatoire de l'hôte va avoir un impact sur les traits d'histoire de vie du parasite. Ainsi, nous pouvons nous poser la question des coûts et bénéfices pour les parasites immunomodulateurs dans cet environnement inflammatoire.

L'environnement inflammatoire auquel les parasites doivent faire face est très variable de par la plasticité de l'immunité et de l'état inflammatoire de l'hôte à leur arrivée mais aussi tout au long de l'infection durant laquelle des modifications de cet état inflammatoire peuvent survenir. Par ailleurs, les parasites contre lesquels les hôtes doivent lutter ne sont pas tous identiques et arborent de nombreuses tactiques d'échappement de l'immunité.

Ainsi, étudier les relations hôte-parasites avec une approche écologique et évolutive, autrement dit (1) en faisant varier les environnements des hôtes et parasites par une approche de normes de réaction, ou de variations temporelles de l'environnement, (2) mais aussi en terme de coûts et bénéfices pour chacun des protagonistes, est essentielle à la compréhension des compromis liés à l'immunité et à sa régulation aussi bien pour l'hôte que pour le parasite.

L'hôte

Dans le chapitre 1 nous avons pu montrer que la résistance était un trait variable en fonction du génotype de l'hôte et de la charge parasitaire (comme approximation d'un environnement plus ou moins dangereux) et qu'il y avait un découplage entre la résistance et le coût de l'infection. En effet, nous avions soumis trois souches de souris, CBA, BALB/c et SJL, à l'infection par *Heligmosomoides polygyrus*. Nous avions constaté que lors de l'augmentation du gradient de charge parasitaire, les souris SJL montraient une forte augmentation du nombre d'œufs de parasite retrouvés dans leurs fèces, jusqu'à rejoindre la production d'œufs des parasites infectant les souris CBA. Par ailleurs, la mortalité était aussi plus forte chez les souris SJL au fur et à mesure de l'augmentation de la charge parasitaire. Cela montre que la mise en place que la réponse inflammatoire chez l'hôte à de fortes charges parasitaires a causé des dégâts imputables à l'immunopathologie et que le coût de de l'infection est indépendant de la résistance au parasite. De plus, les profils de production des cytokines le long du gradient de charge parasitaire montrent clairement une augmentation constante de la production de la cytokine pro-inflammatoire IL-6 alors que la production de la cytokine anti-inflammatoire IL-10 ou de l'IL-4 avait un profil en cloche avec une diminution à des fortes valeurs de charges parasitaires. L'ensemble de ces observations démontre le coût immunopathologique que les souris les plus résistantes au parasite paient lors d'une infection importante. Ce déséquilibre dans la production de cytokines vers un profil pro-inflammatoire suivi d'un échec dans la régulation de cette inflammation est régulièrement source de problèmes inflammatoires durant une infection (Wallace et al. 2004). Divers exemples ont déjà été abordés dans l'introduction comme le cas du choc septique ou de la fibrose hépatique (Graham et al. 2005 ; Hoffmann et al. 2002).

Dans ce premier chapitre nous avons pu observer que la mortalité des individus été liée à la densité de parasite les infectant. C'est aussi le constat auquel nous arrivons dans le chapitre 7 avec notre analyse bibliographique des associations entre parasitémie et survie dans les modèles KO. Dans ce chapitre notre but était de comprendre pourquoi l'immunopathologie est un trait qui n'a pas été contre sélectionné. Nous avons travaillé sur la relation entre survie et densité parasitaire dans des modèles de cytokines KO lors d'infections par divers taxons de parasites (bactéries, virus, protozoaires et champignons). Nous avons trouvé que la mortalité était avant tout bien associée à la densité parasitaire ce

qui confirme le modèle principal expliquant l'évolution de la virulence selon lequel la mortalité est liée à la charge parasitaire (Alizon et al. 2009).

Néanmoins, dans le chapitre 1 nous pouvons bien voir que le coût de l'infection en termes de survie varie avec la résistance, c'est-à-dire avec l'investissement fait par les hôtes dans la réponse immunitaire pour expulser les parasites. Ainsi, et comme observé dans d'autres cas (Cf. infection à haanta virus puumala en France (Charbonnel et al. 2014), l'immunopathologie peut avoir de graves conséquences. Certes, une critique qui peut être faites sur les travaux du chapitre 1 est que les doses utilisées sont au-dessus de ce que nous observons dans la nature et que le coût de l'immunopathologie dans cette étude est exacerbée à cause de ces charges parasitaires importantes. Cependant, toutes choses étant égales par ailleurs, les CBA supportent plutôt bien ces charges importantes de par leur phénotype tolérant au parasite. Comme discuté dans le chapitre 7, l'effet immunopathologique ne mène pas forcement à la mort et aussi bien dans le chapitre 1 que le chapitre 7 nous nous sommes basé sur ce critère pour évaluer l'immunopathologie. Ainsi, dans le chapitre 1, les coûts immunopathologiques à plus faibles doses ne se voient pas en termes de survie mais pourraient être observés en termes de dégâts histologiques et/ou d'altération de fonctions physiologiques chez les individus les plus résistants comparés aux plus tolérants. C'est pour cela que nous souhaitons continuer notre étude bibliographique en y incluant des données physiologiques, histologiques, etc, dans le but de pouvoir aborder la question de la place de l'immunopathologie non plus uniquement en termes de survie mais en termes de dommages infligés à l'hôte sur ces fonctions biologiques.

Par ailleurs, la régulation de l'immunité est essentielle. Différents mécanismes ont été énumérés dans l'introduction. Ces mécanismes sont supportés par des messagers que sont les cytokines, et un dérèglement dans leur production ou dans l'équilibre dans leur production mène aux problèmes immunopathologiques énumérés précédemment. Ainsi, dans le chapitre 1, nous avons pu voir que le coût de l'infection pour les SJL venait en partie d'un problème d'équilibre entre les cytokines pro et anti inflammatoires, résultat similaire à ceux obtenus par Guerreiro et al. (2012).

