



Ecologie évolutive des interactions Hôte / Moustique / Plasmodium : sources d'hétérogénéité de l'infection des vecteurs

Romain Pigeault

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THÈSE

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Docteur

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Présentée par **Romain Pigeault**

Ecologie évolutive des interactions
Hôte / Moustique / *Plasmodium* :
Sources d'hétérogénéité de l'infection
des vecteurs

Soutenue le 17 Décembre 2015 devant le jury composé de

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Introduction

Introduction

1- La malaria

1-1 Présentation

La malaria est une maladie infectieuse causée par des parasites de l'ordre des haemosporidiens et du genre *Plasmodium*. Ces protozoaires hétéroxènes réalisent leur reproduction asexuée au sein de vertébrés terrestres et leur reproduction sexuée au sein d'insectes diptères haematophages : les moustiques. Ces arthropodes jouent également le rôle de vecteur et permettent la transmission de ces parasites. *Plasmodium* possède une répartition quasi-mondiale et infecte toutes les classes de vertébrés terrestres (Levine, 1988).

1-2 Un peu d'histoire

Le paludisme est une très ancienne maladie dont les premières traces écrites, provenant de documents Chinois, remontent à environ 2700 ans avant JC (Cox, 2010). Le terme malaria (privilegié par les Anglo-Saxons), quant à lui, apparaît pour la première fois en 1717. Il est proposé par un médecin italien Giovanni Maria Lancisi dans un ouvrage sur le paludisme « *De noxiis paludum effluviis eorumque remediis* ». Il vient de l'italien « *mal aria* » signifiant « mauvais air ». Le terme paludisme, vient du latin « *paludis* » qui signifie « marais ». En effet, la cause de cette maladie a longtemps été attribuée au fait de respirer l'air humide des marécages. Il faudra attendre plus d'un siècle et demi avant que cette maladie soit associée à un micro-organisme. En effet, ce n'est qu'en 1880 que le médecin français Charles Louis Alphonse Laveran observe pour la première fois des parasites à l'intérieur des globules rouges de patients souffrant de malaria. Ce parasite sera appelé en 1885 *Plasmodium*. En 1890, trois espèces de *Plasmodium* humain sont identifiées. Chacune de ces espèces présentent des caractéristiques différentes avec notamment des périodicités spécifiques. Ces trois espèces, *Haemamoeba vivax*, *Laverania malariae* et *Haemamoeba malariae* sont désormais connues sous le nom de *Plasmodium vivax*, *P. falciparum* et *P. malariae*. Dès le début des années 1880, de nombreux chercheurs ont identifié les moustiques comme jouant un rôle essentiel dans la diffusion de cette maladie. Cependant, ce n'est qu'en 1897, grâce à l'observation de l'intestin de moustiques nourris sur un patient souffrant de paludisme, que le médecin britannique Ronald Ross avance l'hypothèse selon

laquelle ce sont ces insectes qui transmettent directement le parasite. Utilisant la malaria aviaire (*P. relictum* – *Culex sp*), il validera cette théorie par l'intermédiaire d'infection expérimentale. Il décrira par la suite l'ensemble des grandes étapes du développement de *Plasmodium* au sein de son vecteur. En effet il montrera que les moustiques nourris sur des oiseaux infectés développent des « spores pigmentés » à la surface de leur intestin. R. Ross en conclut qu'en se nourrissant sur des hôtes porteurs de *Plasmodium*, ces insectes hématophages ingèrent des parasites mâles et femelles qui se fertilisent dans leur tube digestif et se développent en « spores » sur la surface de leur intestin. Puis il observe que des structures en forme de « tige » sont produites, envahissent les glandes salivaires et sont transmises à un nouvel hôte lors de la piqûre de ces moustiques. Ses résultats sont rendus publics en 1898, ce qui lui vaudra un prix Nobel de médecine en 1902 (Ross, 1902). En 1899, Ross démontre que le développement de *Plasmodium vivax*, *P. falciparum* et *P. malariae* dans les moustiques du genre *Anopheles* suit le même schéma que celui observé dans le cadre de la malaria aviaire.

Rapidement, de nouvelles espèces de *Plasmodium* infectant notamment des primates non humains sont découvertes (*P. cynomolgi*, *P. innui* and *P. pitheci*), cependant leur utilisation en laboratoire fut relativement compliquée notamment en raison de l'élevage de l'hôte vertébré. Le premier parasite de rongeurs (*P. berghei*) a, quant à lui, été identifié tardivement. En effet il n'a été isolé qu'en 1948. Ce parasite a rapidement été injecté à des souris, des rats et des hamsters afin d'être maintenu au laboratoire. Ne réussissant pas à élever le vecteur naturel de *P. berghei* (*Anopheles durenii*), les chercheurs ont alors utilisé une autre espèce de moustique provenant du continent asiatique, *Anopheles stephensi* (Vincke & Lips, 1948). Par la suite, trois autres espèces de malaria des rongeurs ont été identifiées et installées au laboratoire (*P. yoelii*, *P. vinckei* et *P. chabaudi*). Ces nouveaux modèles expérimentaux ont rapidement remplacé la malaria aviaire comme modèle expérimental de référence et ont notamment permis des avancées rapides dans le développement de traitements antipaludéens (Cox, 2010).

2- Cycle de vie

2-1 Chez l'hôte vertébré

Lorsqu'un moustique infecté pique un hôte vertébré, les structures en forme de « tige » observées par R. Ross, plus tard appelées **sporozoïtes**, sont injectées dans le sang de l'hôte en même temps que sa salive. Les sporozoïtes vont alors rapidement s'établir dans les

cellules hépatiques et réticuloendothéliales de l'hôte et entrer dans une première phase d'amplification (multiplication asexuée), connue sous le nom de **schizogonie**. Cette étape constitue la période de prépatence durant laquelle les parasites ne sont pas détectables dans le sang. Cependant, chez certaines espèces de *Plasmodium*, notamment *P. vivax* et *P. ovale*, certains sporozoïtes intra-hépatiques restent quiescents (**hypnozoïtes**). Suite à cette première phase d'amplification, les parasites entrent alors en *mérogonie exoerythrocytaire* et produisent une nouvelle génération de parasites, les **mérozoïtes**. Ces derniers sont libérés dans le flux sanguin et deviennent infectieux pour les globules rouges de l'hôte. Par la suite, les mérozoïtes entrent dans une seconde phase d'amplification: la *mérogonie erythrocytaire*. Les parasites peuvent alors soit s'engager dans un nouveau cycle de réplication en infectant de nouveau les globules rouges, ou alors se différencier en stades sexuels (**gamétocytes**). Ce stade est le seul infectieux pour les moustiques.

La **phase aiguë** de l'infection, caractérisée par un taux élevé de parasites dans la circulation sanguine est généralement atteinte 9 à 14 jours après le début de l'infection. L'éclatement des globules rouges, provoqué par le parasite, ou leur destruction par le système immunitaire, entraîne une sévère anémie qui est l'un des symptômes caractéristiques de la malaria (Menendez *et al.*, 2000). Parallèlement, l'activation du système immunitaire de l'hôte entraîne également une baisse de la parasitémie sanguine en seulement quelques jours. A la suite de ce pic infectieux, la parasitémie diminue et restera à des taux très faibles ce qui est caractéristique de la **phase chronique** de l'infection, qui peut durer de plusieurs semaines à plusieurs mois. Cette phase chronique peut être entrecoupée par des **phases de réactivation** du parasite causées soit par une recrudescence de *Plasmodium* (réactivation du stade erythrocytaire) ou bien par une **rechute** à partir des hypnozoïtes. Cette forme latente du parasite n'a été identifiée qu'au début des années 1980 et a permis d'expliquer pourquoi des individus qui apparaissaient guéris du paludisme rechutaient plusieurs années après que le parasite ait disparu de leur sang (Krotoski *et al.*, 1982; Meis *et al.*, 1983).

2-2 Chez le moustique

La transmission de la malaria repose sur le succès de développement de *Plasmodium* au sein de son vecteur, ce processus est connu sous le nom de **sporogonie**. La sporogonie implique une succession de stades de développement morphologiquement distincts du parasite (**Figure 1**) dont les localisations anatomiques au sein de l'insecte sont spécifiques

(intestin, hémolymphe et glande salivaire, Ghosh *et al.*, 2000). Il faut noter que le développement du parasite au sein des moustiques est très similaire pour toutes les espèces de *Plasmodium* (Baton & Ranford-Cartwright, 2005; Vaughan, 2007). Quelques différences ont toutefois été mises en évidence, notamment en ce qui concerne la durée de chaque phase de développement (Vaughan, 2007). Le cycle sporogonique est composé de trois grandes étapes. La première phase appelée **sporogonie précoce** est brève et commence lorsque le moustique ingère un repas de sang contenant les gamétocytes mâles et femelles. Le refroidissement du sang et la modification de pH dans l'intestin du moustique induisent la maturation des gamétocytes en gamètes matures (Sinden, 2002). Les microgamètes (gamètes mâles) fécondent rapidement les macrogamètes (gamètes femelles) à l'intérieur du repas de sang du moustique, pour former des zygotes, le seul stade diploïde du cycle de *Plasmodium* (Janse *et al.*, 1985; Sinden, 1985). Survient ensuite une étape de méiose sans division cellulaire aboutissant à la formation de cellules tétraploïdes, les ookinètes (McKenzie *et al.*, 2001). Les ookinètes sont mobiles et secrètent des substances lytiques leur permettant de traverser les deux barrières physiologiques constituées par la matrice péritrophique et l'épithélium stomacal du moustique. Une fois la lame basale de l'épithélium du mésentéron atteinte, chaque ookinète forme un **oocyste** inerte. Durant cette première phase de développement, qui peut durer deux jours, (Sinden, 1985; Billingsley & Hecker, 1991) l'abondance des parasites diminue fortement. En effet, dans le cas de la malaria humaine (*P. falciparum* / *Anopheles*) cette diminution est estimée à plus de 90% depuis l'ingestion des gamétocytes jusqu'à la formation des oocystes (Vaughan, 2007). Puis débute la seconde phase du développement, les oocystes subissent alors plusieurs cycles de mitoses et voient donc leurs taille s'accroître (**Figure 2**). Cette étape de multiplication est suivie d'une différenciation cellulaire aboutissant à la formation de plusieurs milliers de cellules filles : les **sporozoïtes** (Baton & Ranford-Cartwright, 2005).

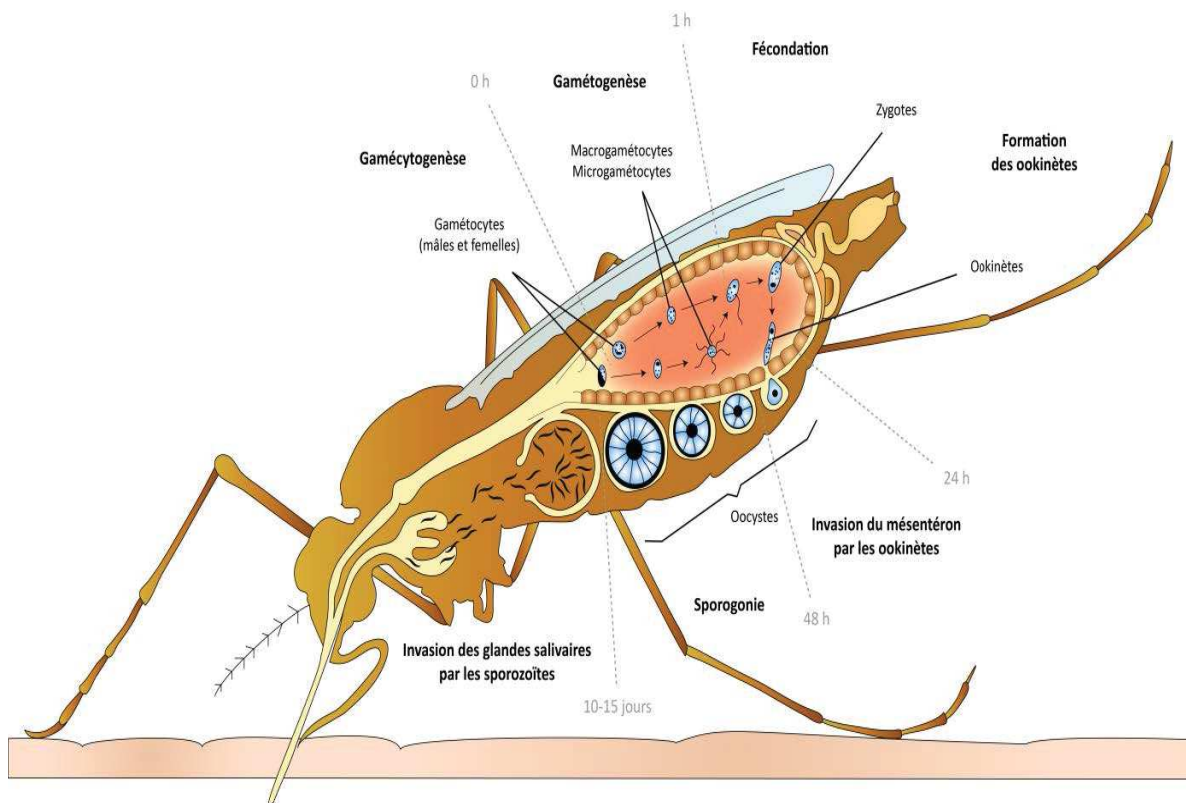


Figure 1. Développement de *Plasmodium* au sein du moustique. Les durées de développement données entre les différents stades correspondent à celles observées lors de l'infection du moustique *Cx. pipiens* par *P. relictum* (modifié par J. Denoyelle d'après Peter, 1992).

La phase finale appelée **sporogonie tardive** implique la libération des sporozoïtes dans l'hémolymphe du moustique et leur migration vers les glandes salivaires de l'insecte. Cette phase s'achève lorsque les sporozoïtes envahissent les glandes salivaires et que le moustique, suite à un nouveau repas de sang, injecte de manière concomitante sa salive et les sporozoïtes dans l'hôte vertébré, initiant alors une nouvelle infection.

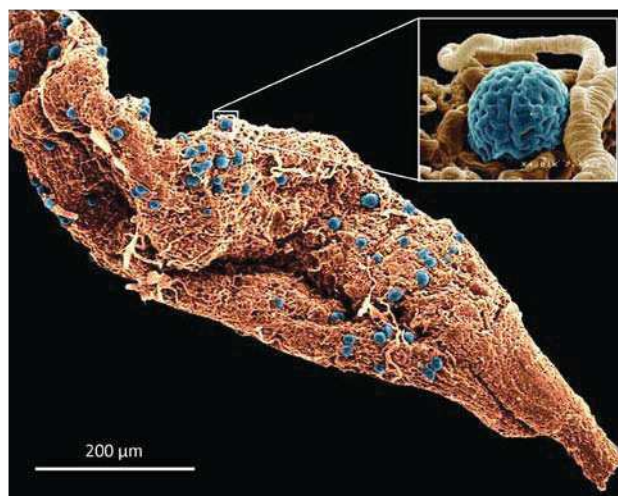


Figure 2 : Oocystes de *Plasmodium relictum* dans la paroi du mésentéron d'une femelle *Cx. pipiens*. Observation au microscope électronique à balayage (photo : A. Nicot, couleur : J Denoyelle).

Le temps nécessaire à *Plasmodium* pour achever son développement depuis l'ingestion des gamétocytes jusqu'à l'arrivée des sporozoites dans les glandes salivaires du moustique est appelé **période d'incubation intrinsèque** du parasite. La durée du cycle varie de 8 jours à 14 jours selon les espèces et les conditions environnementales (Noden *et al.*, 1995; Beier, 1998; Dawes *et al.*, 2009).

Il faut noter que l'infection des moustiques par *Plasmodium* n'est pas anodine. En effet, elle entraîne des coûts importants sur la fitness du vecteur. Les femelles infectées souffrent d'une forte réduction de leur fécondité (Hurd *et al.*, 1995; Hogg & Hurd, 1997; Ahmed *et al.*, 1999) pouvant atteindre les 40% (Vézilier *et al.*, 2012). D'un point de vue mécanistique, il a été mis en évidence que l'infection par *Plasmodium* induisait une perturbation de l'apport en protéine vitelline au niveau des œufs ainsi qu'une stimulation de l'apoptose de ces derniers (Hurd, 2003; Hurd *et al.*, 2006). Bien qu'aucune molécule originaire du parasite n'ait été identifiée permettant de parler de manipulation, il a été proposé que cette réduction de fécondité soit une stratégie adaptative du parasite dans le but d'augmenter la longévité des moustiques (Hurd, 2009). Une étude récente a en partie confirmé cette hypothèse en montrant que la réduction de fécondité observée chez *Cx. pipiens* infecté par *P. relictum* était associée à une augmentation de la longévité des femelles (Vézilier *et al.*, 2012). Alors que la fécondité n'a pas de conséquence directe pour le parasite, la survie des moustiques elle, est essentielle pour deux raisons. Tout d'abord, suite à un repas de sang infecté, les moustiques doivent vivre assez longtemps pour permettre au parasite de se développer et d'envahir les glandes salivaires de ces derniers ; dans un second temps plus la longévité des moustiques sera importante plus la probabilité qu'une femelle infectée se nourrisse sur un nouvel hôte augmentera.