Le système immunitaire, de par le coût de son activation et des dégâts occasionnés pendant la réponse, mais aussi de par sa tendance à arborer un profil pro-inflammatoire

avec l'âge pourrait très bien être une fonction pléiotrope antagoniste. Comme stipulé dans l'intro, certains papiers se sont penchés sur ce potentiel rôle mais ce sujet est jusqu'alors resté plutôt théorique en se basant sur quelques observations et de rares expériences (Ungewitter et Scrable 2009 ; Pursall et Rolff 2011). Ainsi notre but est ici de tester expérimentalement cette hypothèse de pléiotropie antagoniste. Nos travaux sont cependant encore en cours et seuls quelques éléments de réponses sont disponibles. Une fonction qui agirait de façon pléiotrope antagoniste doit donner un bénéfice à un jeune âge et être délétère à un âge avancé. Dans notre étude le bénéfice se mesure à la réussite de la lutte contre le parasite *Plasmodium yoelii*. Nous nous attendions donc à observer une charge parasitaire moins importante chez les souris qui ont reçu un traitement par un anticorps anti-IL-10 (limitant la régulation de l'immunité) pendant les premiers jours de l'infection et à ce que le coût de l'infection soit moindre chez ces mêmes souris. En effet, la charge parasitaire était moins élevée chez les souris parasitées auxquelles nous avions injecté l'anticorps anti-IL-10. D'autre part, la réduction de globules rouges en circulation était aussi moins importante chez ces souris traitées avec l'anticorps anti-IL-10. Ces deux observations confirment la première partie de l'hypothèse de la pléiotropie antagoniste postulant qu'une réponse immunitaire moins régulée, donc plus forte, est plus efficace dans la lutte contre le parasite et permet à l'hôte de subir moins de spoliation de ce dernier.

Dans un second temps nous avons testé le coût à un âge avancé, en termes de survie, de reproduction et de variation du poids. A ce jour, seulement 52% des souris sont mortes ce qui nous empêche de conclure quant à un coût plus important en termes de survie pour les individus parasités contrairement aux individus témoins. Pour le moment les groupes témoins ont une survie d'environ 36% alors que les groupes parasités sont encore à 73 et 64% respectivement pour les individus infectés et les individus infectés et traités avec l'anticorps. Cette observation ne va pas dans le sens d'un coût à long termes de la lutte contre l'infection. Le second trait que nous avons mesuré est le succès reproducteur. Ce succès reproducteur a été faible de par le fait que nos souris étaient déjà sénescentes. Cependant, un succès reproducteur légèrement plus important chez les groupes contrôles que chez les groupes infectés peut laisser suggérer un coût en termes de reproduction pour les individus ayant dû lutter contre le parasite. Finalment, le poids des individus après avoir

montré un plateau entre la première et deuxième année de vie, retombe de façon similaire entre les groupes.

Le parasite

Durant les dernières décennies, les maladies inflammatoires telles que la maladie de Crohn ou les allergies n'ont cessées d'augmenter en prévalence dans les populations humaines principalement dans les pays les plus fortement industrialisés (Bach 2002 ; Hovde et Moum 2012). Cette augmentation rapide laisse plutôt penser à une cause environnementale (Ananthakrishnan 2015) et l'absence de certains de nos parasites et commensaux dans les dans ces pays pourraient en être la cause (Strachan 1989 ; Rook et al. 2010, 2013). En effet, un certain nombre d'études sur l'animal ou l'humain démontrent l'avantage d'être parasité par certains helminthes comme traitement préventif et curatif de ces maladies inflammatoires (revues dans Heylen et al. 2014). Cependant, mis à part les bienfaits sur l'hôte, peu d'études se sont penchées sur les conséquences de ces environnements inflammatoires sur le parasite (e.g. Donskow-Lysoniewska et al. 2013). Donskow-Lysoniewska et ses collaborateurs ont induit chez la souris une inflammation du colon grâce à un sucre complexe, le Dextran Sulfate Sodium (DSS), produisant des symptômes proches de la colite ulcérante chez l'homme, peu avant l'infection par *Heligmosomoides polygyrus* et jusqu'à 15 jours post-infection. Ils ont pu ainsi montrer que cet environnement inflammatoire altérait les traits d'histoire de vie du parasite aussi bien en termes de survie, de taille des vers et de fécondité.

Ainsi nous nous sommes intéressés à l'impact de l'environnement inflammatoire sur le parasite aussi bien lors d'une réponse exacerbée par l'hôte (chapitre 1), que lors de changements dans le profil pro-inflammatoire de l'hôte suite à l'induction d'une colite (inflammation de la partie distale du gros intestin - chapitre 2), que lors d'une modification ponctuelle de l'environnement inflammatoire par un challenge LPS lors de l'infection (chapitre 3).

Dans le chapitre 1, notre approche en normes de réactions nous permet de tester les éventuelles interactions génotype – environnement. Grâce à cette approche nous avons pu observer que la production d'œufs dépendait de l'interaction entre la dose infectieuse et le génotype des hôtes. Alors que la production totale d'œufs sur la période étudiée était

supérieure pour les génotypes les plus tolérants (CBA) à faible dose, les parasites infectant le génotype le plus résistant (SJL) voient leur production d'œufs augmenter jusqu'au niveau des parasites infectant les CBA en même temps que le gradient de la densité de parasite augmente. Par ailleurs, cette augmentation est aussi liée à l'augmentation de la production de cytokine IL-6 (pro-inflammatoire généraliste) et d'une diminution de la production de cytokines IL-4 (pro-inflammatoire spécifique Th2, fortement impliquée dans la lutte contre le parasite). Ainsi cette modification du profil inflammatoire vers une réaction exacerbée suite à l'augmentation de la charge parasitaire, favorise le parasite en termes de fécondité. Dans le chapitre 2, nous avons confronté *Heligmosomoides polygyrus* à un environnement inflammatoire (provoqué par l'ingestion de DSS) dès son arrivée dans l'hôte (phase larvaire) ou à partir de la phase adulte. Nous avons trouvé que les parasites confrontés à cet environnement inflammatoire avaient une meilleure performance, aussi bien en termes d'abondance qu'en termes de production d'œufs, comparé au groupe contrôle. Plus précisément, concernant la phase larvaire, l'inflammation a réduit le temps de développement des larves jusqu'à la phase adulte. A l'état adulte, la survenue d'une inflammation favorise la survie des parasites principalement au vingtième jour post-infection où le nombre de vers retrouvés est cinq fois plus important que chez les souris contrôles. Il en va de même pour la production d'œufs qui est supérieure pour les parasites dans des souris traitées au DSS que pour les parasites dans les souris contrôles. Dans le chapitre 3, nous avons soumis des souris infectés par *Heligmosomoides polygyrus* à un challenge LPS (lipopolysaccharides de la bactérie *Escherichia coli*). Nous avons pu noter que la production d'œufs avait aussi augmentée lors de la stimulation de l'immunité par l'injection de LPS.

Ces trois altérations différentes de l'environnement inflammatoire mènent toutes à une amélioration de la fécondité chez le parasite (chapitre 1, 2 et 3), mais aussi à une accélération du temps de développement des larves (chapitre 2) et une augmentation du nombre de vers adultes (chapitre 2) comparés aux parasites dans les souris contrôles. Ainsi, ces observations démontrent l'avantage que confère un environnement inflammatoire à *Heligmosomoides polygyrus*. Mais comment un environnement inflammatoire pourrait être favorable ? Dans le chapitre 1, la production de cytokines pro-inflammatoires semble mal orientée (avec une diminution de la production de cytokines IL-4 et une augmentation de la production de IL-6), et serait plutôt dirigée vers une réponse de type Th1 potentiellement

suite aux blessures occasionnées par le parasite lors de sa traversé de l'épithélium intestinal. Une étude récente montre que des souris KO pour l'IL-1β, cytokines pro-inflammatoire majeure de la réponse Th1, éliminaient plus rapidement *Heligmosomoides polygyrus* comparé aux wild-type (Zaiss et al. 2013). Lors du challenge LPS du chapitre 3, les cytokines Th2 mesurées diminuent légèrement mais pas assez pour soutenir l'hypothèse du relâchement de la pression de l'immunité favorisant la production d'œufs dans cette étude.

Une autre explication à nos observations peut venir du fait que nos études ne couvrent pas la totalité de la vie du parasite suite à l'exposition à l'environnement inflammatoire et les différents traits d'histoires de vie n'ont pas forcement tous été suivis. La production d'œufs a été mesurée dans chacune de ces études mais jusqu'aux jours 28, 12 ou 20 post infection pour les chapitres 1 et 2, et 53 heures post-injection de LPS pour le chapitre 3. Le nombre d'adultes et l'accélération du développement n'ont été mesurés que dans l'étude décrite dans le chapitre 2. De ce fait, certains traits d'histoire de vie manquent et pourraient montrer un coût pour le parasite. Outre cela, le fait de ne pas avoir mesuré les différents traits d'histoire de vie sur une plus longue période cache peut-être un compromis qui pourrait apparaitre plus tardivement. On pourrait imaginer qu'un environnement inflammatoire (même non spécifique au parasite) soit un signal d'un environnement dangereux et que ce signal fasse modifier au parasite ses traits d'histoire de vie de façon plastique en favorisant une fécondité plus importante dès que le danger est détecté. C'est le cas de l'escargot *Biomphalaria glabrata* infecté par les miriacidies de *Schistosoma mansoni.* Cet escargot, augmente sa fécondité lors de l'infection puisque cette dernière va réduire considérablement sa durée de vie (Minchella and Loverde 1981 ; Thornhill et al. 1986). Nous ne pouvons malheureusement pas répondre à cette interrogation avec les données dont nous disposons. Cependant, une étude de Guivier et al. 2016, montre que suite à un challenge LPS la production d'œufs des parasites était plus faible pour les parasites infectant les souris ayant subit le challenge contrairement aux parasites dans les souris contrôles à 3 et 7 mois post-infection. Ainsi cet environnement pourrait ne pas être si favorable que cela à long terme.

D'ailleurs, lorsque nous nous intéressons à l'immunomodulation du parasite, nous observons dans les chapitres 2 et 3 que ce dernier augmente l'expression d'un homologue

du TGF-β (cytokine anti-inflammatoire) et de cystatines (réduisant l'induction des cellules T helpers par les cellules présentatrices d'antigènes, Klotz et al. 2011). De surcroît, Donskow-Lysoniewska et al. (2013), montrent que dans un environnement inflammatoire induit par la consommation de DSS, les parasites allaient modifier leur profil protéomique et ainsi réduire leur immunogénicité. Pourquoi investir dans de l'immunomodulation et du camouflage si l'environnement est favorable ? Cela laisse tout de même penser qu'il doit y avoir un coût sous-jacent que l'on ne détecte pas ici. Il se pourrait que les parasites s'adaptent plastiquement à l'environnement qu'ils détectent. Des signaux de danger, comme l'augmentation de l'état inflammatoire de l'hôte, induiraient des modifications de la dynamique des traits d'histoire de vie du parasite au profit d'une survie et fécondité augmentée tôt dans leur vie. Cette modification serait permise grâce à une surexpression de l'immunomodulation et camouflage au détriment du maintient de leurs performances (survie et reproduction) à long terme comme le laisse penser l'étude de Guivier et al. (2016). Une autre possibilité est que le coût soit payé à la génération suivante. Cette hypothèse a été suggérée par Babayan et al. (2010) lors d'une infection par des filaires *Litomosoides sigmodontis.* En effet, en manipulant l'immunité spécifique pour lutter contre ces filaires (modification dans la production d'IL-5 et d'éosinophiles), ils ont pu montrer que les filaires confrontées à un environnement inflammatoire plus dangereux pour elles modifiaient leur traits d'historie de vie en ajustant la production des stades de transmission plus tôt et en plus grande quantité. De futures études centrées sur cette interrogation pourraient apporter une réponse en comparant les performances des descendants issus de parents confrontés ou non à un environnement inflammatoire.

Parallèlement à ce questionnement, je me suis posé la question de comment l'environnement inflammatoire peut produire des réponses microévolutives. Je me suis intéressé à la possibilité de sélectionner des parasites dans un environnement inflammatoire induit par l'ingestion de DSS ou débridé par l'inhibition d'une cytokine anti-inflammatoire, le TGF-β. Quelques études de sélection sur *Heligmosomoides polygyrus* ont été réalisées par l'équipe de Dobson (Dobson et Owens 1977 ; Dobson et Tang 1991 ; Su et Dobson 1997) et ont montré que la sélection de ce parasite dans différents environnement inflammatoires était possible en seulement quelques générations (chapitre 3 et 4).

Dans le premier cas j'ai donc soumis deux lignées de parasites soit à des souris traitées au DSS, soit à des souris contrôles, pendant 9 générations. Dans le second cas nous avons soumis les parasites à 3 environnements de sélections : des souris qui ont reçu des injections de PBS (comme contrôle), de TGF-β (cytokines anti-inflammatoire) et d'anticorps anti-TGF-β, pendant 4 générations. Comme constaté lors de l'étude de la plasticité des traits d'histoire de vie, nous observons, après sélection, un avantage en termes de fécondité pour les parasites sélectionnés dans un environnement inflammatoire (chapitre 4 : DSS) et en termes de production d'œufs (chapitre 3 : anti-TGF-β). Dans le chapitre 4, seul la fécondité per capita répond à la sélection au bout de 9 générations, montrant une fécondité per capita plus importante au jour 26 post-infection pour la lignée de parasites venant de souris exposées au DSS. Par ailleurs, nous avons aussi une réponse plastique sur la production d'œufs totale : les parasites dans des souris traités aux DSS ont une production d'œufs totale supérieure aux parasites dans les souris contrôles à la neuvième génération, ce qui coïncide avec les observations faites lors des chapitres sur la plasticité des traits d'histoire de vie du parasite. Dans le chapitre 4, la lignée de parasites sélectionnée dans des souris traitées à l'anticorps anti-TGF-β ont une meilleure production d'œufs que les autres lignées. Cette différence s'accentue avec le temps et les parasites de la lignée anti-TGF-β maintiennent une production d'œufs quasiment constante jusqu'au jour 74 post-infection. Malheureusement nous n'avons pas mesuré le nombre d'adultes. Ainsi nous ne savons pas si cette production d'œufs est due à une survie des parasites ou à une fécondité per capita qui augmente avec le temps. En faisant le rapprochement avec le chapitre 4 (DSS), bien que les environnements de sélection n'aient pas grand-chose à voir entre eux, l'augmentation de la fécondité per capita pourrait être une explication au haut niveau de production d'œufs observé au chapitre 3 (anti-TGF-β). Si cette hypothèse est vraie, alors la survie diminue au court du temps pour la lignée de parasites anti-TGF-β. Un compromis entre fécondité et survie, favorisant la fécondité apporterait une réponse à la question de l'existence d'un changement en faveur d'une fécondité accrue suite à la détection d'un signal de danger que représente l'environnement inflammatoire. Changement qui, semble t-il, pourrait être sélectionné (lignée de parasite anti-TGF-β qui garde une production d'œufs plus élevée dans des souris non traitées après 4 générations). Cependant cela reste une hypothèse et nous n'avons malheureusement pas les éléments pour y répondre. Cependant une étude réalisée