3- Le moustique : Une simple seringue volante ?

3-1 Transmission de la malaria

Le moustique, à la fois vecteur et lieu de reproduction sexuée de *Plasmodium*, joue un rôle plus qu'essentiel dans la dynamique de transmission du paludisme. En effet le **taux reproductif de base** du parasite (R_0 , MacDonald, 1957), qui se définit comme le nombre d'infections secondaires produites par une seule infection dans une population hôte entièrement susceptible, est fortement lié à la **capacité vectorielle individuelle** (CI) des moustiques.

Le R_0 peut s'écrire de cette manière :

$$R_0 = \frac{mCI}{r}$$

Avec $1/r$ qui représente la durée prétendue de la maladie chez l'hôte vertébré, m le nombre de vecteurs adultes par hôte et CI la capacité vectorielle individuelle du moustique (Rivero *et al.*, 2010). CI se définit comme :

$$CI = \frac{a^2 b c e^{-gn}}{g}$$

Dans cette équation, g est le taux de mortalité du moustique ($1/g$ est la durée de vie du vecteur), a^2 est le nombre de piqûres du moustique (par moustique, par jour), n est la durée d'incubation du parasite à l'intérieur du vecteur ; et b et c sont respectivement la probabilité qu'un hôte devienne infecté suite à la piqûre d'un moustique infecté et la probabilité qu'un moustique s'infecte suite à une piqûre sur un hôte infecté. On observe alors que trois paramètres, très étroitement liés aux vecteurs, vont influencer directement la transmission de *Plasmodium*. Le premier paramètre qui paraît, de prime abord, le plus important concerne le **taux d'infection** des moustiques. En effet, si un parasite présent au sein d'un hôte vertébré est incapable d'infecter un moustique à la suite d'un repas de sang alors la transmission de ce dernier est immédiatement stoppée. Un second paramètre essentiel concerne la **longévité** des vecteurs (Ferguson & Read, 2002b; Vézilier *et al.*, 2012). Comme vu précédemment, ce trait d'histoire de vie va fortement impacter la transmission de *Plasmodium* (Vézilier *et al.*, 2012). Enfin le **comportement de piqûre** des femelles est également un paramètre essentiel dans la dynamique de transmission du parasite. Plus le moustique se nourrira sur un nombre important d'hôtes différents, plus le taux de transmission du parasite sera susceptible d'augmenter.

3-2 Sources de variations

Ces trois paramètres, essentiels pour la transmission de *Plasmodium*, vont être impactés par un ensemble de facteurs génétiques et environnementaux (biotiques et abiotiques) pouvant induire des variations quant à la dynamique de transmission du parasite.

3-2-1 Facteurs génétiques

Il y a plus de 80 ans, Clay Huff (Huff, 1929, 1931) identifie, grâce à des stratégies sélectives d'accouplement, l'importance des facteurs génétiques dans la résistance des moustiques face à une infection par *Plasmodium*. Ce résultat s'est vu confirmé par la suite par Collins et collaborateurs (1986), qui, en utilisant ce même procédé expérimental, ont créé une lignée de vecteurs résistante à plusieurs espèces de *Plasmodium*. Plus récemment, une résistance partielle à la malaria a également été observée au sein de différentes populations de moustiques sur le terrain (Niaré *et al.*, 2002). Grâce au développement des nouvelles techniques de biologie moléculaire, les facteurs génétiques responsables de cette résistance (totale ou partielle) ont pu être identifiés. En effet, le gène APL1 (pour *Anopheles Plasmodium*-responsive Leucine-rich repeat 1, Riehle *et al.*, 2006; Mitri *et al.*, 2009), localisé dans une petite région du chromosome 2L, ainsi que plusieurs gènes intervenant dans la réponse immunitaire humorale des moustiques (Harris *et al.*, 2010) ont été caractérisés et associés aux différents phénotypes de résistances observés.

L'immunité des vecteurs n'est pas le seul paramètre influencé par des facteurs génétiques. En effet, la variation observée au niveau de la durée de vie des femelles serait également largement sous tendue par une composante génétique (Lehmann *et al.*, 2006). A l'inverse, concernant le comportement de piqûre, actuellement, aucune étude ne s'est intéressée aux variations génomiques pouvant expliquer les différences observées quant à l'expression de ce comportement (i.e. nombre de piqûres par jour). Il est tout de même important de noter que des niveaux d'attractions variables pour un hôte donné ont été observés entre des populations génétiquement distinctes d'une même espèce de moustique (Kilpatrick *et al.*, 2007; Fernández-Grandon *et al.*, 2015). Cette variabilité d'attraction pourrait être engendrée par des modifications de l'expression de gènes codant pour des récepteurs olfactifs (McBride *et al.*, 2014).

3-2-2 Facteurs environnementaux

3-2-2-1 Facteurs biotiques

De nombreux facteurs biotiques vont pouvoir impacter de manière importante la probabilité de transmission de *Plasmodium* en agissant à la fois sur le taux d'infection des moustiques, sur leur comportement de piqûre et sur leur longévité. La densité globale de vecteurs au sein d'un environnement va également pouvoir être fortement influencée par différents paramètres modifiant alors le ratio de moustiques par hôte dans une population donnée.

Dès le stade larvaire, la compétition intra- et inter-spécifique va impacter le taux de survie des larves et donc la taille des populations de moustiques (Agnew *et al.*, 2000; Koenraadt & Takken, 2003; Braks *et al.*, 2004; Bédhomme *et al.*, 2005). A long terme, cette compétition va également avoir des répercussions importantes puisque des coûts sur la longévité des adultes (Reiskind & Lounibos, 2009) ainsi que sur leur successibilité à différents agents pathogènes ont été observés (Alto *et al.*, 2005, 2008; Bevins, 2007). Au sein des gîtes larvaires, les larves de moustiques sont également soumises à une forte pression de prédation (Ellis & Borden, 1970; Dida *et al.*, 2015). Les prédateurs aquatiques vont jouer un rôle important dans la régulation de la densité des larves (Tuno *et al.*, 2005; Shaalan *et al.*, 2007). Cependant, après l'émergence, peu d'informations sont disponibles concernant la pression de prédation exercée sur ces vecteurs (Medlock & Snow, 2008). Néanmoins, une source importante de mortalité des adultes a été clairement identifiée et est liée au comportement de défense de l'hôte vertébré lors du repas de sang, mais ce risque va varier de manière importante en fonction de l'hôte visé (Day & Edman, 1984; Kweka *et al.*, 2010). Au cours de leur vie, les moustiques vont également être exposés à un ensemble d'agents pathogènes plus ou moins virulents. Des bactéries ainsi que des virus et des champignons vont pouvoir influencer la probabilité de survie de ces insectes (Christensen, 1978; Scholte *et al.*, 2005; Costanzo *et al.*, 2014). Concernant le cas de la malaria, l'impact de *Plasmodium* sur la longévité des femelles reste une question non résolue. Dans la littérature, on observe qu'en fonction des espèces de moustiques ou de parasite utilisées, de l'intensité de l'infection ou des conditions expérimentales (température, humidité, accès au sucre) l'impact de *Plasmodium* sur la survie des femelles varie de manière importante (Ferguson & Read, 2002b).

L'infection par différents agents pathogènes, en plus d'impacter négativement la longévité des moustiques, va également influencer la probabilité d'infection des vecteurs par *Plasmodium*. Il a en effet été montré que l'infection par des microsporidies, des champignons, des filaires et des bactéries endosymbiotiques exogènes (i.e. *Wolbachia*) peut avoir un fort impact négatif sur le développement du parasite de la malaria (Blanford *et al.*, 2005; Bargielowski & Koella, 2009; Moreira *et al.*, 2009; Kambris *et al.*, 2010; Aliota *et al.*, 2011 mais voir Zélé *et al.*, 2014). D'autre part, la co-infection par plusieurs souches de *Plasmodium*, qu'elle soit concomitante ou consécutive, semble également avoir un impact négatif sur le développement du parasite (Paul *et al.*, 2002; Rodrigues *et al.*, 2010).

Certains de ces agents pathogènes sont également décrits comme responsables de modification du comportement de piqûre des moustiques. Il a été montré que des femelles infectées par un arbovirus (La Crosse virus) se nourrissent de manière plus fréquente mais avec des repas de sang plus petits que les femelles non infectées (Grimstad *et al.*, 1980). L'infection par *Plasmodium* affecte également le comportement de piqûre (Koella & Packer, 1996; Cator *et al.*, 2012), mais les effets vont varier drastiquement en fonction du stade de développement du parasite. Durant la phase pré-infectieuse (oocystes), les femelles sont moins attirées par les hôtes vertébrés (Cator *et al.*, 2012) et moins persistantes dans leur tentative d'alimentation (Anderson *et al.*, 1999). Cependant, une fois les glandes salivaires des femelles colonisées par les sporozoïtes elles sont davantage attirées par les hôtes vertébrés, sont plus insistantes lors de leur tentative d'alimentation (Anderson *et al.*, 1999) et se nourrissent sur davantage d'hôtes différents (Koella *et al.*, 1998). Ces résultats suggèrent fortement l'existence d'une manipulation du vecteur par *Plasmodium* (Schwartz & Koella, 2001; Cator *et al.*, 2012 mais voir Cator *et al.*, 2013). En effet, diminuer les tentatives d'alimentation des vecteurs lorsque le parasite n'a pas terminé son cycle sporogonique permettrait de limiter les risques de mortalité causés par le comportement de défense de l'hôte. Par la suite, une fois le parasite installé dans les glandes salivaires, l'augmentation du nombre de repas de sang sur plusieurs hôtes vertébrés permettrait d'augmenter le taux de transmission de *Plasmodium*.

Enfin, depuis quelques années les chercheurs s'intéressent également à la composition de la flore intestinale des moustiques et à l'impact qu'elle pourrait avoir sur leur susceptibilité à l'infection par *Plasmodium* (Dong *et al.*, 2009; Cirimotich *et al.*, 2011; Boissière *et al.*, 2012). Au cours de leur développement larvaire, les moustiques se nourrissent de micro-organismes et de matières organiques présentes dans les gîtes aquatiques. L'une des réserves nutritives largement exploitée par les larves est le biofilm, riche en bactéries, présent à la surface de l'eau (Wotton *et al.*, 1997). Certaines bactéries ingérées sont capables de coloniser leur intestin et sont ensuite transmises au stade adulte (Wang *et al.*, 2011; Boissière *et al.*, 2012). L'impact de ces bactéries intestinales sur la résistance des femelles au parasite de la malaria n'est actuellement pas bien établi. Alors que certaines études mettent en évidence un effet bénéfique du microbiome (Dong *et al.*, 2009), d'autres, au contraire, ont montré que l'élimination de la flore microbienne de l'eau des gîtes larvaires réduit la charge en oocystes chez les moustiques infectés par *Plasmodium* (Okech *et al.*, 2007).

3-2-2-2 Les facteurs abiotiques

Au cours de leur vie, les moustiques vont également être exposés à un ensemble de facteurs abiotiques liés d'une part au climat (i.e. température, humidité et photopériode) et d'autre part à l'acquisition des ressources nutritives. Ces différentes variables de l'environnement vont, tout comme les facteurs biotiques, fortement influencer les traits d'histoires de vie des moustiques.

Sachant que ces insectes hématophages sont ectothermes, il est bien évident que la température va être l'une des composantes environnementales la plus importante. En effet, elle va impacter la durée de développement des larves (Rueda *et al.*, 1990), le taux de survie des adultes (Rueda *et al.*, 1990; Ciota *et al.*, 2014), leur comportement (Ciota *et al.*, 2014), mais également leur système immunitaire (Suwanchaichinda & Paskewitz, 1998; Murdock *et al.*, 2012) et donc potentiellement la susceptibilité des femelles à *Plasmodium*. L'expression optimale de ces paramètres va être observée au sein d'une gamme de température donnée qui va varier d'une espèce de moustiques à une autre. Ce facteur environnemental, en plus d'impacter le moustique, semble également avoir un effet sur *Plasmodium*. L'augmentation de la température accélère la vitesse de développement du parasite au sein des moustiques mais cela jusqu'à atteindre un certain seuil au-delà duquel sa survie décroît nettement (Mitzmain, 1917; Noden *et al.*, 1995; Okech *et al.*, 2004; Mordecai *et al.*, 2013). Concernant les autres facteurs environnementaux, il semble qu'une valeur seuil d'humidité relative (variable en fonction des espèces) doit être atteinte pour permettre une longévité maximale, en dessous de cette valeur la survie des femelles décroît (Gaaboub *et al.*, 1971; Bayoh, 2001; Costa *et al.*, 2010). La photopériode semble également impacter la durée de vie des femelles avec une meilleure survie lorsque les journées sont courtes (L:10 D:14, Costanzo *et al.*, 2015).

Concernant l'alimentation, que ce soit au stade larvaire ou adulte, ce paramètre va avoir un impact important sur la probabilité de transmission de *Plasmodium*. En effet, il va pouvoir agir à la fois sur les traits d'histoires de vie des moustiques, mais également potentiellement directement sur le parasite. Dans un premier temps il a clairement été mis en évidence que l'alimentation des larves avait un impact important sur la durée de vie des moustiques : Les femelles issues de larves bien nourries ont une durée de vie plus longue que les femelles provenant de larves ayant grandi dans des conditions moins favorables (Araujo *et al.*, 2012; Yamany & Adham, 2014). Au stade adulte l'apport en sucre est essentiel à la survie des moustiques (Day *et al.*, 1994; Foster, 1995; Joy *et al.*, 2010; Xue *et al.*, 2010;

Yamany & Adham, 2014). Cependant, la longévité des femelles serait maximisée par la prise d'un repas de sang (Gary & Foster, 2001). La quantité et la qualité des ressources alimentaires acquises au cours de la vie de ces insectes va également avoir des effets importants sur leur réponse immunitaire et donc sur leur susceptibilité à *Plasmodium* (Suwanchaichinda & Paskewitz, 1998; Ferguson & Read, 2002a; Koella & Sørensen, 2002; Lambrechts *et al.*, 2006) ainsi que sur leur comportement (Araujo *et al.*, 2012). Concernant ce dernier paramètre, il a été montré qu'il existait une corrélation positive entre la quantité de nourriture disponible dans le milieu larvaire et la fréquence de piqûre des femelles (Araujo *et al.*, 2012). Ceci pourrait s'expliquer par le fait que les larves nourries avec des quantités importantes de nourriture produisent des individus plus grands (Nasci & Mitchell, 1994; Araujo *et al.*, 2012) ; or une corrélation positive entre taille des femelles et fréquence de piqûre a été observée (Klowden & Lea, 1978; Xue *et al.*, 1995). Il faut tout de même noter que ce résultat est controversé. En effet certaines études montrent que les larves peu nourries produisent des femelles plus petites qui vont alors devoir piquer davantage dans le but de « rattraper leur retard » du fait de leur faible réserve énergétique (Nasci, 1986; Farjana & Tuno, 2013).

Enfin, l'alimentation des moustiques va également pouvoir avoir un effet direct sur *Plasmodium*. Le sucre par exemple, primordial pour la survie des moustiques, est également essentiel au développement du parasite (Scott & Takken, 2012). Des études ont d'ailleurs montré que les femelles infectées par le parasite de la malaria consommaient davantage de sucre que les femelles non infectées (Rivero & Ferguson, 2003; Scott & Takken, 2012; Nyasembe *et al.*, 2014).

Il existe donc de nombreux paramètres pouvant influencer à la fois la longévité, le comportement et le taux d'infection des moustiques. La compétence vectorielle individuelle des vecteurs va donc varier d'un individu à l'autre en fonction de l'environnement, des conditions de croissance des individus mais également de leur fond génétique. Ceci va alors pouvoir influencer la dynamique de transmission de *Plasmodium*.

4- Sources d'hétérogénéité d'infection chez le vecteur : sujet de thèse

Au cours de ces trois années de thèse, je me suis principalement intéressé à l'un des paramètres intervenant dans la dynamique d'infection de *Plasmodium* : la probabilité

d'infection des vecteurs. En effet j'ai étudié l'impact de différents paramètres, peu pris en compte jusqu'à présent, pouvant induire une réelle hétérogénéité au niveau de l'infection des moustiques.

Avant de m'intéresser aux sources de variations de l'infection, j'ai tout d'abord débuté ma thèse en essayant de caractériser la **dynamique d'infection de *Plasmodium*** au sein des moustiques (**Chapitre 1**). En effet, bien que cela puisse paraître surprenant, peu de données sont disponibles quant à la dynamique d'infection des vecteurs par ce parasite (Dawes *et al.*, 2009). Cette première partie m'a notamment permis d'identifier certains moments clés du développement de *Plasmodium* comme la période de temps nécessaire pour atteindre le pic d'oocystes ou encore le nombre de jours séparant la prise du repas de sang infecté et l'apparition des premiers sporozoïtes. Parallèlement, j'ai également cherché à déterminer si l'intensité d'infection de l'hôte vertébré pouvait avoir un impact sur cette dynamique d'infection.

La première source potentielle d'hétérogénéité d'infection à laquelle je me suis intéressé concerne **l'effet de l'âge** des moustiques (**Chapitre 2**). En effet, une immunoscnescence a été observée chez de nombreuses espèces d'insectes (Li *et al.*, 1992; Adamo *et al.*, 2001; Doums *et al.*, 2002; Schwartz & Koella, 2002; Wilson-Rich *et al.*, 2008; Prasai & Karlsson, 2012; Cornet *et al.*, 2013a). Ce résultat laisse alors penser que les insectes plus âgés devraient être plus sensibles à une infection que leurs homologues plus jeunes. Cette prédiction est cependant contestée par de nombreuses études montrant que l'âge semble impacter positivement la résistance des individus face à de nombreux agents pathogènes (Walshe *et al.*, 2011; Chaimanee *et al.*, 2013; Khan & Prasad, 2013; Ariani *et al.*, 2015). Etudier l'effet de l'âge des moustiques sur leur successibilité à une infection par *Plasmodium* est particulièrement pertinent étant donné que, comme vue précédemment, la longévité des vecteurs joue un rôle essentiel dans la dynamique de transmission du paludisme (MacDonald, 1957). Cependant, très peu d'études, qu'elles soient expérimentales ou théoriques, se sont intéressées à ce sujet (Terzian *et al.*, 1956; Reiner *et al.*, 2013).

Par la suite je me suis intéressé à une source d'hétérogénéité d'infection non plus située à l'échelle de la vie d'un individu mais à l'échelle de la génération. Alors que le système immunitaire des invertébrés a très longtemps été caractérisé comme non adaptatif (Hoffmann *et al.*, 1996), depuis une quinzaine d'années des preuves évidentes de l'existence d'un amorçage immunitaire se sont accumulées (Schmid-Hempel, 2005; Best *et al.*, 2013). Cette modification de la réponse immunitaire en réponse aux expériences passées semble

persister durant toute la vie des individus (Jacot *et al.*, 2005; Thomas & Rudolf, 2010) mais semble également avoir des effets sur la génération suivante. En effet, un nombre croissant d'études a mis en évidence des transferts parentaux d'immunité (Moret & Schmid-Hempel, 2001; Little *et al.*, 2003; Moret, 2006; Freitak *et al.*, 2009; Zanchi *et al.*, 2011) pouvant aboutir à une meilleure résistance des descendants suite à une infection par un agent pathogène (Roth *et al.*, 2010; Eggert *et al.*, 2014). Les **effets transgénérationnels d'une infection** peuvent donc avoir des conséquences importantes sur la dynamique de transmission des parasites. Il est alors particulièrement pertinent d'étudier ces effets dans le cadre des maladies à transmission vectorielle. Pour ma part, je me suis intéressé aux effets de l'infection des moustiques par *Plasmodium* sur la fitness et la résistance de leurs descendants (**Chapitre 3**). En menant cette étude je me suis alors rendu compte que, chez les invertébrés, nos connaissances sur les effets transgénérationnels d'une infection, et notamment sur le transfert de protection, étaient limitées à un nombre restreint d'espèces. Parallèlement, j'ai pu constater que les conditions nécessaires à l'évolution et au maintien de ce mécanisme n'avaient que très peu été étudiées. Après avoir réalisé une revue de la littérature j'ai alors cherché à déterminer si, comme chez les vertébrés, la présence ou l'absence de protection transgénérationnelle pouvait être gouvernée par différents facteurs écologiques (**Chapitre 4**).

Après avoir étudié des sources d'hétérogénéité d'infection observées à l'échelle de la vie d'un individu (i.e. âge), puis à l'échelle de la génération (i.e. effets maternels de l'infection), je me suis intéressé au **rôle de la variation génétique sur l'hétérogénéité d'infection des vecteurs**. Comme nous l'avons vu précédemment les traits d'histoire de vie des moustiques sont fortement impactés par de nombreux paramètres environnementaux. Cependant une part non négligeable de la variation phénotypique observée au niveau de ces traits est sous-tendue par une base génétique (Sorci *et al.*, 1997; Mpho *et al.*, 2002; Schneider *et al.*, 2011). A partir de moustiques capturés sur le terrain, j'ai alors cherché à déterminer la part de variance, observée au niveau de la susceptibilité des femelles à *Plasmodium*, expliquée par de la génétique additive (**Chapitre 5**). Je me suis également intéressé à la variation phénotypique observée au niveau de différents traits d'histoire de vie des vecteurs intervenant notamment dans la dynamique de transmission de *Plasmodium*. A partir de ces données de génétique quantitative, j'ai cherché à approximer le taux d'héritabilité de ces différents paramètres, ce qui m'a notamment permis d'estimer leur réponse potentielle à la sélection (Bosio *et al.*, 1998).

Au cours des cinq premiers chapitres de la thèse je me suis principalement focalisé sur différents paramètres propres aux vecteurs (âge, transfert d'immunité). Cependant, la prévalence et l'intensité d'infection au sein des populations d'hôtes vertébrés vont également pouvoir impacter l'hétérogénéité d'infection des moustiques. Alors que dans la grande majorité des études expérimentales les protocoles mis en place, ou les analyses statistiques réalisées, permettent de contrôler la part de variation induite par cet **effet hôte vertébré** (Bonnet *et al.*, 2000; Ferguson & Read, 2002b), j'ai, durant le **Chapitre 6 et 7**, travaillé sur cette source potentielle d'hétérogénéité d'infection des vecteurs.

A partir des données collectées par l'équipe d'Ana Rivero et Sylvain Gandon depuis plus de cinq ans j'ai pu identifier de nombreuses relations entre différents paramètres liés aux infections des hôtes vertébrés, telles que la parasitémie, la gamétocytémie, la morbidité de l'hôte et le taux de transmission au vecteur (**Chapitre 6**). Parallèlement, à partir de ce jeu de données j'ai également pu m'intéresser à l'impact du maintien de *Plasmodium* au laboratoire sur **l'évolution** de sa virulence à la fois envers l'hôte vertébré mais également envers le vecteur (**Chapitre 6, Annexe 1**).

Enfin, bien qu'il existe une hétérogénéité d'infection entre hôtes vertébrés, elle existe également au sein de ces derniers. En effet, la charge en parasites au sein de l'hôte vertébré va pouvoir varier dans le temps (Hawking *et al.*, 1972; Garnham & Powers, 1974; Gautret & Motard, 1999) et dans l'espace (Smalley *et al.*, 1981 mais voir Pichon *et al.*, 2000). Dans cette dernière partie je me suis donc penché sur la dynamique d'infection du parasite au sein de l'hôte vertébré. J'ai également essayé de déterminer si une **rythmicité journalière**, quant à la multiplication de *Plasmodium*, pouvait être observée et si elle pouvait avoir un impact sur la transmission du parasite (**Chapitre 7**).

Afin d'étudier ces différents points, j'ai utilisé un modèle biologique offrant de nombreux avantages et revenant sur le devant de la scène depuis une dizaine d'années, la malaria aviaire.

5- La malaria aviaire

La malaria aviaire est le plus ancien système expérimental non humain utilisé pour étudier la biologie et la transmission de *Plasmodium* (Ross, 1902). Comme vu précédemment, tout démarre avec les travaux de Ronald Ross à la fin du 19^{ème} siècle. Par la

suite, durant près de 50 ans, la malaria aviaire apparaît comme le modèle expérimental de choix pour étudier le paludisme. Il a notamment permis d'élucider les principaux aspects de la biologie de la transmission du parasite (Huff & Bloom, 1935; Raffaella & Marchiafava, 1944), ainsi que le développement des premiers médicaments antipaludéens (Marshall, 1942) et vaccins (Russell & Mohan, 1942). Cependant, la découverte en 1948 de *Plasmodium berghei*, parasite responsable de la malaria des rongeurs, ainsi que l'infection réussie de singes par des parasites humains en 1966 (Young *et al.*, 1966) marquent le déclin de ce modèle expérimental (Pigeault *et al.*, 2015, **Figure3**). Néanmoins, au cours des quinze dernières années, une augmentation significative du nombre d'études utilisant la malaria aviaire a été constatée. Ce regain d'intérêt a notamment été impulsé par le dépistage sanguin de routine des oiseaux sauvages à travers le monde. Des études moléculaires ont mis en évidence un niveau de diversité des parasites inattendu avec plus de 600 séquences mitochondriales de cytochrome b décrites (base de données Malavi, Bensch *et al.*, 2009). De plus la malaria aviaire est beaucoup plus fréquente (la prévalence pouvant atteindre 80% dans certaines régions, Bichet *et al.*, 2014, Pigeault *et al.* données non publiées) et généralisée que tout autre paludisme vertébré. Il faut tout de même noter que, malgré une augmentation exponentielle des informations acquises sur les interactions *Plasmodium*/oiseaux, très peu d'informations sont disponibles concernant les vecteurs (Ventim *et al.*, 2012; Zélé *et al.*, 2014b). Plusieurs dizaines d'espèces vectrices sont actuellement inscrites dans la base de données Malavi, bien que pour *Plasmodium relictum*, les moustiques du genre *Culex* sont, de loin, les vecteurs les plus communs (Ventim *et al.*, 2012). En plus des travaux réalisés sur le terrain, la malaria aviaire a récemment fait son retour en laboratoire. En effet, depuis quelques années, cette ancien modèle expérimental est de nouveau utilisé pour étudier l'écologie évolutive de la malaria (Vézilier *et al.*, 2010, 2012; Cornet *et al.*, 2013b; Cornet & Sorci, 2014; Zélé *et al.*, 2014a). Ce modèle présente la possibilité attrayante d'éviter les deux principaux inconvénients liés à l'utilisation de la malaria des rongeurs : l'utilisation de souches qui ont été conservées au laboratoire depuis les années 50 et le fait que leurs vecteurs naturels ne puissent pas être conservés au laboratoire, ce qui oblige l'utilisation de combinaisons moustiques/parasites artificiels.

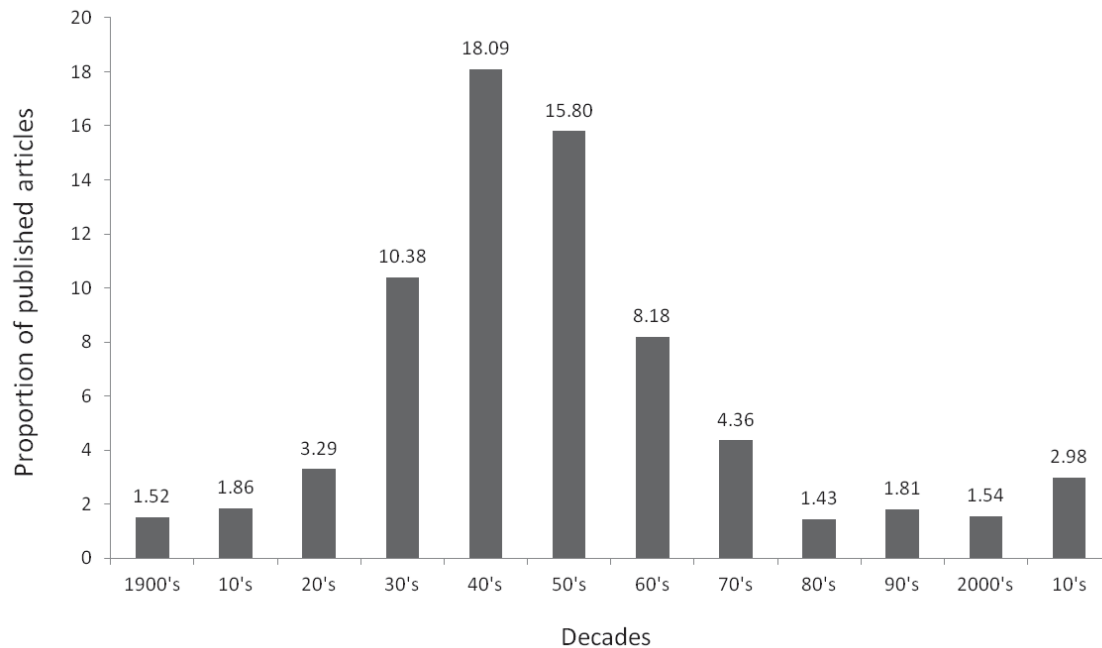


Figure 3. Proportion du nombre total d'articles sur le paludisme publiés entre 1900 et 2014 qui utilisent la malaria aviaire. Recherche effectuée sur le site Web of Science en utilisant les termes de recherche "*Plasmodium* ou malaria" (pour le nombre totaux d'articles traitant du paludisme) et "((*plasmodium* or malaria*) et (avian or bird*)) ou (*Plasmodium* et (*gallinaceum* ou *relictum* ou *elongatum* ou *cathemerium* ou *lophurae* ou *fallax*))" pour les articles traitant de la malaria aviaire.

6- Système expérimental : *Plasmodium relictum* - *Culex pipiens*

J'ai utilisé comme système expérimental le complexe *Plasmodium relictum* (SGS1) – *Culex pipiens*. *Plasmodium relictum* est une morpho-espèce décrite chez plus de 300 espèces d'oiseaux en Eurasie, Afrique et sur tout le continent Américain (Valkiunas, 2004). Cette morpho-espèce est divisée en 9 différentes lignées sur la base de leurs séquences pour le gène mitochondrial du *cytochrome b*. Plusieurs études suggèrent que ces lignées sont des entités reproductivement isolées et, de ce fait, peuvent être considérées comme des espèces différentes (Schrenzel *et al.*, 2003; Bensch *et al.*, 2004, 2009; Beadell *et al.*, 2006). La lignée SGS1 a été trouvée chez plus de 40 espèces d'oiseaux différentes, au sein de 15 familles dont la plupart appartiennent à l'ordre des Passeriformes. SGS1 est de loin la lignée de malaria aviaire la plus commune en Europe. En moyenne elle représente environ 20% des infections d'hémosporidiens diagnostiquées chez les passereaux sauvages (Dimitrov *et al.*, 2010). Cependant, dans certaines populations connues pour être fortement touchées par la malaria aviaire (Sainte marie de la mer, France, prévalence de 70%) le taux d'infection par SGS1 est de plus de 80% (Pigeault et al données non publiés). Concernant l'infection chez le moustique *Culex pipiens*, le principal vecteur de *Plasmodium relictum* en Europe, il a été montré que dans le sud de la France près de 40% des femelles infectées le sont par la lignée

SGS1 (Zélé *et al.*, 2014b). Au cours de mes différents travaux, j'ai utilisé deux lignées de *Plasmodium relictum* SGS1. La première lignée, isolée en 2009 par G. Sorci (Université de Bourgogne) à partir d'un moineau sauvage (*Passer domesticus*), a été utilisée pour les expériences présentées dans **les Chapitres 1 à 6**. La deuxième lignée a été isolée en avril 2015 à partir d'un moineau sauvage (*Passer domesticus*) capturé sur le site de Pont de Gau (France) et a été utilisée pour le **Chapitre 7**. Ces isolats de parasites ont été transférés sur des canaris naïfs (*Serinus canaria*) par injection intrapéritonéale de sang infecté. Ces deux lignées ont ensuite été maintenues dans notre animalerie par passages réguliers de sang entre canaris infectés et naïfs. Les passages en série sont réalisés tous les 15 à 20 jours, ce qui coïncide avec la phase aigue d'infection (Vézilier *et al.*, 2010). Toutes les 30 semaines environ, un cycle complet du parasite est réalisé. Des moustiques prennent un repas de sang sur un canari infecté puis, 15 jours plus tard, infectent un canari naïf. Pour toutes les études réalisées au cours de ma thèse les moustiques *Cx. pipiens*, qu'ils proviennent de la lignée de laboratoire (Slab) ou bien du terrain (Ssau), ont été infectés à la suite d'un repas sanguin durant la phase aigue de l'infection chez les canaris. Le protocole de piqûre des moustiques sur les canaris que nous avons développé permet de nourrir jusqu'à 180 femelles sur le même oiseau. Suite à la prise d'un repas de sang infecté 60 à 90% des femelles développent une infection par *Plasmodium*. Cette infection est déterminée par un comptage d'oocystes dans le mésentéron des moustiques 7-9 jours après le repas de sang.

Chapitre 1 :

Dynamique d'infection de *Plasmodium* chez son vecteur

Chapitre 1 : Dynamique d'infection de *Plasmodium* chez son vecteur

Short note soumise dans "Transactions of the Royal Society of Tropical Medicine and Hygiene"

Contexte

- Tandis que la dynamique d'infection de *Plasmodium* au sein de l'hôte vertébré est relativement bien documentée, peu d'informations sont disponibles quant à la dynamique d'infection chez les vecteurs.
- En utilisant comme modèle biologique la malaria aviaire nous avons suivi pendant 26 jours la production des oocystes et des sporozoïtes au sein de moustiques nourris sur des hôtes infectés.

Résultats

- Les premiers sporozoïtes sont observés 8 jours seulement après la prise du repas de sang.
- La parasitémie de l'hôte vertébré semble impacter fortement la charge en oocystes mais pas la densité de sporozoïtes finale observée au sein des moustiques âgés.

Conclusion

- Alors que la période d'incubation intrinsèque de *Plasmodium* au sein des moustiques est généralement décrite comme durant entre 12-14 jours, il semblerait que dans le cas de la malaria aviaire cette période soit plus courte.
- Vu que la densité en sporozoïtes, au sein des moustiques âgés de plus de 20 jours, est similaire et ne semble pas liée à la densité d'oocyste il est alors possible de se questionner sur l'impact de l'intensité d'infection des vecteurs quant à la dynamique de transmission de *Plasmodium*.