par Dobson et Owens en 1977, montre qu'après 30 générations de sélection dans des souris non consanguines, l'infectivité, autrement dit le nombre de parasites adultes retrouvés dans la lumière de l'intestin, augmente en début de cycle (jour 11 post-infection) comparé à la génération initiale pour ensuite retomber plus vite en fin de cycle vers le jour 28 post-infection comparé à la génération initiale. Ces résultats pourraient soutenir la possibilité du rôle de la fécondité per capita dans la production d'œufs observée au chapitre 3 (anti-TGF-β).

Par ailleurs, nous avons mesurés les capacités d'immunomodulation des parasites directement en mesurant l'expression des gènes de l'homologue du TGF-β et du gène d'une cystatine chez *Heligmosomoides polygyrus* dans le chapitre 3, et indirectement par la capacité que le parasite a à atténuer les symptômes de l'hôte soumis au DSS dans le chapitre 4. Dans le chapitre 3, l'expression des deux gènes immunomodulateurs ne change pas quel que soit la lignée. Ce trait semble plus répondre de façon plastique, ce qui *in-fine* sera sans doute meilleur pour le parasite confronté à des environnements inflammatoires très variables entre hôtes (Robinson et Delvig 2002 ; Agrawal et al. 1998), ou encore pour éviter les infections opportunistes qui pourraient tuer l'hôte (Cornet et Sorci 2010 ; Guivier et al. 2016).

A l'inverse, confronté à un environnement inflammatoire provoqué par l'ingestion de DSS, les parasites sélectionnés dans cet environnement montrent une plus grande capacité à atténuer les symptômes de l'hôte confronté au DSS. Cette observation est un pas de plus vers le fait que cet environnement inflammatoire aurait bel et bien un coût pour le parasite puisque ce trait a été sélectionné pour immunomoduler l'inflammation de façon plus importante quand les parasites se trouvent dans un environnement inflammatoire provoqué par l'ingestion de DSS chez l'hôte. Cependant, au fur et a mesure de la sélection, un phénomène de bottleneck peut se produire, ce qui diminue la diversité des parasites et augmente leur immunogénicité (Dobson et Owens 1977). Ainsi cette augmentation de la régulation pourrait aussi être due à une plus forte pression de l'immunité sur les parasites sélectionnés qui présentent moins de diversité et donc immunomodulent plus pour leur propre survie. Cette hypothèse pourrait être facilement vérifiée par des études de diversité

génétiques chez les vers au cours des générations en suivant le même protocole de sélection.

Conclusion générale

Les relations hôte-parasites sont complexes et soumettent les deux protagonistes à un ensemble de compromis aussi bien plastiques qu'évolutifs. Bien que l'immunité soit indispensable pour la lutte contre les parasites, elle peut aussi causer de nombreux dégâts à l'hôte et se montrer délétères avec l'âge ou au travers de divers disfonctionnements menant à des maladies auto-immunes ou allergiques. De l'autre coté du front, les parasites doivent faire face à une diversité d'hôte et d'environnements inflammatoires qui altèrent leurs traits d'histoire de vie.

Dans cette thèse j'ai choisi d'aborder ces compromis à travers deux modèles de laboratoires : H*eligmosomoides polygyrus* et *Plasmodium yoelii* infectant *Mus musculus domesticus*. Grâce à différentes approches expérimentales et bibliographiques nous avons pu montrer que l'immunopathologie est un trait qui perdure grâce aux bénéfices de la réponse immunitaire dans la lutte contre les parasites. Cependant, une approche plus raffinée, en s'intéressant aux symptômes plutôt qu'à la survie, pourrait montrer un rôle plus important de l'immunopathologie sur la fitness des individus. D'ailleurs, une inflammation exacerbée, peut aussi affecter les traits d'histoire de vie du parasite. Ainsi, concernant le parasite *Heligmosomoides polygyrus*, j'ai pu mettre en évidence, par plusieurs approches, qu'une inflammation provoquée par l'ingestion de DSS ou par l'injection de LPS altérait positivement les traits d'histoire de vie du parasite aussi bien de façon plastique que lors de processus de sélection. Cependant, le parasite semble tout de même investir d'avantage dans l'immunomodulation et le camouflage quand il est confronté à ces environnements, laissant planer la question du coût de cette inflammation à long terme et sur plusieurs générations.

Ainsi, de futurs travaux devraient être menés pour éclaircir les compromis de l'environnement inflammatoire chez le parasite qui pour le moment semble principalement se retrouver dans un compromis entre reproduction précoce et tardive, voire intergénérationnel.

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Chapitre Annexe : L'âge des parasites influence des traits d'histoire de vie liés à la fitness chez leurs descendants

Title: Aging parasites produce offspring with poor fitness prospects

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Abstract

Senescing individuals have poor survival prospects and low fecundity that directly impinge on Darwinian fitness. Old individuals might also produce offspring with reduced survival and reproductive success. While this has been shown in free-living organisms, we do not know if senescing parasites also produce propagules with poor fitness prospects. This is particularly relevant for macroparasites that establish long-lasting, chronic infections. We tested the effect of parental age on the performance of descendants in the nematode *Heligmosomoides polygyrus*, an intestinal parasite of rodents. We found that propagules of senescing worms had reduced within-host survival, and reduced egg shedding over the first month post-infection compared to propagules produced by young parents. These results suggest that offspring quality might set a limit to the length of chronic infection in terms of transmission potential.