Temporal dynamics of *Plasmodium* development in mosquitoes

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Background: Little attention has been given to the dynamics of *Plasmodium* within the vector. Yet, fluctuating parasite densities in the vector can have consequences for estimating the efficacy of novel vector-based control strategies or to estimates epidemiological parameters in the field.

Methods: The temporal dynamics of oocysts and sporozoites in mosquitoes were closely monitoring during 26 days.

Results: Although birds' parasitaemia was quite different, temporal dynamics of *Plasmodium* inside the mosquito were consistent across host. We have observed that the infections in mosquitoes were highly dynamic.

Conclusions: A single snapshot in time of the prevalence and/or intensity of the mosquito infection may not provide a good approximation of what it is in fact a temporally complex process.

Keywords: Plasmodium, Transmission, Temporal dynamics, Sporogony

Introduction

There is a growing interest on the role of within-host dynamics on the evolution and epidemiology of malaria. Most studies are focused on the dynamics and synchronicity of the parasite blood stages, and comparatively little attention has been given to the dynamics of the parasite within the vector. Fluctuating parasite densities in the vector can have consequences for the accurate estimate of epidemiologically relevant parameters in the field as well as for estimating the efficacy of novel vector-based control strategies in the laboratory.¹

Despite this, few studies of the temporal dynamics of *Plasmodium* parasites in mosquitoes exist that explicitly include sporozoites.² Here we use an avian malaria system to closely monitor the temporal dynamics of both oocysts and sporozoites of malaria parasites in a natural mosquito-*Plasmodium* combination. Our results reveal a temporally dynamic pattern both for the oocysts and sporozoites. We discuss how this pattern may originate and what consequences it may have for the interpretation of malaria-mosquito experiments based on a single snapshot in time of *Plasmodium* intensity or prevalence.

Methods

Experiments were carried out using *Plasmodium relictum* and its natural vector the mosquito *Culex pipiens* (Pigeault et al 2015). Birds (*Serinus canaria*) were infected using standard protocols (Pigeault et al 2015). Ten days later, at the peak of the acute infection, each bird was placed overnight in a separate cage with 180 7-day old female mosquitoes.³ Blood-fed mosquitoes were kept with 10% *ad libitum* sugar at standard temperature (25°) and humidity (70%) conditions. Every two days, starting on day 4 and finishing on day 26 post-blood meal (day 22 for Bird 3 due to high mosquito mortality), twelve mosquitoes were haphazardly sampled from each cage. Each mosquito was dissected to count the number of oocysts in the midgut, and its head-thorax was preserved at -20°C for the quantification of the transmissible sporozoites.

Developing oocysts were counted under the microscope. Sporozoites were quantified using real-time quantitative PCR as the ratio of the parasite's *cytb* gene relative to the mosquito's *ace-2* gene.³

Results and Discussion

The temporal dynamics of *Plasmodium* inside the mosquito were consistent across birds. Oocysts first become visible in the midgut on days 4-6 after the blood meal and were thereafter continuously present in the mosquito midgut for the next two weeks albeit at low levels (**Figure 1**). The peak oocyst burdens were reached on days 8-10. Thereafter a rapid decrease in the number of oocysts is observed so that by day 12, only between 2-20% of the peak oocyst burdens remain. By day 22, however, mosquitoes still contained an average of 5.17 ± 2.91 , 21.67 ± 5.31 , and 8.0 ± 4.37 oocysts.

The first sporozoites appear on the head-thorax homogenate on days 8-10, only 2-4 days after the first oocysts. Sporozoites reach their peak on day 12, except for the bird with the lowest parasitaemia (Bird 3) where the peak is reached much later (day 16). There onwards the number of sporozoites decreases steadily, but sporozoites are still detectable, albeit at low numbers, 22-26 days after the initial infection (Figure 1).

Hillyer et al (2007) showed that *P. berghei* sporozoites injected directly into the haemocoel of *An. gambiae* mosquitoes reached a peak in the salivary glands within the first 24 hours and remained constant thereafter for the next seven days. In contrast, sporozoites in the head-thorax fraction were rapidly and entirely degraded by an, as yet undescribed, immune mechanism.⁴ The drastic reduction in sporozoites we observe from peak day onwards suggests—that the large majority of these sporozoites may have been in the haemocoel, rather than in the salivary glands. The quantification of sporozoites in whole head-thorax extracts is standard practice in malaria,⁵ because it is less labour and skill intensive than dissecting the salivary glands, particularly when large numbers of mosquitoes are needed.⁵

In conclusion, our results show that malaria infections in mosquitoes are highly dynamic. We therefore contend that a single snapshot in time of the prevalence and/or intensity of the infection 6-7 days (oocyst) or 14-15 days (sporozoite) post-infection, the gold standard in mosquito-malaria research, may not provide a good approximation of what it is in fact a temporally complex process. Here all mosquitoes were kept under the same experimental conditions, but it is worth considering that the parasite dynamics in the mosquitoes may shift in time in response to a given experimental treatment or intervention, further adding to the risk of wrongly interpreting results based on a single time point. Future work should therefore consider spreading mosquito dissections over several consecutive days in order to avoid these pitfalls.

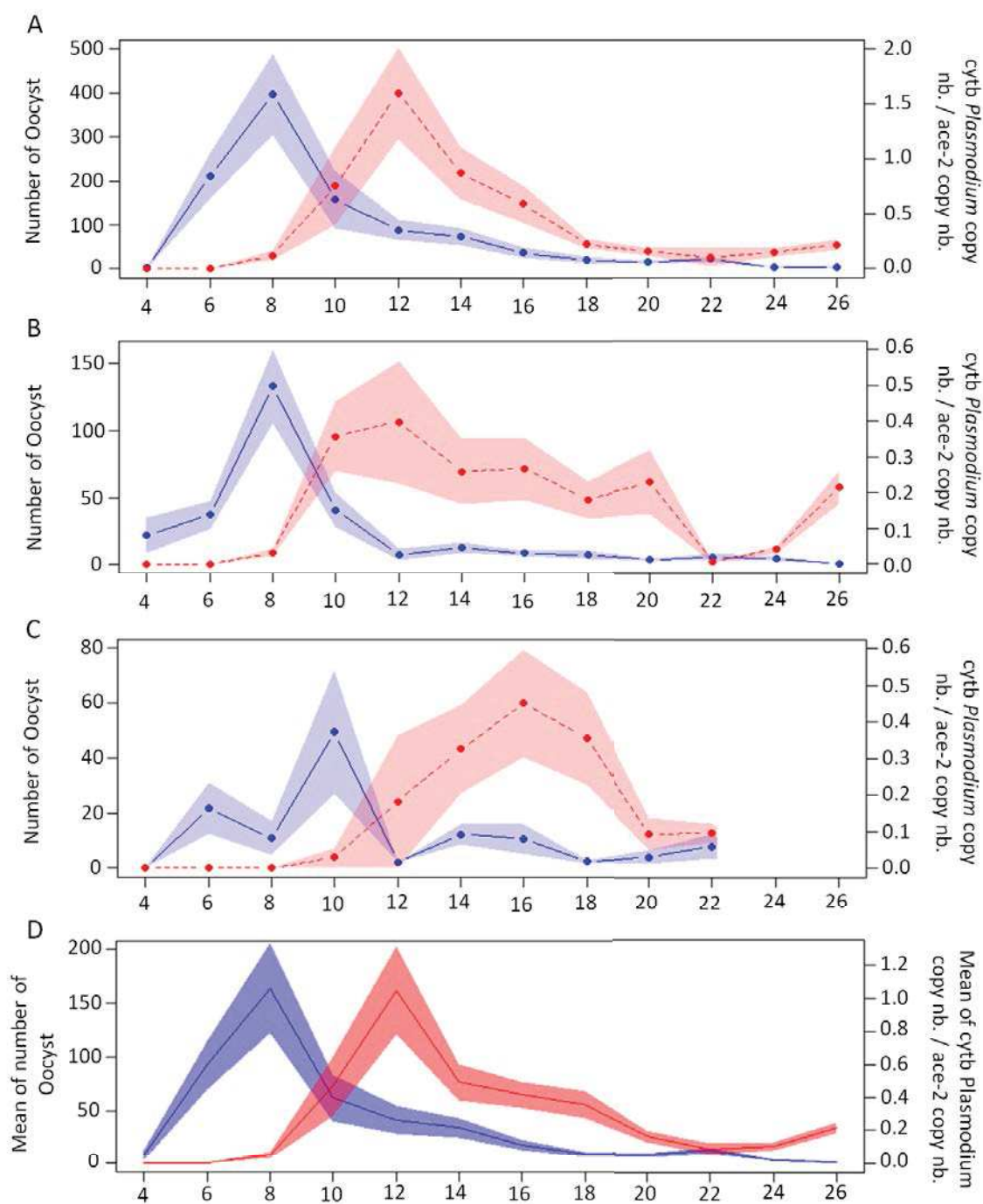


Figure 1: Mean normalised oocyst (blue) and sporozoite (red) counts at each dissection day, for each of the three birds (A: Bird 1, B: Bird 2, C: Bird 3) and mean across birds (D). Shadows represent standard error. Parasitaemias and gametocytaemias for each of the birds are as follows: Bird 1: 5.12% and 1%, Bird 2: 7.33% and 0.4%, and Bird 3: 4.48% and 0.3%, respectively.

Authors' contributions: RP, AN, SG and AR conceived and designed the experiment. RP and AN performed the experiment. RP analysed the data. RP, SG and AR wrote the paper.

Conflicts of interest: None declared.

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Chapitre 2 :

Âge des moustiques et infection par
Plasmodium

Chapitre 2 : Âge des moustiques et infection par *Plasmodium*

Article publié dans « malaria journal »

Contexte

- Une immunosenescence est observée chez de nombreuses espèces d'invertébrés.
- Cependant, dans de nombreux cas il a été mis en évidence une relation positive entre âge des organismes et résistance à un agent pathogène.
- Etudier les effets de l'âge sur la susceptibilité des moustiques à une infection est particulièrement pertinent étant donné leur rôle de vecteur dans la transmission de nombreuses maladies.
- Nous nous sommes intéressés à l'impact du vieillissement sur (1) l'immunité des moustiques et (2) sur leur susceptibilité à *Plasmodium*. Nous avons également essayé de déterminer si (3) la prise d'un premier repas de sang avant le repas infecté pouvait influencer le taux d'infection des vecteurs.

Résultat

- Une immunosenescence est observée chez les moustiques.
- Cependant l'infection des femelles par *Plasmodium* décroît drastiquement avec l'âge.
- La prise d'un premier repas de sang avant le repas infecté annule la protection conférée par l'âge.

Conclusion

- Nos résultats suggèrent que des altérations physiques et physiologiques, associées au vieillissement des moustiques, pourraient être davantage responsables de l'augmentation de résistance à *Plasmodium* que leur système immunitaire.
- L'inversion de l'effet protecteur de l'âge, suite à la prise d'un premier repas de sang non infecté, laisse penser qu'en milieu naturel le vieillissement des moustiques est peu susceptible d'avoir un impact sur la transmission de *Plasmodium*.

RESEARCH

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Mosquito age and avian malaria infection

Romain Pigeault^{1*}, Antoine Nicot^{1,2}, Sylvain Gandon² and Ana Rivero¹

Abstract

Background: The immune system of many insects wanes dramatically with age, leading to the general prediction that older insects should be more susceptible to infection than their younger counterparts. This prediction is however challenged by numerous studies showing that older insects are more resistant to a range of pathogens. The effect of age on susceptibility to infections is particularly relevant for mosquitoes given their role as vectors of malaria and other diseases. Despite this, the effect of mosquito age on *Plasmodium* susceptibility has been rarely explored, either experimentally or theoretically.

Methods: Experiments were carried out using the avian malaria parasite *Plasmodium relictum* and its natural vector in the field, the mosquito *Culex pipiens*. Both innate immune responses (number and type of circulating haemocytes) and *Plasmodium* susceptibility (prevalence and burden) were quantified in seven- and 17-day old females. Whether immunity or *Plasmodium* susceptibility are modulated by the previous blood feeding history of the mosquito was also investigated. To ensure repeatability, two different experimental blocks were carried out several weeks apart.

Results: Haemocyte numbers decrease drastically as the mosquitoes age. Despite this, older mosquitoes are significantly more resistant to a *Plasmodium* infection than their younger counterparts. Crucially, however, the age effect is entirely reversed when old mosquitoes have taken one previous non-infected blood meal.

Conclusions: The results agree with previous studies showing that older insects are often more resistant to infections than younger ones. These results suggest that structural and functional alterations in mosquito physiology with age may be more important than immunity in determining the probability of a *Plasmodium* infection in old mosquitoes. Possible explanations for why the effect is reversed in blood-fed mosquitoes are discussed. The reversal of the age effect in blood fed mosquitoes implies that age is unlikely to have a significant impact on mosquito susceptibility in the field.

Keywords: Immune senescence, Ecological immunity, *Culex*, Avian malaria, Haemocyte

Background

Ever since Manson [1] and Ross [2] demonstrated over a century ago that some of the key human diseases are not transmitted through contaminated air or water, but through the bite of infected mosquitoes, a great deal of effort has been invested in identifying the genetic and environmental determinants of mosquito competence for these parasites. Mosquito immunity has taken centre stage in these efforts. Laboratory gene silencing studies have provided key insights into immune pathways that

limit the prevalence and intensity of parasitic infections in mosquitoes [3–5]. More recently, the burgeoning field of ecological immunology has shifted the attention to the causes and consequences of variation in immune function in insects and, in particular, to the role played by non-genetic factors such as temperature [6], nutrition [7], reproductive status [8, 9], and previous infectious history [10, 11].

One of the best documented sources of heterogeneity in the immune system of multicellular organisms is their age. In virtually all species, immune function decreases drastically with age, a process that has been termed immune senescence and for which both mechanistic and

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evolutionary explanations have been proposed [12, 13]. Insects are no exception to this rule. Significant decreases in the antibacterial activity, melanization potential and number of haemocytes with age have been reported in a range of widely different insect taxa, including bees [14±16], butterflies [17, 18], scorpionflies [19], crickets [20], damselflies [21], flies [22, 23], and also mosquitoes [22±27].

The ubiquity of immune senescence leads to the general prediction that older insects should be more susceptible to infection than their younger counterparts, i.e., that individuals exposed to parasites later in life should have a higher probability and/or intensity of infection. This prediction has been confirmed in some cases, but not in others. Hillyer et al. [25] and Roberts and Hughes [28] both found that, as expected, a decrease in immunity with age is associated with an increase in parasite susceptibility. Surprisingly, however, in many insect species individuals seem to become more resistant to parasites as they age (see Additional file 1). Unfortunately, however, few of these studies have concomitantly measured immune function and parasite susceptibility, so the potential role played by the immune system cannot be established.

Understanding the effects of mosquito age on parasite transmission is particularly relevant for malaria. Early on, MacDonald [29] used mathematical epidemiology to show that vector lifespan is one of the most important determinants of malaria transmission. Indeed, the time lag between adult emergence and the activation of the host-seeking behaviour [30] combined with the long extrinsic incubation period of *Plasmodium* (approximately 14 days), entail that infectious mosquitoes are, necessarily, old. Consequently, old mosquitoes are often considered to be epidemiologically more important vectors of malaria than their younger counterparts. The age structure of the mosquito population also has important applied implications because it has been suggested that effective malaria control, with only weak selection for insecticide resistance, could be achieved by using insecticides that target only old mosquitoes [31, 32]. Yet, aside from a few studies that have shown that age-dependent mortality could affect malaria epidemiology [33±35], most epidemiological models of malaria do not take age structure explicitly into account [36]. Specifically, the potential for age-dependent susceptibility to *Plasmodium* in mosquitoes has been largely overlooked. Yet, early studies showed that *Aedes aegypti* mosquitoes become less susceptible to a *Plasmodium gallinaceum* infection as they age [37]. This effect challenges the claim that old females are more important vectors of *Plasmodium* and could have far reaching consequences for malaria transmission. Further experimental studies monitoring the

effects of mosquito age on immunity and susceptibility to *Plasmodium* parasites are therefore required to evaluate the importance of mosquito age-structure on malaria epidemiology.

Here, the effect of age on both mosquito immunity and susceptibility to a *Plasmodium* infection are quantified using an experimental system consisting on the avian malaria parasite *Plasmodium relictum* and its natural vector in the field, the mosquito *Culex pipiens*. This parasite is ubiquitous in natural populations of birds [38] and is known to incur an important fecundity cost on their vectors [39]. The aim of this study is three-fold. First to establish the existence of immune senescence in *Cx pipiens* by quantifying the number of circulating haemocytes in the haemolymph of three, seven and 17-day old mosquitoes. Haemocytes are a crucial component of the mosquito immune system that kill pathogens via phagocytic, lytic and melanization pathways [40] and play a key role in the defence against *Plasmodium* [41], although the precise relationship between haemocyte density and *Plasmodium* protection has not been established, and may not necessarily be linear. Second, to establish whether changes in haemocyte numbers with age are associated with changes in the susceptibility to a *Plasmodium* infection: are old mosquitoes more or less susceptible to a *Plasmodium* infection? And third, to establish whether the effect of age on either the immune function or parasite susceptibility is modulated by the previous blood feeding history of the mosquitoes. Indeed, in practice, old mosquitoes are likely to have taken at least one previous uninfected blood meal. Previous blood meals drastically alter the physiology of the mosquitoes [42] and could potentially reset the effect of age on susceptibility to malaria [37, 43].