Introduction

Senescence refers to the progressive decline of physiological functions, leading to accelerated mortality and reproductive failure with age. Although it was generally believed that senescence rarely occurs in nature, because sources of external mortality (predation, starvation, infectious diseases, etc.) prevent individuals to reach old ages, evidence is now rapidly accumulating suggesting that aging is a common phenomenon across a large spectrum of organisms (Nussey et al. 2013; Jones et al. 2014).

The study of senescence has attracted considerable attention to identify the molecular and physiological mechanisms underpinning the age-associated decline in performance (Olshansky et al. 2016) and the selective forces underlying its evolution (Rose 1991). Classical theories on the evolution of aging share the same basic assumption: the strength of selection declines with age (Hamilton 1966). Declining selective forces imply that accumulating deleterious mutations cannot be purged (Medawar 1952), and that genes conferring benefits at late age cannot be selected for (Williams 1957). Life-history models have also provided useful predictions on the onset and rate of age-associated decline in performance, depending on the pattern of resource allocation to reproduction vs. maintenance (Kirkwood 1981; Davison et al. 2014). For instance, individuals that invest a lot of resources into reproduction have been shown to have earlier and more rapid senescence in several species (Jones et al. 2008; Ricklefs 2010; Preston et al. 2011; Boonekamp et al. 2014).

As mentioned above, declining reproductive output with age has been reported in a wide spectrum of organisms, both vertebrates and invertebrates (Nussey et al. 2013; Jones et al. 2014). However, reduced fecundity at old age does not necessarily translate into deteriorating fitness because old parents might produce fewer offspring of better quality. Improved offspring quality from experienced parents has been reported, for instance, in long-lived species of birds (e.g., Limmer and Becker 2010). Therefore, a proper assessment of the fitness consequences of reproductive senescence should include evaluating offspring quality in addition to the number of descendants.

The effect of parental age on offspring performance (survival and reproduction) has been explored in several species, both animals and plants, and in many cases old parents do

indeed produce offspring of poorer quality compared to young individuals (Lansing 1947; Hercus and Hoffmann 2000; Kern et al. 2001; Descamps et al. 2008; Bouwhuis et al. 2010, Gillespie et al. 2013; Barks and Laird 2015). More recently, parental age has also been shown to affect gamete quality in a bird species, offering a mechanistic explanation for the observed fitness reduction of offspring produced by old parents (Preston et al. 2015).

Many helminth parasites establish long-term, chronic infections. Given that the great majority of helminths do not multiply within their definitive hosts but release propagules to infect new hosts, the establishment of chronic infections implies that individual worms can live for months or even years. For instance, filarial parasites of humans, such as *Loa loa*, can live more than 10 years and have very long reproductive lifespan (Coutelen 1935 cited in Gems 2000).

As for free-living organisms, macroparasites have to cope with environmental hazards and their age-specific survival depends on both environmental and intrinsic factors. Macroparasites do have a declining fitness as age progresses, both in terms of reduced egg output and increased mortality (reviewed in Gems 2000). However, assessing patterns of senescence in parasitic organisms is challenging for several reasons. First, age-dependent mortality and age-dependent decline of egg output may reflect host immunity (an extrinsic source of mortality) rather than an endogenous deterioration of organismal functions. Second, using the time of parasite persistence within the host (patent period) as a proxy of parasite longevity can be misleading if hosts get new infections. These two shortcomings can be avoided by using hosts that do not mount a specific immune response against the parasite and where re-infection is not possible.

A few years ago, Gardner et al. (2006) published a seminal paper on the aging of a nematode species (*Strongyloides ratti*). *Strongyloides ratti* has both parasitic and free-living adults; this offers the unique opportunity to compare aging in the external environment and within the host for the same species. Using immune deficient (*nude*) rats, Gardner et al. (2006) showed that parasite mortality rate increased exponentially approximately at the age of 250 days, with maximum lifespan being 400 days. This was strikingly different from the senescence pattern of free-living adults that only lived for about 5 days, even under optimal culture conditions. Age-specific variation in per capita fecundity

mirrored mortality, with stable fecundity up to the age of 250 days and then an exponential decline. Interestingly, parasitic *Strongyloides ratti* showed little evidence of age-associated degenerative changes up to 11 months and female worms had morphologically normal embryos in the gonad (Gardner et al. 2006). However, the performance of such lately produced offspring was not assessed. Declining offspring quality as parental age progresses can have profound fitness consequences for parasites that establish chronic infections. Poor quality offspring can fail to infect novel hosts and therefore late produced propagules might have no contribution to parental fitness. Even if they successfully manage to infect a new host, they might have reduced reproductive output. All this might, therefore, have consequences for the evolution of the optimal allocation of reproductive effort over different ages (Charlesworth 1994; Davison et al. 2014), and potentially set a limit to the length of chronic infection in terms of transmission efficacy.

We investigated the effect of parental age on the infection dynamics of propagules using the intestinal nematode *Heligmosomoides polygyrus bakeri*. *Heligmosomoides polygyrus* is a natural parasite of rodents with a direct life cycle. Eggs are shed with the host feces, larvae develop within the shed eggs and hatch around two days after being laid (first stage larvae, L1), after one more day, L1 larvae molt in L2 larvae, and one more day (and molt) is required to reach the L3 which is the infective stage. Larval survival (up to the L3 stage) in the external environment strongly depends on the moisture of the background, since larvae do not survive desiccation (Lettini and Sukhdeo 2006). Mice become infected upon ingestion of L3 larvae. Once in the gut, larvae penetrate the intestinal wall where they molt twice to reach the adult stage (day 7-8 post-infection). At day 8 post-infection, adults return in the intestinal lumen where they attach to the villi and start reproducing. No “dormant” stages (with long encystment in the intestinal wall) have been described for this species. *Heligmosomoides polygyrus* establishes long term infections which last for months depending on the genetic background of the host (Reynolds et al. 2012).

We infected a highly tolerant strain of mice that fails to mount a Th2 response (the immune response that confers resistance to *H. polygyrus* (Filbey et al. 2014; Lippens et al. 2016)), and we harvested the larvae produced at days 15, 45, 105 and 165 post-infection. This corresponds to the worm age because secondary infection was not

possible, given the unfavorable environmental conditions encountered by the larvae in the cage. The litter at the bottom of the cage is a dry environment not suitable for larval development, and even though mice can eat their own feces, eggs are not infective. Reaching the infective stage would require approximately a four day survival in a very unfavorable environment. In agreement with this, previous work has shown that uninfected male mice housed with *H. polygyrus* infected females do not get the infection and that offspring of infected females kept in the same maternal cage until weaning (when 20 day old) are parasite free (Kristan 2004).

Larvae produced by worms of different ages were used to infect mice and their egg shedding monitored over 28 days post-infection. At day 28 post-infection, we also counted the number of adult worms in the intestinal lumen to infer infection success and compute per capita fecundity.