Methods

Plasmodium strain and bird infections

Plasmodium relictum (lineage SGS1) is the aetiological agent of the most prevalent form of avian malaria in Europe [44]. The biology of avian malaria is similar to that of human malaria, both in the vertebrate host and in the mosquito, which is why avian malaria has historically played a key role in human malaria research [45]. The lineage was isolated from infected sparrows in 2009 [45] and passaged to naïve canaries (*Serinus canaria*). Since then it has been maintained by carrying out regular passages between the stock canaries through intraperitoneal injections. Experimental canaries (n = 5) were infected by injecting them with ca. 80 µL of blood from the infected canary stock. Mosquito blood feeding took place 12 days after the injection, to coincide with the acute phase of the *Plasmodium* infection [46].

Oocyst prevalence and burden

Experiments were carried out using a laboratory line of *Cx pipiens* (SLAB, [46]). The experimental design is shown in Fig. 1. To test the effect of mosquito age on the probability and intensity of a *Plasmodium* infection, two mosquito cohorts were generated: **Old** (17-day old) mosquitoes (which was later split between **Blood fed** and **Unfed**—see below) and **Young** (7-day old) mosquitoes. The larvae for both of these cohorts were raised in an identical way following previously published protocols [46]. Larval trays ($n = 6$ for the young and $n = 10$ for the old cohorts) were placed individually inside an emergence cage ($40 \times 28 \times 31$ cm) and emerged adults were allowed to feed ad libitum on a 10 % glucose water solution.

The experiment proceeded as follows. For the old cohort, on the day of emergence (Day 0, Fig. 1) mosquitoes were haphazardly sampled from the emergence cages (c.a. 20 mosquitoes from each emergence cage) and placed in six different experimental cages. Seven days later (Day 7) the cages were randomly allocated to either

the **Old-unfed** treatment or the **Old-blood fed** treatment, and the latter were provided with an uninfected canary to blood feed (a different canary per cage) following previously published protocols [46]. Blood feeding success was confirmed through the visual inspection of the mosquito abdomen. Unfed mosquitoes (<7 %) were discarded. The young cohort was launched 10 days after the old cohort (Day 10, Fig. 1) in an identical way. All mosquitoes were given a *Plasmodium*-infected blood meal on Day 17. For this purpose, 50 mosquitoes from each of the three experimental treatments (young, old-unfed, old-blood fed) were placed together inside a cage ($n = 5$ different cages) and allowed to blood feed from a *Plasmodium*-infected bird for 12 h. To distinguish between the three treatments, mosquitoes were previously marked using three different fluorescent colour powders and the colour allocated to each treatment changed in each of the cages (for details, see [47]).

To obtain an estimate of blood meal size, 1 day after the infected blood meal, all blood-engorged females were

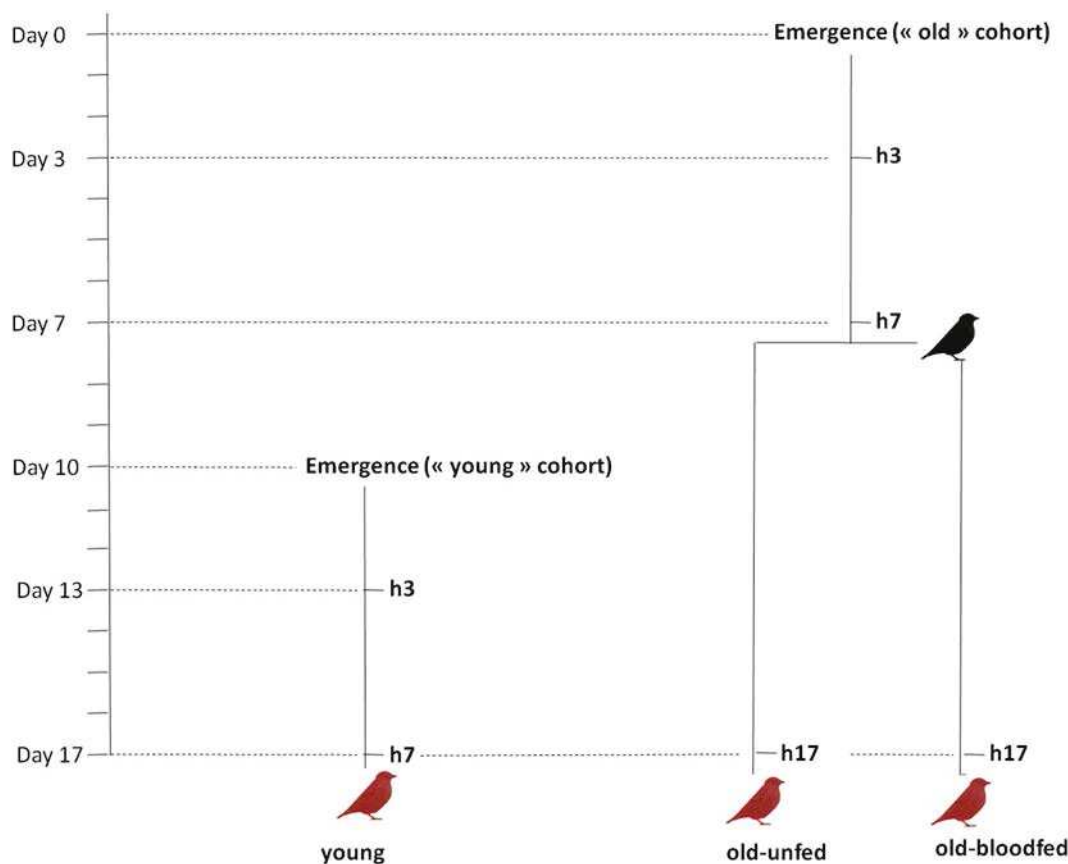


Fig. 1 Schematic representation of the experimental design. *Black bird* uninfected blood meal; *red birds* *Plasmodium*-infected blood meal; h3, h7, h17: haemolymph sampling from 3, 7 and 17-day old mosquitoes, respectively. Haemolymph was sampled immediately before the blood meal on a separate sample of mosquitoes (haemocyte extraction is destructive). Young, old-unfed and old-blood fed mosquitoes are colour marked and fed from the same bird ($n = 5$ different birds)

placed individually in numbered plastic tubes (30 ml) covered with a mesh. Food was provided in the form of a cotton pad soaked in a 10 % glucose solution. Seven days later (Day 7 post-blood meal) the females were taken out of the tubes and amount of haematin excreted at the bottom of each tube was quantified as an estimate of the blood meal size [46]. Females were then dissected and the number of *Plasmodium* oocysts in their midguts counted with the aid of a binocular microscope [46]. The wings of ten haphazardly chosen females from each size and treatment combination were removed prior to the midgut dissection and measured along its longest axis as an index of body size.

As the young and old cohorts are not launched simultaneously, there is a risk that small differences in the larval rearing conditions may result in adults that varied in their body condition independently of their age, thereby potentially confounding the results. To account for this, the entire experiment was repeated 4 months later (the experiments are hereafter called Blocks 1 and 2).

Haemolymph collection and haemocyte quantification

To establish whether the patterns of oocyst prevalence and burden in old and young mosquitoes are correlated with their immune activity, a sub-sample of ten to 15 mosquitoes from each cage and treatment combination was taken at regular intervals throughout the experiment and their haemolymph extracted to count the number of circulating haemocytes. The soft exoskeleton of newly emerged mosquitoes made the task of sampling the haemolymph on the day of emergence impossible. Old and young mosquitoes were therefore sampled 3 days after emergence (once their exoskeleton had hardened) and immediately before each of the blood meals: on Days 7 (old mosquitoes only) and 17 (old and young mosquitoes) of the experiment (see Fig. 1). The wing of haphazardly sampled females from each cages were measured as above to control for body size, and haemolymph were extracted individually following the protocols described below.

The haemolymph collection protocol was adapted from one described by Qayum and Telang [48]. Mosquitoes were injected between the seventh and eighth abdominal segments with an anticoagulant solution (70 % Schneider® Insect medium and 30 % citrate buffer) and placed on ice for 5 min to allow the anticoagulant solution to dislodge haemocytes adhering to the internal tissues. The tip of the abdomen was then removed and, using a Hamilton™ syringe, 10 µL of anticoagulant buffer was injected in the lateral side of mosquito mesothorax. This caused the diluted haemolymph to flow out of the abdomen. Eight microlitre of diluted haemolymph were collected with a pipette equipped with a sterile tip and

placed on a glass slide. Slides were placed in the dark for 20 min to allow the haemocytes to adhere to the slide® surface. The haemocytes were then fixed and stained using the Microscopy Hemacolor® staining kit as follows. Each glass slide was dipped five times for 1 s in the fixative solution, then three times for 1 s in both the solution I and in the solution II. Finally the glass slide was rinsed one time for 1 s in the solution III. Haemocytes were counted under the optical microscope (40× objective). The technique was repeated in an identical way in both experimental blocks. In Block 2, however, due to a problem with a one of the batches of the anticoagulant solution, the number of haemocytes in the young cohort could not be quantified.

Haemocytes were classified based on their morphological appearance into granulocytes, easily distinguishable by their ability to spread on glass surfaces, and oenocytoids, characterized by their spherical shape (see Additional file 2). According to Hillyer et al. [25] these are the only two types of circulating haemocytes capable of adhering to glass slides. A third population of haemocytes called prohaemocytes has also been identified in some studies [40]. Prohaemocytes are morphologically similar to oenocytoids, only smaller. In addition, their origin and functional role are still under discussion: they may be multipotent stem cells that give rise to the other haemocyte types [26] or may arise through the asymmetrical division of granulocytes [49]. As these prohaemocytes may constitute a very small fraction of the total number of circulating haemocytes [40] and since no clear size threshold existed to confidently distinguish between oenocytoids and prohaemocytes, no such distinction was made. It is thus worth bearing in mind that the population referred to as being oenocytoids may have included a small fraction of prohaemocytes.

Statistical analyses

Analyses were carried out using the R statistical package (v. 3.1.1). The different statistical models built to analyse the data are described in Additional file 3. The analysis of response variables, which may depend on which bird the mosquitoes fed on, such oocyst prevalence and oocyst burden was carried out using mixed model procedures, fitting bird as a random factor in the models and treatment (young, old-unfed, old-blood fed) and blood meal size (haematin) as fixed factors. Oocyst prevalence (presence/absence of oocysts) was analysed using the *lmer* mixed model procedure and binomial errors, while oocyst burden (number of oocysts in individuals with more than one oocyst) was normalized using a logarithmic transformation and analysed using the *lme* procedure. All other variables were analysed using standard general linear models (glm) with an appropriate error

distribution: wing size and haemocyte counts were analysed with a normal error distribution.

Maximal models, including all higher order interactions, were simplified by sequentially eliminating non-significant terms and interactions to establish a minimal model [50]. The significance of the explanatory variables was established using either a likelihood ratio test (which is approximately distributed as a Chi square distribution [51]) or an F test. The significant Chi square or F values given in the text are for the minimal model, whereas non-significant values correspond to those obtained before the deletion of the variable from the model. A posteriori contrasts were carried out by aggregating factor levels together and by testing the fit of the simplified model using a likelihood-ratio test [50].

Ethical statement

Animal experiments were carried out in strict accordance with the National Charter on the Ethics of Animal Experimentation of the French Government, and all efforts were made to minimize suffering. Experiments were approved by the Ethical Committee for Animal Experimentation established by the authors@institution (CNRS) under the auspices of the French Ministry of Education and Research (permit number CEEA- LR-1051).

Results

Haemocyte density

As there were no significant differences in size between the females in the different treatments (treatment effect, Block 1: model 5: $F_{1,73} = 0.066$, $p = 0.417$, Block 2: model 19: $F_{1,37} = 0.089$, $p = 0.328$), female size was not included in subsequent analyses. In Block 1, the old mosquito cohort was analysed to establish whether granulocytes, oenocytoids or the total haemocyte counts change with time. For this purpose, differences between old-unfed and old-blood fed females on Day 17 were first tested. As neither total haemocyte (model 6: $F_{1,19} = 0.819$, $p = 0.377$), granulocyte (model 9: $F_{1,19} = 0.1582$, $p = 0.224$) or oenocytoid numbers (model 12: $F_{1,19} = 0.002$, $p = 0.999$) differed between blood fed and unfed individuals (Fig. 2), the effect of time within the old mosquito cohort on these variables was carried out pooling these two data points together. This analysis showed that time has a significant effect on the total number of haemocytes (model 7: $F_{2,47} = 8.007$, $p = 0.001$, Fig. 2a) and oenocytoids (model 13: $F_{2,47} = 20.820$, $p < 0.0001$, Fig. 2c), but not on the number of granulocytes (model 10: $F_{2,47} = 1.320$, $p = 0.277$, Fig. 2b). While total haemocytes and oenocytoids remained stable at around 586 ± 25 and 325 ± 26 cells, respectively, between Days 3 and 7, their numbers decreased by $\sim 40\%$ thereafter (contrast analyses Day 7 vs 17, total haemocytes: $F_{1,48} = 13.793$, $p < 0.001$,

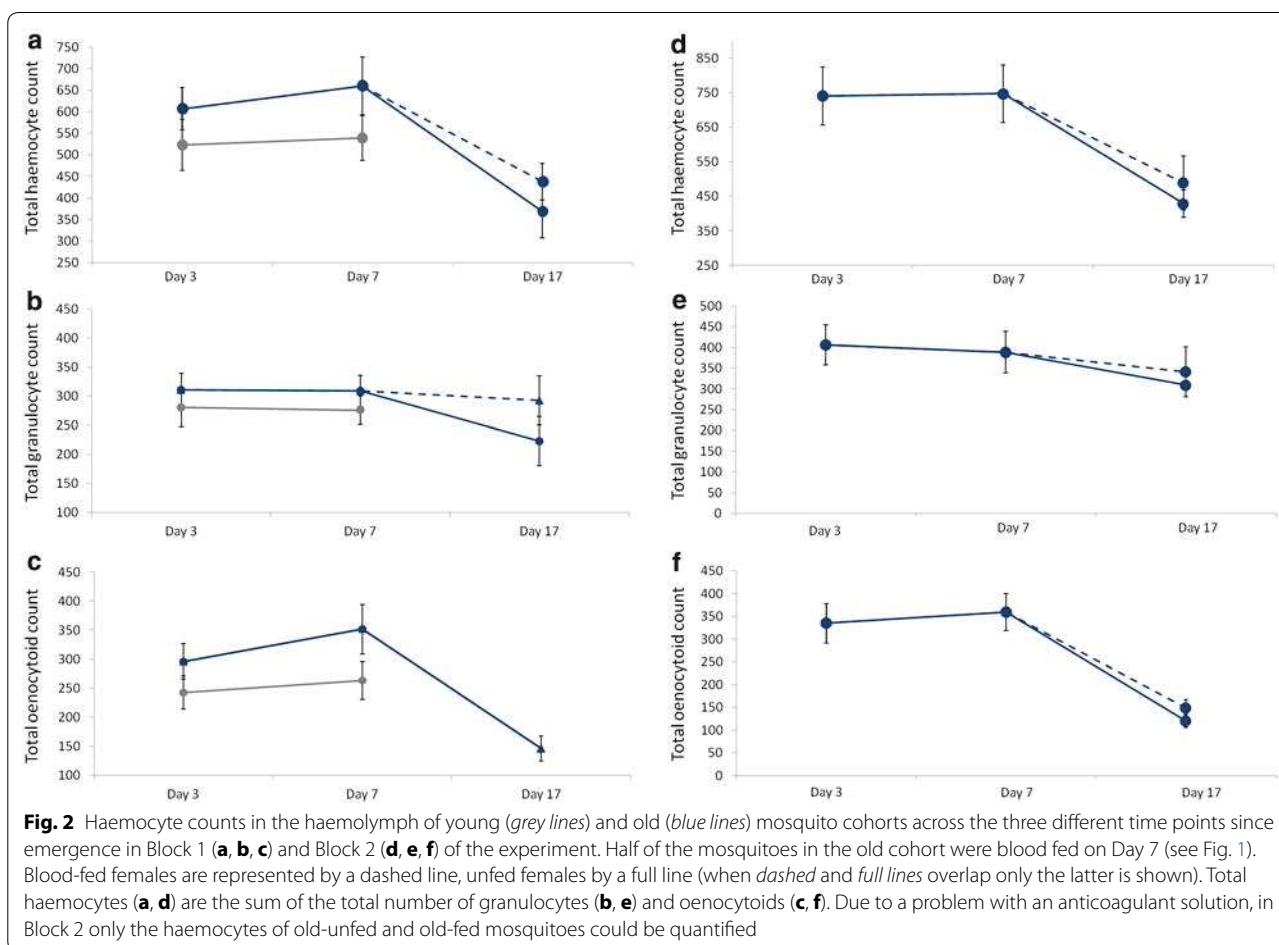
oenocytoids: $F_{1,48} = 34.755$, $p < 0.0001$). These analyses also revealed no significant effects of either treatment or time on the number of either total haemocytes, granulocytes or oenocytoids between the old and young females on Days 3 and 7, the time points shared by the two cohorts (interaction treatment $\times$ time effect, total haemocytes: model 8: $F_{2,50} = 3.119$, $p = 0.083$, granulocytes: model 11: $F_{2,50} = 0.003$, $p = 0.956$, oenocytoids: model 14: $F_{2,50} = 0.034$, $p = 0.854$, Fig. 2).