We predicted that the performance (infection success, egg shedding) of worms produced by old, senescing parents should be lower compared to worms of young parents.

Materials and methods

Ethics statement

All animal experiments were approved by the Comité d'Ethique de l'Expérimentation Animale Grand Campus Dijon (CNREEA n° C2EA – 105) and by the "Ministère de la Recherche et de l'Enseignement Supérieur" (project N°01867.01) according to the national guidelines ("Charte nationale portant sur l'éthique de l'expérimentation animale", Ministère de la Recherche et de l'Enseignement Supérieur) on the use of animals for research purposes.

Mice, parasites and treatments

All mice were purchased from JanvierLABS (JRj) and housed at the Université de Bourgogne. They were maintained under constant temperature (24°C) and photoperiod (12L:12D), fed with standard mouse pellets *ad libitum* and had *ad libitum* access to filtered tap water. When 7 week old, 8 unrelated B6CBAF1 female mice (F1 of ♀ C57BL/6JRj and ♂ CBA/JRj cross), kept in group in a 38 cm (L) × 22.5 cm (W) × 18.5 cm (H) plastic cage with sawdust bedding, were infected with 500 (± 35) L3 stage larvae of *Heligmosomoides*

polygyrus bakeri. Mice were infected by gavage with 0.1 ml of drinking water, using a blunted tip needle (18 g) on an insulin syringe of 1 ml.

At days 15, 45, 105 and 165 post-infection (corresponding to worm age), we collected fresh feces. Feces were mixed with distilled water and coal in powder, spread on a Whatmann paper (grade 40) kept wet by daily humidification in a Petri dish at ambient temperature. L3 larvae were harvested 8 days after feces collection. Fifty larvae (± 10) for each parental age were then used to infect five female CBA mice. CBA mice were kept in groups of five in 38 cm (L) × 22.5 cm (W) × 18.5 cm (H) plastic cages with sawdust bedding.

We used fecal egg count and number of adult worms in the intestine as proxies of parasite fitness. At day 11, 14, 18, 20, 25, 28 post-infection, each mouse was transferred, at 9 am, in an individual cage. The cage had a humidified towel paper at the bottom to avoid feces desiccation and a grid to prevent the mouse shredding the paper. Mice were left four hours in these conditions and then put back in their shared cages. Feces produced during this four-hour period were collected and 200 mg were smashed and suspended in 2.5 ml of water. Thereafter, 5 ml of salted water (75% of saturation) were added to allow eggs to float. After agitation, a fraction of this suspension was transferred into a McMaster chamber for egg count. We performed two counts per sample and used the mean values. Fecal egg count is expressed as number of eggs per mg of feces. In addition to daily egg count, we also computed the total egg production as the sum of each fecal egg count throughout the study. At day 28 post-infection, mice were euthanized by cervical dislocation. The abdomen was opened and the intestine removed and placed in a Petri dish. The intestine was longitudinally opened with a small scissor and the number of adult worms in the intestinal lumen counted using a NIKON AZ100 Multi-Purpose Zoom Microscope.

Statistical analyses

The number of adults harvested at day 28 post-infection across parental ages was analyzed using a generalized linear model (GLM) with a Poisson distribution of errors and a log link function.

Fecal egg count was log-transformed and analyzed using a linear mixed model with a normal distribution of errors and with mouse identity as a random factor. Fixed factors were

time, parental age and their interaction. We also compared per capita fecundity (number of eggs/number of worms) at day 28 post-infection, and the overall egg output (log-transformed) over the 28 days post-infection across parental ages.

All statistical analyses were performed with R software (version 30.2) with library nlme for linear mixed models. The statistical significance was fixed at the threshold of 0.05.

Results

Parental age had a strong effect on the number of adult parasites recovered in the intestinal lumen at day 28 post-infection (z = -6.536, p < 0.0001, n = 20), with almost a two-fold reduction in the number of parasites from the oldest parents, compared to the youngest ones (Figure 1).

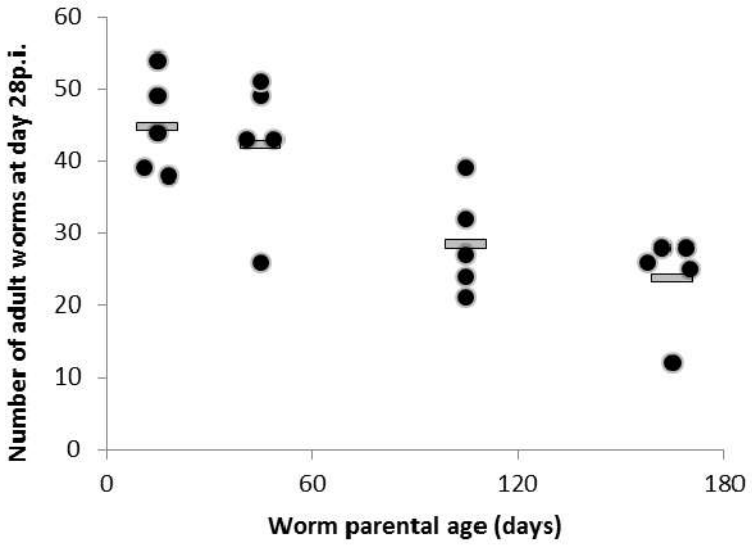

Figure 1. Number of adult *H. polygyrus* worms recovered in the intestinal lumen as a function of worm parental age. Grey bars indicate mean number of adult at each worm parental age.

Fecal egg count varied with time post-infection as a function of parental age, as shown by a statistically significant time * parental age interaction (time post-infection: t = 2.91, df = 1,97, p = 0.0045; parental age: t = 0.41, df = 1.97, p = 0.6878; time post-infection * parental age: t = -2.55, df = 1,97, p = 0.0123; Figure 2). Worms from the youngest parents had high initial fecundity and maintained high fecundity throughout the first month post-infection (Figure 2A); whereas worms from the oldest parents had a declining fecundity through time (Figure 2D).