The results of Block 2 for the old-unfed and old-fed treatments (the haemocyte quantification for the young treatment could not be carried out due to a problem with one of the anticoagulant batches, see above) were highly consistent with those obtained in Block 1 (Fig. 2). As above, neither total haemocyte (model 20: $F_{1,16} = 0.470$, $p = 0.503$), granulocyte (model 22: $F_{1,16} = 0.2284$, $p = 0.639$) or oenocytoid numbers (model 24: $F_{1,16} = 1.354$, $p = 0.262$) differed between blood fed and unfed individuals so the data were pooled for subsequent analyses. Time has a significant effect on the total number of haemocytes (model 21: $F_{2,36} = 6.891$, $p = 0.003$, Fig. 2d) and oenocytoids (model 25: $F_{2,36} = 17.716$, $p < 0.0001$, Fig. 2f), but not on the number of granulocytes (model 23: $F_{2,36} = 1.191$, $p = 0.315$, Fig. 2e).

Plasmodium prevalence and intensity

As there were no differences in size between the females in the three different treatments (treatment effect Block 1, model 1: $\chi^2_2 = 2.848$, $p = 0.609$; Block 2, model 11: $\chi^2_2 = 2.881$, $p = 0.0747$), female size was not included in subsequent analyses. The amount of blood ingested, however, varied significantly between the treatments (Block 1, model 2: $\chi^2_5 = 7.6836$, $p < 0.0001$; Block 2, model 16: $\chi^2_5 = 41.851$, $p < 0.0001$): young females took significantly larger blood meals than old-unfed (contrast analyses: Block 1: $\chi^2_4 = 14.988$, $p < 0.0001$, Block 2: $\chi^2_4 = 69.009$, $p < 0.0001$) and old-fed females (Block 1: $\chi^2_4 = 3.221$, $p = 0.073$, Block 2: model 13: $\chi^2_4 = 32.622$, $p < 0.0001$). Old-fed females also took larger blood meals than old-unfed ones (Block 1: $\chi^2_4 = 4.866$, $p = 0.027$, Block 2: $\chi^2_4 = 13.106$, $p < 0.0001$). As blood meal size can be a strong predictor of both the prevalence and the intensity of the infection, this explanatory variable was introduced into all subsequent analyses.

The results of Blocks 1 and 2 are highly consistent in showing a strong effect of female age on the probability of becoming infected by *Plasmodium* (treatment effect Block 1, model 3: $\chi^2_5 = 13.08$, $p = 0.001$; Block 2, model 17: $\chi^2_5 = 21.123$, $p < 0.001$, Fig. 3a, b). In both blocks, the probability of infection of old females was a roughly a third lower than that of young females (contrast analyses young vs old-unfed Block 1: $\chi^2_4 = 9.433$, $p = 0.002$; Block 2: $\chi^2_4 = 22.561$, $p < 0.001$). However, taking an uninfected



blood meal 10 days before the infected one reverses the protective effects of age so that the prevalence of infection in old-blood fed females was no different to that of young females (contrast analyses Block 1: $\chi^2_4 = 0.071$, $p = 0.790$, Block 2: $\chi^2_4 = 0.023$, $p = 0.880$, Fig. 3a, b). Including haematin in the analyses did not alter the significance of these results.

A similar trend was observed in the intensity of the infection: old-unfed females have roughly three times fewer oocyst burdens than young and old-blood fed ones, although the results are only statistically significant in the second experimental block (treatment effect Block 2, model 18: $\chi^2_2 = 14.405$, $p < 0.001$, Fig. 4b). Including haematin in the analyses did not alter the significance of these results. Block 1 followed a similar trend, with lower oocyst burdens in old-unfed females than in the other two treatments, but the results were not statistically significant (treatment effect Block 1, model 11: $\chi^2_2 = 3.607$, $p = 0.165$, Fig. 4a). Here, however, a significant positive correlation between haematin and oocyst burden was found (haematin effect, model 11: $\chi^2_4 = 18.515$, $p < 0.001$).

Discussion

The results of these experiments show that: (1) haemocyte numbers decrease drastically as mosquitoes age; (2) this effect is not correlated with an increased susceptibility to *Plasmodium*, rather, older mosquitoes are significantly more resistant to malaria parasites than their younger counterparts; and, (3) the increased resistance with age is reversed when mosquitoes have previously taken an uninfected blood meal. Below each of these results separately are discussed separately.

Immunity wanes with age

Immune defences tend to decline as individuals age. This decline may be the result of the generalized, and inevitable, process of physiological wear and tear, or an adaptive adjustment of immune function with age [12, 16]. Age-related declines in immune function have been documented in several insect species using a variety of immune indicators, such as phenoloxidase (PO) activity, encapsulation rate and haemocyte numbers [25, 52]. The results of the current experiments show that 17-day old

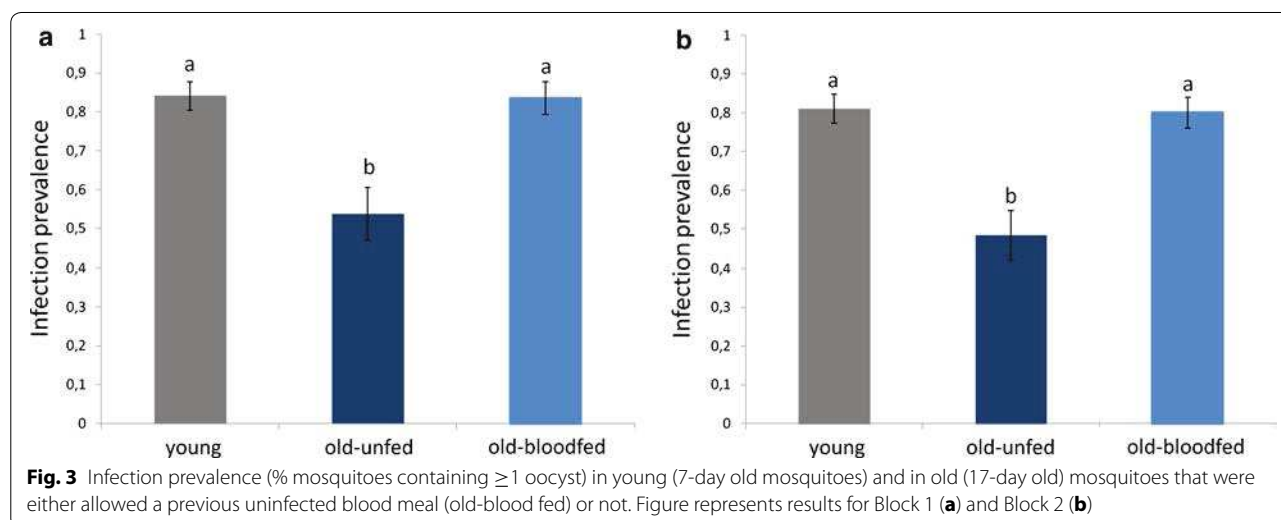


Fig. 3 Infection prevalence (% mosquitoes containing ≥ 1 oocyst) in young (7-day old mosquitoes) and in old (17-day old) mosquitoes that were either allowed a previous uninfected blood meal (old-blood fed) or not. Figure represents results for Block 1 (a) and Block 2 (b)

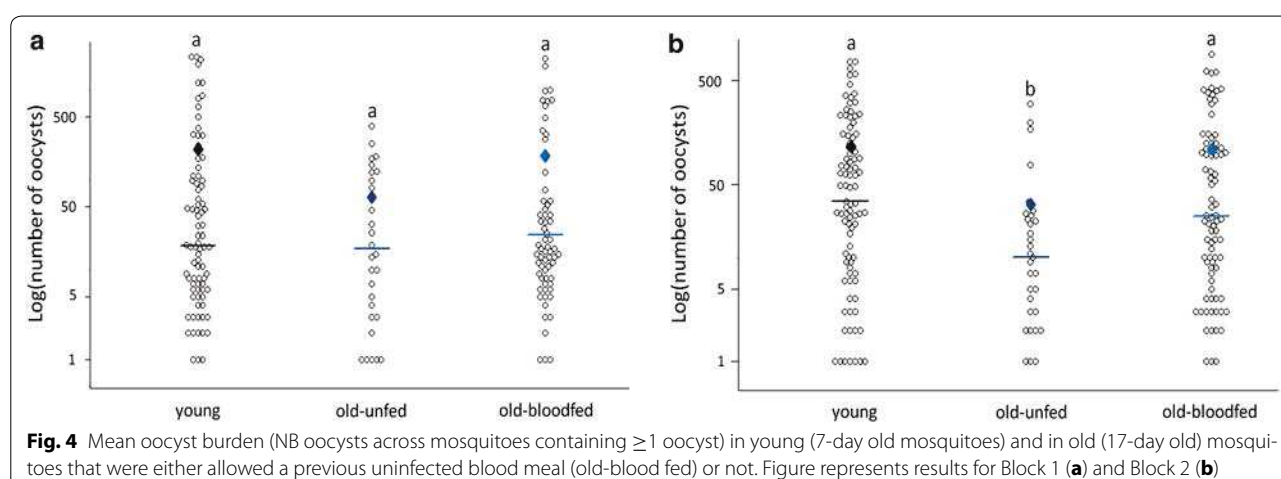


Fig. 4 Mean oocyst burden (NB oocysts across mosquitoes containing ≥ 1 oocyst) in young (7-day old mosquitoes) and in old (17-day old) mosquitoes that were either allowed a previous uninfected blood meal (old-blood fed) or not. Figure represents results for Block 1 (a) and Block 2 (b)

Cx pipiens mosquitoes have 40 % fewer haemocytes than their younger (7-day old) counterparts. Interestingly, this decline in haemocytes with time is entirely due to a drastic decrease in the number of oenocytoids, while granulocytes remain largely unchanged. Granulocytes and oenocytoids play different roles in the immune system. Granulocytes primarily kill pathogens through phagocytosis or lysis, while oenocytoids are the major producers of the enzymes required for melanization, including PO [40]. The drastic decrease in oenocytoids observed agrees with previous results showing that PO activity declines drastically with age so that, by Day 14, *Cx pipiens* females have lost around two-thirds of their PO at emergence [27]. Immune decay with age has also been observed in the two other major mosquito vector species, *Ae. aegypti* and *Anopheles gambiae*, where decreases in haemocyte numbers [25, 26] and melanization potential [23, 24] have

been documented. Taking a previous blood meal did not alter the course of immune senescence in mosquitoes. Previous work has shown that blood feeding activates the mosquito innate immune system [26, 53, 54]. The effect is however transient, which probably explains why no differences in mosquitoes were found 10 days after the uninfected blood meal.

Susceptibility to *Plasmodium* decreases with age

Immune decay has led to predictions that as insects age their ability to clear infections should also decrease [55, 56]. The results of the current experiments contradict this prediction. Despite a drastic decrease in the immune system with age, old *Cx pipiens* females are significantly more resistant to a *P. relictum* infection than younger ones. The probability of becoming infected by *P. relictum* is 30 % lower in 17-day old than in seven-day old

mosquitoes. These results are consistent across two different experimental blocks carried out several weeks apart under identical experimental conditions. Two other studies to date have investigated the role of mosquito age on *Plasmodium* infection. Terzian et al. [37], who carried out what is probably the first investigation into the effect of insect age on susceptibility to infection in any invertebrate, also reported that as *Ae. aegypti* age they become more resistant to a *P. gallinaceum* infection. More recently, however, Okech et al. [57] found no effect of age on the prevalence of *Plasmodium falciparum* infections in *An. gambiae*, although the low infection rates in these experiments (<4 % of the membrane-fed mosquitoes became infected) may have significantly reduced the statistical power of their experiments.

Interestingly, a decrease in the probability of infection with age seems to be a common feature in insects (see Additional file 1). It has been reported in bacteria-challenged drosophila flies [58], *Trypanosoma*-challenged tsetse flies [59], *Microsporidium*-challenged bees [60, 61] and virus and *Filaria*-challenged mosquitoes [43, 62, 63]. The mechanisms underlying these increased susceptibilities to infection later in life are not known, but none of these studies measured immunity, so a re-allocation of resources towards a particular arm of the immune system cannot be entirely excluded [16]. Unfortunately, only a handful of studies have concomitantly measured immunity and parasite infection rates in aging insects. Hillyer et al. [25] found that a decrease in haemocyte counts with age increases the susceptibility of *Ae. aegypti* mosquitoes to injected *Escherichia coli* bacteria. Similarly, Roberts and Hughes [28] found that a decrease in PO level in older bumblebees was associated with increased microsporidian infection rates. Clearly, more studies are needed that investigate the effect of age not only on immunity but also on parasite infection rates since, as the results presented here show, both are not necessarily correlated.

The fact that a decrease in PO [27] and PO-producing oenocytoids (Fig. 2) does not result in an increase in *Plasmodium* infection rates could be partly explained by PO playing no role in *Plasmodium* protection in this system. Current knowledge of the role of PO in *Plasmodium* protection is largely based on studies using genetically selected resistant and susceptible mosquitoes and gene-silencing procedures [3] but there is hardly any evidence that melanization plays a role in defence against *Plasmodium* in the field (e.g., [24]), and *P. relictum* oocysts in *Cx pipiens* mosquitoes are rare. Melanization, however, is the most apparent, but not the only, outcome of the PO cascade. The PO enzymatic cascade also produces many cytotoxic intermediates that generate reactive oxygen and nitrogen intermediates [64] which may kill the

parasite without encapsulating it [65]. Alternatively, the possibility that the decrease in haemocyte counts and PO activity with age in *Cx pipiens* mosquitoes is correlated with the upregulation of another unmeasured, immune effector, cannot be entirely excluded. Potential candidates are antimicrobial peptides, which are produced by the mosquito fat body [25] so their titers may be uncorrelated with haemocyte densities [16]. Indeed, Moret and Schmid-Hempel [16] found that, as bumblebees age, a decrease in haemocyte numbers and PO activity was associated with an increase in antimicrobial activity. However, Lambrechts et al. [66] however, found a positive correlation between PO activity and antibacterial defence. In addition, previous results showing that insecticide-resistant *Cx pipiens* mosquitoes show no differences in *P. relictum* prevalence or intensity, despite having significantly higher levels of antimicrobial peptide expression [46, 67], suggest that these peptides may not play a key role in *Plasmodium* defence in this system. It is thus highly likely that immunity is not directly responsible for the lower infection rates observed in old mosquitoes and that an explanation must therefore be found elsewhere.

The journey of *Plasmodium* through the mosquito requires the complex interplay of a suite of finely tuned physiological, immunological and molecular events, many of which take place in the mosquito midgut [68, 69]. In recent years, the role played by the different arms of the mosquito immune system in limiting *Plasmodium* development has received a great deal of attention (recently reviewed by Clayton et al. [3]). Fewer efforts have been directed at investigating how other physiological events, which are essential for the successful transformation and passage of the different *Plasmodium* stages through the mosquito, vary across individuals and experimental conditions. Most of these events take place in the mosquito midgut and include: the production of large amounts of xanthurenic acid, a by-product of the synthesis of the eye pigment, which is essential for the exflagellation of gametocytes in the midgut [70], the secretion of digestive trypsin-like enzymes which aid ookinetes to break down and traverse the chitinous peritrophic membrane [71], the binding of ookinetes to specific midgut epithelial ligands [69] and to structural proteins in the basal lamina [72], and the secretion of digestive carboxypeptidase enzymes which may provide *Plasmodium* with essential amino acids for oocyst development [68]. It is likely that some of these factors are disturbed as mosquitoes age. Recent work has indeed shown that in *Drosophila*, ageing is accompanied by both a structural and a functional degeneration of the fly's midgut [73]. In particular, a decrease in trypsin production with age has been observed not only in *Drosophila* [73], but also

in *Anopheles* [74]. Equivalent changes in the *Culex* midgut could explain the decrease in *Plasmodium* infection with age observed in this system. In addition, recent work has demonstrated the crucial role played by the midgut microbial flora in the success of *Plasmodium* infections in mosquitoes [75–77]. In flies, the number of microorganisms found in the lumen of the gut increases significantly with age [78]. Further work is needed on how the gut microbiota changes as mosquito ages and on the consequences this may have for *Plasmodium* transmission.