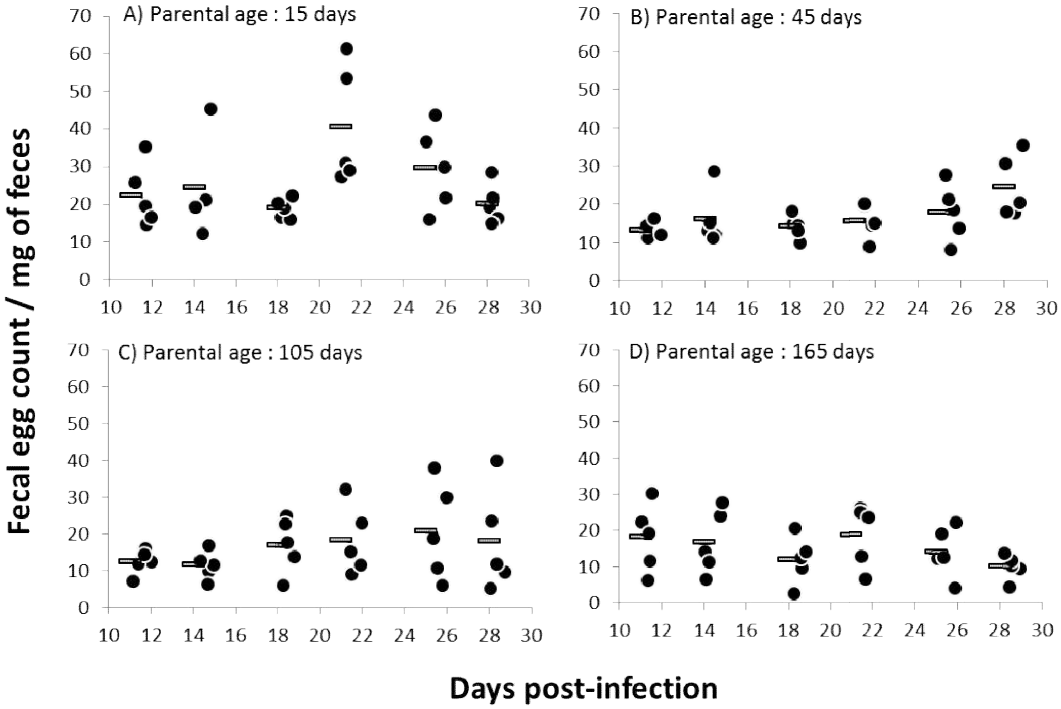

Figure 2. Fecal egg count of *H. polygyrus* over the first 28 days post-infection for the different worm parental ages. A) parental age = 15 days; B) parental age = 45 days; C) parental age = 105 days; D) parental age = 165 days. Grey bars indicate mean number of eggs at each worm parental age.

At day 28 post-infection, parental age had a strong effect on fecal egg count (t = -3.07, df = 1,18, p = 0.0066). However, this was due to differences in parasite population size, since the per capita fecundity was similar across parental ages (t = -0.50, df = 1,18, p = 0.622; Figure 3).

Figure 3. Per capita fecundity at day 28 post-infection (fecal egg count/mg of feces/number of adult worms) for the different worm parental ages. Grey bars indicate mean per capita fecundity at each worm parental age.

We also computed the total egg production up to day 28 post-infection. Parental age was negatively correlated with total egg count (t = -2.17, df = 1,17, p = 0.0443).

Discussion

Intestinal worms have long lifespan and can shed eggs for years (Gems 2000); however, the contribution to parental fitness of late produced offspring is still unknown. In this study, in agreement with the predictions, we found that parental age has an effect on offspring performance, in terms of infection success, and as a consequence of reduced adult population size, in terms of overall reproductive output.

Why organisms age has been a major evolutionary biology question for decades. Recent review papers have stressed that senescence is a widespread phenomenon across the tree of life, even though variation in the shape of the relationship between age and mortality exists among species (Jones et al. 2014). Helminths are no exception to this general pattern, and aging has been reported in a few species of nematodes (Gems 2000, Gardner et al. 2006). Here, we go beyond this previous work investigating how parental age affects the future performance of offspring.

The relevance of our work relies on a few assumptions: 1) patent period (in other terms the duration of the infection) does not depend on external factors, such as host immunity; 2) patent period accurately reflects worm age (in other terms there is no risk of secondary infection); 3) host health status does not vary with time post-infection. We briefly discuss each of these points and how we addressed them in our study.

Extensive work has shown that the Th2 response is essential for host immunity to *H. polygyrus*. Urban et al. (1995) infected immunocompetent (BALB/c) and immune deficient (SCID) mice with *H. polygyrus* and treated them with the Th2 cytokine IL-4. In both mouse strains, IL-4 treatment reduced parasite fecundity and survival during the first month post-infection. Further work has shown the different inbred strains of mice can be ranked with respect to their susceptibility/tolerance to the infection with *H. polygyrus* and this depends on the Th2 response, the activation of innate lymphoid cells and macrophage populations (Filbey et al. 2014, Lippens et al. 2016). We used very tolerant strains of mice with the lowest expression of immune-associated resistance to *H. polygyrus* (Filbey et al. 2014), including the

production of the hallmark of Th2 response, IL-4. Therefore, we are confident that the duration of the infection in these hosts reflects intrinsic characteristics of the parasite rather that external, immune-mediated, mortality.

Another potential shortcoming in the study of aging in parasitic organisms arises from secondary infections, potentially flawing the estimation of worm age. *Heligmosomoides polygyrus* allows preventing this potential bias because the infective stage (L3 larvae) is reached after about four days eggs are released in the external environment. Larvae are very sensitive to the soil moisture and previous work has shown that they fail to survive a desiccation episode (Lettini and Sukhdeo 2006). Given that the bottom of the housing cages is covered by sawdust bedding, this represents a very unfavorable environment for larval development since feces become very dry already a few hours after being excreted. In support to the idea that inter-individual transmission is not possible in this system in the lab, non-infected males housed with infected females do not contract the infection and offspring born from parasitized mother and kept in the same cage until weaning are parasite free (Kristan 2004). Moreover, no “dormant” stage has been described for *H. polygyrus*, the interindividual variation in developmental rate (the time needed to emerge in the intestinal lumen as adults) being in the order of a few days.

Finally, another potential bias could come from a concomitant deterioration of host health as the infection goes on. In this case, poor worm offspring performance could simply be due to a “senescing host” effect rather than to a “worm parental age” effect. However, as mentioned above, we used strains of mice that are very tolerant to the infection and that do not have any obvious sign of declining health as the infection goes on. We maintain the population of parasites in B6CBAF1 mice in our lab since 2012 (with naïve hosts being infected every 2-3 months) and we never observed any host morbidity (reduction of body mass, distress or discomfort, mortality). Moreover, at the end of the experiment, hosts were about 30 week old, which is well before the onset of senescent decline in this strain of mice (see Hackbarth et al. 1982 for an example). For all these reasons, we believe that our experiment provides reliable and unbiased information on the effect of worm parental age on offspring future performance.

Although the decrease in the number of parasites harvested in the intestinal lumen across parental age does indicate a relative failure to establish a successful infection as parental age progresses, we do not know at which stage of the worm development mortality occurred. In other terms, we do not know if failure to establish a successful infection was due to enhanced larval or early adult mortality. Further work should clarify this point. Nevertheless, the finding that egg shedding progressively declined with time for worms issued from the oldest parents, while the per capita fecundity stayed constant, indicates that adult survival was reduced in this group.