A prior blood meal reverses the effect of age on *Plasmodium* susceptibility

The fact that the age effect is reversed when old mosquitoes have taken one previous non-infected blood meal is significant and may provide further clues about the mechanisms underlying the effect of mosquito age on infection. Interestingly, Terzian [37] and Ariani et al. [43] also found that a previous blood meal reverses the negative effect of age on the development of two very different parasites: *Plasmodium* and the filarial nematode *Brugia malayi*, respectively. Both speculated that a long stretch without a blood meal may have depleted the older females of key nutrients required for parasite development, and that the additional blood meal may have provided the extra resources. Terzian et al. [37] explored the possibility that nitrogen or protein depletion were responsible for their results by substituting the first blood meal with a diet of chicken plasma and haemoglobin. Puzzlingly, this diet resulted in an even larger decrease in *Plasmodium* oocyst numbers than in sugar-fed mosquitoes. Complementing the diet with a raisin infusion did, however, restore oocyst numbers to those obtained in younger mosquitoes [37]. Clearly, more work is needed on the nutritional needs of *Plasmodium* inside the mosquito and on whether key limiting nutrients for *Plasmodium* development are acquired by the mosquito during their adult or larval lives [79].

Aside from their nutritional contents, blood meals also trigger a whole set of physiological events in the mosquito, which could impact the infection success of a subsequent infected blood meal. Taking a warm blood meal induces a temperature stress that results in the synthesis of *hsp70* [80], a heat shock protein that has been shown to lead to drastic increases in oocyst burdens in *Plasmodium*-infected *Anopheles* [81]. In addition, ingestion of a first blood meal may alter the kinetics of digestion of subsequent blood meals [82]. In *An. gambiae*, ingestion of a prior blood meal accelerates the digestive process of subsequent infected blood meals, thereby reducing the probability of a *Plasmodium* infection [82], while in *Ae. aegypti*, subsequent

digestions are slowed down [83]. Because parasite fertilization, transformation and penetration through the midgut require time, a slower blood meal digestion in previously blood-fed *Cx pipiens* females would have afforded the parasite more time to establish a successful infection in the mosquito [82].

Conclusion

Mosquito populations are structured both by age and by the time since they took their infectious blood meal [35]. The results of these experiments show that age significantly impacts the susceptibility of mosquitoes to a *Plasmodium* infection: older females have a lower probability of becoming infected with the parasite. However, the dramatic effects of age are reversed in previously blood-fed females. This point is crucial because most old mosquitoes in the field are likely to have taken at least one previous blood meal. Indeed, up to 80 % of malaria vectors collected in the field are parous: i.e., have taken a blood meal and laid a batch of eggs (e.g., [84, 85]). As a consequence, in the field, age is unlikely to have a significant impact on the susceptibility of mosquitoes to a malaria infection. However, investigating the mechanisms underlying both the increased resistance of old unfed mosquitoes, and its reversal when mosquitoes are previously given blood meal, may unravel key insights about the biology and development of *Plasmodium* parasites in the mosquito.

Additional files

Additional file 1: Impact of age on parasite prevalence and parasite intensity in experimentally infected insects, and correlated measurements of immune senescence, when available.

Additional file 2: Optical microscope (x400) image of *Cx pipiens* haemocytes showing the two different morphotypes described in this study: granulocytes and oenocytoids (see main text for details).

Additional file 3: Description of statistical models used to analyse the data.

Authors' contributions

RP, SG and AR conceived and designed the experiments. RP and AN performed the experiments. RP analysed the data. RP, SG and AR wrote the paper. All authors read and commented on earlier drafts of the paper. All authors read and approved the final manuscript.

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Compliance with ethical guidelines

Competing interests

The authors declare that they have no competing interests.

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Supplementary materials

Table S1: Impact of age on parasite prevalence and parasite intensity in experimentally infected insects, and correlated measurements of immune senescence, when available. WNV: West Nile Virus, SLE: Saint Louis Encephalitis Virus, JEV: Japanese Encephalitis Virus, ×: not reported. Superscripts: ¹immune senescence is quantified in uninfected individuals, ²the age effect differs between genetic lines, ³the age effect depends on temperature and virus dose, ⁴the age effect depends on the immune parameter measured

	Age range	Parasite	Parasite prevalence	Parasite intensity	Immune senescence ¹	REF
Flies						
<i>D. melanogaster</i>	3-13 days	bacteria (<i>S. marescens</i>)	×	decreases	×	[1]
<i>D. melanogaster</i>	8-75 days	bacteria (several species)	×	no effect	×	[2]
<i>D. melanogaster</i>	3-40 days	bacteria (<i>E. coli</i>)	×	no effect	×	[3]
<i>D. melanogaster</i>	1-4 weeks	bacteria (<i>E. coli</i>)	×	decreases / increases ²	×	[4]
<i>M. domestica</i>	1-14 days	fungus (<i>B. basana</i>)	no effect	×	×	[5]
<i>G. morsitans</i>	24-48 hours	trypanosome (<i>T. brucei</i>)	decreases	×	×	[6]
<i>G. morsitans</i>	24-48 hours	trypanosome (<i>T. congolense</i>)	decreases	×	×	[6]
<i>G. palpalis</i>	24-48 hours	trypanosome (<i>T. brucei</i>)	decreases	×	×	[6]
Mosquitoes						
<i>C. pipiens</i>	4-10 days	virus (WNV)	decreases / increases ³		×	[7]
<i>C. pipiens</i>	3-12 days	virus (SLEV)	decreases	×	×	[8]
<i>C. pipiens</i>	2-22 days	filaria (<i>Brugia</i> sp.)	increases	×	×	[9]
<i>C. tritaeniorhynchus</i>	10-24 days	virus (JEV)	no effect	×	×	[10]
<i>C. tritaeniorhynchus</i>	4-12 days	virus (WNV)	decreases	×	×	[11]
<i>A. aegypti</i>	1-5 days	bacteria (<i>E. coli</i>)	×	increases	yes	[12]
<i>A. aegypti</i>	1-6 weeks	filaria (<i>B. malayi</i>)	decreases	decreases	×	[13]
<i>A. aegypti</i>	1-4 weeks	apicomplexa (<i>P. gallinaceum</i>)	×	decreases	×	[14]
<i>A. gambiae</i>	2-12 days	fungus (<i>M. anisopliae</i>)	×	no effect	×	[15]
<i>A. gambiae</i>	1-11 days	apicomplexa (<i>P. falciparum</i>)	no effect	no effect	×	[16]
Bees						
<i>A. mellifera</i>	1-14 days	microsporidia (<i>N. ceranae</i>)	×	increases	yes / no ⁴	[17]
<i>A. mellifera</i>	1-12 days	microsporidia (<i>N. ceranae</i>)	decreases	decreases	yes / no ⁴	[18]
<i>B. terrestris</i>	2-10	microsporidia (<i>N. bombi</i>)	decreases	×	×	[19]

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Table S2: Description of statistical models used to analyze the data. The response variable was not transformed unless otherwise stated. N gives the number of mosquitoes included in each analysis. "Maximal model" gives the complete set of explanatory variables (and their interactions) included in the model. "Minimal model" gives the model containing only the significant variables and their interactions. Round brackets indicate variables fitted as random factors (1/bird denotes that the effect of bird is fitted as a random variable). Square brackets indicate the error structure used (n: normal, b: binomial). treat: treatment ("young", "old-unfed", "old-bloodfed"), hm: haematin excreted (blood meal size), time: (time since adult emergence: D3, D7 or D17) .

Variable of interest	Response variable	Model nb	N	Maximal model	Minimal model	R subrout [error]
Block 1						
<i>Plasmodium prevalence and burden</i>						
Wing size	size	1	229	treat	1	glm [n]
Blood meal size	hm	2	229	treat + (1/bird)	treat + (1/bird)	lme [n]
Oocyst prevalence	prev	3	229	treat*hm + (1/bird)	treat + hm + (1/bird)	lmer [b]
Oocyst burden	log (burd)	4	229	treat*hm + (1/bird)	hm + (1/bird)	lme [n]
<i>Hemocyte density</i>						
Wing size	size	5	75	treat	1	glm [n]
Nb total hemocytes, day 17	hct	6	21	treat	1	glm [n]
Nb total hemocytes through time ("old" cohort)	hct	7	50	time	time	glm [n]
Nb total hemocytes, days 3 and 7	hct	8	54	treat*time	1	glm [n]
Nb granulocytes, day 17	grn	9	21	treat	1	glm [n]
Nb granulocytes through time ("old" cohort)	grn	10	50	time	1	glm [n]
Nb granulocytes, days 3 and 7	grn	11	54	treat*time	1	glm [n]
Nb oenocytoids, day 17	log (oen)	12	21	treat	1	glm [n]
Nb oenocytoids through time ("old" cohort)	log (oen)	13	50	time	time	glm [n]
Nb oenocytoids, days 3 and 7	log (oen)	14	54	treat*time	1	glm [n]
Block 2						
<i>Plasmodium prevalence and burden</i>						
Wing size	size	15	271	treat	1	glm [n]
Blood meal size	hm	16	271	treat +(1/bird)	treat +(1/bird)	lme [n]
Oocyst prevalence	prev	17	271	treat*hm + (1/bird)	treat*hm + (1/bird)	lmer[b]
Oocyst burden	log (burd)	18	271	treat*hm + (1/bird)	treat*hm + (1/bird)	lme [n]
<i>Hemocyte density</i>						
Wing size	size	19	39	treat	1	glm [n]
Nb total hemocytes, day 17	hct	20	18	treat	1	glm [n]
Nb total hemocytes through time ("old" cohort)	hct	21	39	time	time	glm [n]
Nb granulocytes, day 17	grn	22	18	treat	1	glm [n]
Nb granulocytes through time ("old" cohort)	grn	23	39	time	1	glm [n]
Nb oenocytoids, day 17	log (oen)	24	18	treat	1	glm [n]
Nb oenocytoids through time ("old" cohort)	log (oen)	25	39	time	time	glm [n]

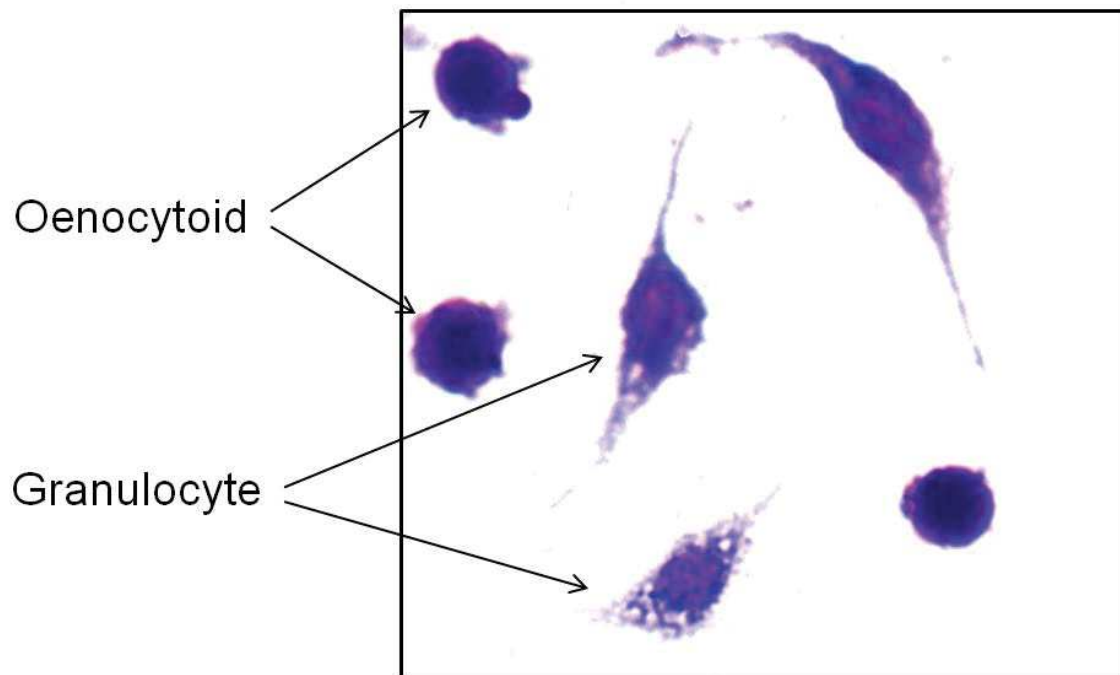


Figure S1: Optical microscope (x400) image of *Cx pipiens* hemocytes showing the two different morphotypes described in this study: granulocytes and oenocytoids (see main text for details).

Chapitre 3 :

Effets transgénérationnels de l'infection

Chapitre 3 : Effets transgénérationnels de l'infection

Article publié dans « *Biology letters* »

Contexte

- Les effets transgénérationnels d'une infection ont été étudiés chez plusieurs espèces d'invertébrés. Il a notamment été mis en évidence que chez certaines d'entre elles le système immunitaire des descendants pouvait être impacté positivement en réponse aux expériences immunitaires des parents.
- Dans le cadre des maladies infectieuses à transmission vectorielle, l'étude des effets transgénérationnels de l'infection chez les vecteurs est particulièrement pertinente. En effet, un transfert de protection pourrait impacter directement la dynamique de transmission des agents pathogènes.
- Nous avons testé les effets transgénérationnels de l'infection des moustiques par *Plasmodium* sur la fécondité et la susceptibilité de leurs descendants à ce même parasite.

Résultat

- Alors qu'aucun transfert transgénérationnel de protection n'a été observé suite à l'infection des moustiques par *Plasmodium*, un fort effet négatif sur la fécondité des descendants a été mesuré.

Conclusion

- Le transfert transgénérationnel de protection ne semble pas présent chez toutes les espèces d'invertébrés.
- L'infection des moustiques par *Plasmodium* va, cependant, pouvoir engendrer des coûts non négligeables sur la fitness des descendants.



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Transgenerational effect of infection in *Plasmodium*-infected mosquitoes

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Transgenerational effects of infection have a huge potential to influence the prevalence and intensity of infections in vectors and, by extension, disease epidemiology. These transgenerational effects may increase the fitness of offspring through the transfer of protective immune factors. Alternatively, however, infected mothers may transfer the costs of infection to their offspring. Although transgenerational immune protection has been described in a dozen invertebrate species, we still lack a complete picture of the incidence and importance of transgenerational effects of infection in most invertebrate groups. The existence of transgenerational infection effects in mosquito vectors is of particular interest because of their potential for influencing parasite prevalence and intensity and, by extension, disease transmission. Here we present what we believe to be the first study on transgenerational infection effects in a mosquito vector infected with malaria parasites. The aim of this experiment was to quantify both the benefits and the costs of having an infected mother. We find no evidence of transgenerational protection in response to a *Plasmodium* infection. Having an infected mother does, however, entail considerable fecundity costs for the offspring: fecundity loss is three times higher in infected offspring issued from infected mothers than in infected offspring issued from uninfected mothers. We discuss the implications of our results and we call for more studies looking at transgenerational effects of infection in disease vectors.

1. Introduction

The environmental conditions experienced by an organism can have a drastic effect on the outcome of infection in its offspring. Factors such as nutritional availability, crowding conditions or the temperature experienced by mothers can affect the ability of their offspring to withstand an infection [1,2]. When such transgenerational maternal effects result in a net increase in the fitness of offspring, they are usually assumed to be adaptive [3]. One particular instance of such adaptive transgenerational maternal effects occurs when infected mothers transfer some level of disease protection to their offspring. In vertebrates such transgenerational disease protection has been extensively investigated and shown to be largely mediated through the transfer of immunoactive compounds such as antibodies [4]. Demonstrating the existence of transgenerational immune protection in invertebrates has, however, been much more slow. Invertebrates have been long thought to have the most basic of immune systems, allowing them to defend themselves against a wide range of pathogens, but lacking the two defining characteristics of the vertebrate immune system: specificity and memory. In the last decade this paradigm has, however, been entirely overhauled.

The first reported instances of transgenerational immune protection in invertebrates, published over 10 years ago, constituted a paradigm shift in our understanding of how the invertebrate immune system works [5]. Since then, transgenerational immune protection has been reported in a range of invertebrate species, including several insects (electronic supplementary material, table S1).

This form of immune protection is, however, not expected to be widespread in invertebrates. Following a well-established evolutionary biology maxim, transgenerational immune protection should evolve only if the benefits of immune transfer (protection against pathogens) outweigh its costs. Two *sine qua non* conditions for the evolution of transgenerational protection are that (i) the pathogen induces a fitness cost on the offspring and (ii) the offspring have a high probability of encountering the same pathogen as their parents. As a result, the overwhelming majority of transgenerational protection studies have focused on aquatic, low-dispersing or eusocial invertebrates (electronic supplementary material, table S1). We therefore lack a complete picture of the incidence and importance of transgenerational protection in other invertebrate groups. More generally, we also lack information about what other transgenerational effects may be passed on from infected mothers to their offspring. There are numerous examples of maternal effects decreasing, as opposed to increasing, offspring fitness [3], resulting in offspring that may be more easily infected or less able to withstand an infection. Either way, it is clear that maternal effects of infection can have drastic consequences on disease dynamics.

Many invertebrates act as vectors of human and animal diseases. Transgenerational effects of infection have a huge potential to influence the prevalence and intensity of infections in vectors and, by extension, disease epidemiology. Despite this, to our knowledge no study has investigated whether having an infected mother influences the outcome of infection in any vector of disease. Here we investigate this question by focusing on the avian malaria parasite *Plasmodium relictum* and its natural vector, the mosquito *Culex pipiens*. Recent work has indeed shown that a *Plasmodium* infection triggers a long-lived response in mosquitoes [6], opening up the possibility that this memory-like response is transmitted to the offspring. Transgenerational immune protection against avian malaria may be expected to evolve in *C. pipiens* mosquitoes because this species is highly ornitophylic and avian malaria infections are extremely prevalent in the field: in some areas, the proportion of infected birds can be as high as 90% (R. Pigeault *et al.* 2014, unpublished data). In addition, avian malaria incurs fitness costs in *C. pipiens* mosquitoes: infected females lay, on average, 30% fewer eggs than uninfected ones [7].