In addition to the effect on the number of worms harvested in the intestinal lumen, we also found that the dynamics of egg shedding across time varied as a function of parental age. Variation in fecal egg count might, however, reflect differences in adult population size rather than a specific effect of parent age on offspring fecundity. In agreement with this view, we found that even though fecal egg count at day 28 post-infection was considerably lower for worms issued from old parents, this effect was entirely due to the number of recovered adult worms. In other words, the per capita fecundity did not vary across parental ages. Nevertheless, the cumulative egg output (over the first 28 days post-infection) was lower for worms produced by old parents, suggesting a poorer transmission potential of parasites produced by old parents.

We focused on two main life history traits of the parasite: egg output and survival in the host. Of course, other fitness-linked traits might vary as a function of parental age. Since eggs are excreted with the feces and the first molts occur in the external environment, it would be very interesting to explore the effect of parental age on the capacity of larvae to resist environmental stress (temperature, desiccation, etc.). Similarly, one important fitness-associated trait is the capacity of *H. polygyrus* to interfere with the host immune response (Grainger et al. 2010). It would be fascinating to investigate if these immunomodulatory properties of *H*. *polygyrus* do show signs of age-associated decline.

Our findings corroborate previous studies on free-living organisms which stressed the role of parental age as a driver of offspring performance (Priest et al. 2002, Tarin et al. 2005, Beamonte-Barrientos et al. 2010; Bouwhuis et al. 2010, Torres et al. 2011, Gillespie et al. 2013, Barks and Laird 2015, Preston et al. 2015). Usually, offspring of old

parents have reduced growth, fecundity or survival prospects and the underlying mechanisms can involve the accumulation of germ-line mutations with age (Kong et al. 2012), or phenotypic adjustment of parental effort as age progresses (Beamonte-Barrientos et al. 2010). Whatever the mechanisms, poor fitness return of late-produced offspring should affect the optimal allocation of resources over somatic maintenance and reproduction across the individual lifespan.

Solving the dilemma between allocating to maintenance or reproduction is particular relevant for parasites that establish chronic infections because their reproductive lifespan can be very long. *Heligmosomoides polygyrus* can persist for months in permissive host strains (Reynolds et al. 2012) showing that under benign environmental conditions, worm longevity is essentially determined by age-dependent deterioration of organismal functions or in other terms aging. In agreement with this, infection of different strains of immune deficient mice (SCID and *nude*) has shown that adult survival is stable up to the age of 9 weeks, whereas worms infecting the corresponding wild-type immunocompetent strains show a decrease in adult survival of about 90% during the same period (Hashimoto et al. 2009). Unfortunately, worms infecting such immune compromised strains were not monitored long enough to know at which age *H. polygyrus* starts to show signs of senescing decline. Even in outbred lines of mice, *H. polygyrus* usually establishes chronic infection. Primary infection of MF1 mice with increasing doses of worms showed that on average 17% of worms (relative to the inoculum size) were still alive 22-23 weeks post-infection (Keymer and Hiorns 1986).

Actuarial and reproductive senescence results in declining fitness at late ages and evolutionary theory has provided a series of explanations and predictions on why, when and how individuals should age (Medawar 1952; Williams 1957; Hamilton 1966). In permissive hosts that exert weak selection pressure in terms of immune-mediated mortality (the main source of extrinsic mortality) parasites are expected to have delayed maturation, larger adult size and extended longevities. Comparative and experimental work has provided general support to these theoretical predictions (Chehresa et al. 1997; Sorci et al. 2003). For instance, lines of *Heligmosomoides polygyrus* that have been isolated from a laboratory population have been shown to differ in most of the life history traits investigated, including

egg production, survival, and lifetime reproductive success, with some parasite lines having up to 50% survival 6 months post-infection (Chehresa et al. 1997). There was nevertheless extensive variation among parasite lines and generations. Interestingly, rapid passages (during the first month post-infection) of these isolated lines produced microevolutionary shifts both in terms of early egg production and short-term survival (Chehresa et al. 1997), suggesting genetic variation of life history traits and rapid parasite adaptation to the prevailing environmental conditions.

To conclude, the performance of offspring produced by aged parents has rarely been taken into account in life history evolution models (but see Moorad and Nussey 2016). Poor performance of lately produced offspring might impinge on parental fitness and have evolutionary consequences for the optimal schedule of age-dependent investment into reproductive effort. For parasitic organisms, the evolutionary consequences extend to the length of chronic infections and the transmission efficiency of propagules produced by old parents.

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Résumé

Les relations hôte-parasite sont complexes et soumettent les deux protagonistes à un ensemble de compromis aussi bien plastiques qu'évolutifs. D'un côté, bien que l'immunité soit indispensable pour la lutte contre les parasites, elle peut aussi causer de nombreux dégâts à l'hôte lors de la réponse et mener à des maladies inflammatoires. De l'autre côté, les parasites, bien que dotés de mécanismes d'évasion, vont être affectés par l'environnement inflammatoire de l'hôte. Cela pose la question des coûts et bénéfices que l'interaction entre ces deux protagonistes va avoir sur chacun d'eux lors de l'apparition de troubles inflammatoires chez l'hôte. Grâce à différentes approches expérimentales et bibliographiques, j'ai pu montrer que l'immunopathologie est un trait qui perdure vraisemblablement grâce aux bénéfices de la réponse immunitaire dans la lutte contre les parasites. Par ailleurs, j'ai pu mettre en évidence qu'une inflammation altérait positivement les traits d'histoire de vie du parasite Heligmosomoides polygyrus aussi bien de façon plastique qu'après sélection expérimentale. Cependant, le parasite investit davantage dans l'immunomodulation et le camouflage lorsqu'il est confronté à cet environnement, laissant planer la question du coût de cette inflammation à long terme et sur plusieurs générations.

Abstract

Host-parasite interactions are characterized by trade-offs that involve both plastic and microevolutionary responses. On one hand, while immunity is essential to fight parasites, it can also cause damage to the host, leading to autoimmunity and inflammatory diseases. On the other hand, parasites have to cope with the immune environnement provided by the host. This raises the question of the costs and benefits of the inflammatory response for the two partners of the interaction. With different experimental and literature-based approaches, I showed that immunopathology is a trait that likely persists because of the immediate benefits of the immune response in terms of protection against parasites. Furthermore, I was able to show that inflammation positively altered the life history traits of the gastrointestinal nematode Heligmosomoides polygyrus both plastically or after experimental evolution. However, the parasite invested more in immunomodulation and camouflage when facing an inflammatory environment, leaving open the question of the costs associated with an inflammatory environment over the entire lifespan of the parasite and/or across generations.