The aim of this study was twofold: (i) to test whether the prevalence and/or the intensity of a *Plasmodium* infection is lower in mosquitoes issued from infected mothers and (ii) to test whether having an infected mother incurs fecundity costs for the offspring.

2. Material and methods

Complete Material and methods are provided in the electronic supplementary material. The experiment spanned two consecutive mosquito generations, henceforth called maternal (or F_0) and offspring (or F_1) generations. The aim of the experiment was to quantify infection success and fecundity in four kinds of F_1 females: uninfected F_1 females issued from either uninfected F_0 mothers or infected F_0 mothers, and infected F_1 females issued from either uninfected F_0 mothers or infected F_0 mothers.

For the maternal (F_0) blood meal, mosquitoes were randomly allocated to feed on either a *Plasmodium*-infected ($n = 4$) or an uninfected bird ($n = 4$). The success of the infection was verified by dissecting a subsample of the mosquitoes. The egg rafts laid

by mosquitoes within a treatment (infected or uninfected) were pooled together in a tray until the eggs hatched. Larvae issued from infected and uninfected mothers were randomly seeded into 18 plastic trays (nine for each treatment) and reared to adulthood in cages following standard protocols [7].

For the offspring (F_1) blood meal, 20 experimental cages were set up, each containing 40 female mosquitoes issued from an infected mother and 40 issued from an uninfected mother randomly sampled from the 18 infected and uninfected cages. Mosquitoes originating from infected and uninfected mothers were marked with different-coloured fluorescent powders for subsequent identification (electronic supplementary material). Cages were then randomly provided with either an infected ($n = 10$) or an uninfected ($n = 10$) bird.

To obtain an estimate of the blood meal size, all engorged F_1 females were placed individually in numbered plastic tubes until all haematin was excreted. Mosquitoes were then transferred to a tube containing mineral water to allow the females to lay their eggs. We quantified female size, number of eggs laid, larvae emerged and haematin excreted as previously published [7].

To estimate the prevalence and intensity of the *Plasmodium* infection in the F_1 generation, 7 days after the blood meal, 20 females (10 issued from uninfected and 10 from infected mothers) from each of the infected cages were randomly sampled and dissected to count the number of oocysts in their midguts.

(a) Statistical analysis

Analyses were carried out using the R statistical package (v. 3.1.0). The statistical models built to analyse the data (numbered m1–m10) are described in the electronic supplementary material, table S2. Briefly, when the response variable was a proportion, the data were analysed using a linear mixed effects model with a binomial error distribution (lmer). Otherwise, data were log or Box Cox transformed and a normal error distribution was used (lme). Count data were analysed using a glm with a quasi-Poisson error distribution, to correct for overdispersion. Laying date was analysed using Cox proportional hazards mixed effects models (coxme). *A posteriori* contrasts were carried out by aggregating factor levels together and by testing the fit of the simplified model using a likelihood ratio test.

3. Results and discussion

The aim of this experiment was to quantify both the benefits (protection from infection) and the costs (effects on fecundity) for mosquitoes of having a *Plasmodium*-infected mother. We found no effect of a *Plasmodium* infection in the maternal generation on either the probability (m1: $\chi^2_1 = 0.752$, $p = 0.386$, figure 1a) or the intensity (m2: $\chi^2_4 = 0.1357$, $p = 0.7126$, figure 1b) of an infection in the offspring. Our results, therefore, conclusively show no evidence for a transgenerational transfer of protection in response to a *Plasmodium* infection in mosquitoes.

Previous work has shown that the offspring of *Aedes aegypti* mosquitoes primed using negatively charged Sephadex beads were no better at melanizing beads than the offspring of naive ones [8], a result that seems to agree with the findings of our study. Whether the quantification of a single immune pathway suffices to prove the existence, or lack thereof, of transgenerational immune protection is, however, debatable. The use of a few immune assays as a proxy for parasite resistance has come under increased scrutiny, as evidence accumulates that they are not necessarily correlated with each other [9]. This question is highly relevant because the overwhelming majority of published studies have used immune measurements taken from the offspring of infected mothers to infer resistance (electronic

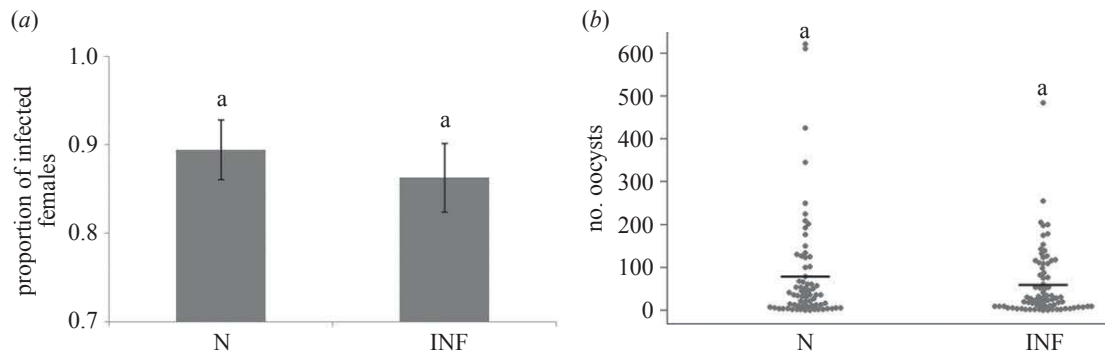


Figure 1. Effect of the infection status of the maternal (F_0) generation on the (a) infection prevalence and (b) oocyst burden in the F_1 offspring. (a) Mean ($\pm$ s.e.) proportion of females infected; (b) number (dots) and median number (horizontal line) of oocysts. N, uninfected mothers; INF, infected mothers.

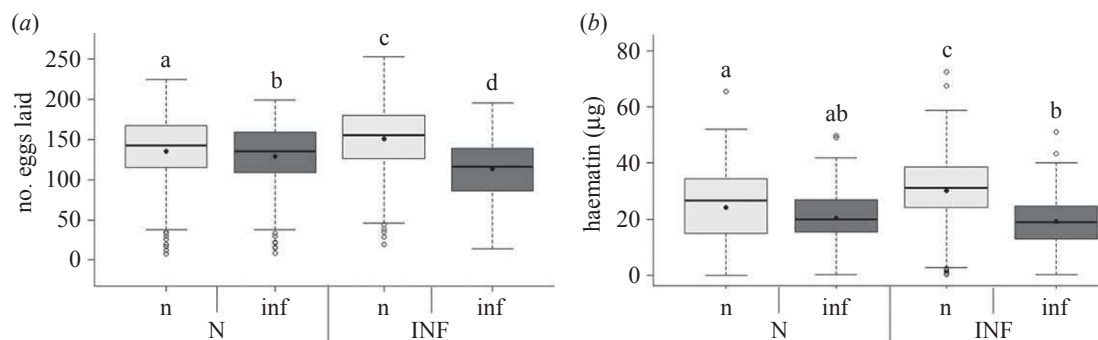


Figure 2. Effect of the infection status of the maternal (F_0) generation on the fecundity (a) and blood meal size (b) of uninfected (light grey) and infected (dark grey) F_1 offspring issued from uninfected (N) or infected (INF) F_0 mothers. Boxplots represent the means (points) and medians (horizontal lines). Boxes above and below the medians show the first and third quartiles, respectively. Dashed lines delimit 1.5 times the inter-quartile range, above and below which individual counts are considered outliers and marked as empty circles.

supplementary material, table S1). Further studies are, in our opinion, needed to establish whether the outcome of these assays translates into increased protection against parasites.

Having an infected mother does not have any measurable benefits in terms of parasite protection, but it does, however, entail considerable costs. Offspring of infected and uninfected mothers have the same probability of laying an egg raft (m3: $\chi^2_1 = 0.35$, $p = 0.5509$), but infected offspring issued from uninfected mothers lay on average 10 ± 3 (mean $\pm$ s.e.) fewer eggs per egg raft than uninfected ones, which represents a fecundity loss of ca 8%. This fecundity loss is tripled in infected offspring issued from infected mothers: infected offspring lose as many as 38 ± 2 eggs (25%) compared with uninfected ones (m4: $\chi^2_1 = 24.46$, $p < 0.0001$, figure 2a). As a result, the highest fecundity costs are paid by infected offspring issued from infected mothers. A similar trend, albeit of smaller amplitude, was observed for the number of hatched larvae (m6: $\chi^2_1 = 13.325$, $p = 0.0002$). One potential explanation for these results is that the offspring of infected mothers avoided feeding on the infected birds, which could be equated to a form of transgenerational behavioural protection. We have, however, shown that both infected and uninfected mosquitoes have a preference for feeding on infected birds [10], making this explanation unlikely, although worth testing in the future.

Previous work on this system has shown that *Plasmodium*-infected mosquitoes lay their eggs significantly earlier than uninfected ones, possibly to compensate for the strong and progressive decrease in the quality of eggs laid by these females [11]. Our results agree with this: infected females lay egg rafts earlier than uninfected ones (m7: $\chi^2_1 = 4.52$, $p = 0.047$,

although this effect is independent of the infection status of the mother (m7: $\chi^2_1 = 0.0181$, $p = 0.8931$).

Parental infection has been reported to impact several life-history traits of the offspring [12]. To our knowledge, however, the actual mechanisms underlying changes in life-history traits in offspring have never been investigated. To deepen our insight into the mechanisms underlying the large differences in fecundity costs in offspring issued from infected and uninfected mothers, we analysed the blood meal size (haematin) of mosquitoes. Blood meal size is a strong predictor of fecundity in this system [7]. Offspring that feed on infected birds secrete significantly less haematin than those that feed on uninfected ones (m8: $\chi^2_1 = 11.49$, $p = 0.0035$). This result may be due to smaller blood meals and/or to the confounding effect of anaemia [7]. The difference in haematin between infected and uninfected offspring is, however, much more marked when the mother is infected (m9: $\chi^2_1 = 24.17$, $p < 0.0001$, figure 2b). These differences in blood meal size cannot be explained by differences in body size (m10: $\chi^2_1 = 2.46$, $p = 0.328$). When haematin was added as a covariate in our statistical model, the significance of the F_0 and F_1 infection treatment on fecundity was maintained (m5: $\chi^2_1 = 10.22$, $p = 0.0014$), implying that the fecundity costs observed are not entirely mediated through an alteration of blood meal size and that other, as yet unknown, mechanisms are at play.

4. Conclusion

Whether the absence of transgenerational immune protection we report is specific to this particular mosquito–parasite

combination or whether it is a general attribute of mosquito vectors requires further investigation, because of its potential epidemiological consequences [13]. Transgenerational acquired immunity has thus far been described in a dozen invertebrate species, the large majority of which are either aquatic, eusocial or stored-product species (electronic supplementary material, table S1), life histories that favour a common mother–offspring environment and thus the evolution of an immune transfer across generations. Whether the scarcity of published negative results indicates that transgenerational protection is the rule rather than the exception in the invertebrate world or whether it reflects a publication bias deserves further consideration.

Either way a more complete picture of the transgenerational effects of infection across the invertebrate world is needed.

Ethics statement. Experiments were approved by the Ethical Committee for Animal Experimentation established by the authors' institution (permit number CEEA- LR-1051).

Data accessibility. Data for this study are available at Dryad (doi:10.5061/dryad.ht79k).

Author contributions. R.P., J.V., S.G. and A.R. conceived and designed the experiment. R.P. and A.N. performed the experiment. R.P. analysed the data. R.P., S.G. and A.R. wrote the paper.

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Competing interests. We declare we do not have any competing interest.

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Supplementary Materials

Detailed description of the materials and methods

We used a lineage of *Plasmodium relictum* known as SGS1, the aetiological agent of the most prevalent form of avian malaria in Europe, to infect canaries (*Serinus canaria*) following our standard laboratory protocols (Vézilier et al 2012). *Culex pipiens* mosquitoes (SLAB strain) were reared and infected following standard protocols (Vézilier et al 2012). Under these conditions 80-90% of blood-fed mosquitoes exposed to an infected bird become infected.

The experiment spanned two consecutive mosquito generations, henceforth called maternal (or F0) and offspring (or F1) generations. The aim of the experiment was to quantify infection success and fecundity in four kinds of F1 females: uninfected F1 females issued from uninfected F0 mothers, uninfected F1 females issued from infected F0 mothers, infected F1 females issued from uninfected F0 mothers and infected F1 females issued from infected F0 mothers.

For the maternal (F0) blood meal, mosquitoes were haphazardly allocated to feed on either a *Plasmodium*-infected or an uninfected bird (n=4 infected, n=4 uninfected birds, 50 mosquitoes per bird). After the blood meal, all cages were provided with a water-filled container for 5 days, to allow oviposition. A subsample of 15 mosquitoes per cage was dissected 7 days after the blood meal to verify the success of the infection (oocyst count). The egg rafts from each of the 4 cages within a treatment (infected or uninfected) were pooled together in a tray until the eggs hatched. Larvae were then haphazardly seeded into plastic trays (9 trays per treatment, 300 larvae per tray). Each tray was placed in an "emergence cage" and larvae were thus raised to adulthood following the standard rearing conditions (Vézilier et al 2012).

For the offspring (F1) blood meal, 20 experimental cages were set up. Each cage contained 40 female mosquitoes issued from an infected mother and 40 issued from an uninfected mother. These females were haphazardly sampled from the emergence cages (roughly 5 females from each of the 9 emergence cages in each treatment). Mosquitoes issued from infected and uninfected mothers were marked using different fluorescent colour powders (Vézilier et al 2012). Cages were then haphazardly provided with either an infected (n= 10) or an uninfected (n=10) bird.

To obtain an estimate of the blood meal size, all engorged F1 females were placed individually in numbered plastic tubes until all haematin (a product of the degradation of haemoglobin) was excreted. Mosquitoes were then transferred to an "oviposition tube" containing 4 ml of mineral water to allow the females to lay their eggs. The quantification of the number of eggs laid, larvae

emerged, and haematin excreted took place following previously published protocols (Vézilier et al 2012). Female size was estimated by measuring its wing length.

To estimate the prevalence and intensity of the *Plasmodium* infection in the F1 generation, seven days after the blood meal, twenty females from each of the infected cages (ten issued from uninfected and ten from infected mothers) were haphazardly sampled and dissected to count the number of oocysts in their midguts.

Table S1 : Summary of invertebrate TGIP reported to date

	Immune stimulation (F0)	Immune stimulation (F1)	Effects on immunity / parasite resistance (F1)	Effects on life history traits (F1)	Refs
Molusca					
<i>Chlamys farreri</i>	HK Bacteria	Bacteria/fungus	(+) AB activity, (+) Immune-related proteins	(+) survival	[1]
Crustacea					
<i>Daphnia magna</i>	Bacteria	same as F0	(-) Parasite prevalence	(+) reproductive output, early reproduction	[2]
<i>Penaeus sp.</i>	Glucane	Virus	<i>ni</i>	(+) survival	[3]
Hymenoptera					
<i>B. terrestris</i>	LPS	none	(+) PO activity	<i>ni</i>	[4]
<i>B. terrestris</i>	HK bacteria	LPS	(+) AB activity	<i>ni</i>	[5]
<i>B. terrestris</i>	HK bacteria	same as F0	(+) AB activity	<i>ni</i>	[6]
<i>B. terrestris</i>	HK bacteria	HK bacteria/Tripanosoma	(+) AB activity (-) Tripanosoma intensity	<i>ni</i>	[7]
<i>A. mellifera</i>	Bacteria	none / same as F0	(+) haemocytes	(+) survival	[8]
Diptera					
<i>D. melanogaster</i>	Bacteria	same as F0	no effect	(-) survival	[9]
<i>Ae. aegypti</i>	Sephadex beads	same as F0	no effect	no effect	[10]
Coleoptera					
<i>T. mollitor</i>	LPS	same as F0	(+) AB activity	(+) survival	[11]
<i>T. mollitor</i>	LPS	same as F0	(+) haemocytes ¹ , (+) PO activity	<i>ni</i>	[12]
<i>T. mollitor</i>	LPS	none ²	(+) AB activity	<i>ni</i>	[13]
<i>T. mollitor</i>	LPS	none ²	(+) AB activity	<i>ni</i>	[14]
<i>T. castaneum</i>	HK Bacteria	Bacteria	(+) PO activity ¹ , (+ -) AB activity ^{3,1} , (-) Parasite prevalence ¹	(+) survival ¹ , (-) fecundity ¹ , (+) develop. time	[15]
<i>T. castaneum</i>	HK Bacteria	Bacteria	(+) PO activity ¹ , (-) Parasite prevalence ¹ , (+) Immune transcripts ¹	(+) survival ¹	[16]
Lepidoptera					
<i>T. ni</i>	Bacteria	none	(+) PO activity, (+) Immune transcripts	(-) developmental time	[17]
<i>P. interpunctella</i>	Virus	same as F0	(-) Parasite prevalence	<i>ni</i>	[18]
<i>M. sexta</i>	PGN	none / PGN	(+) PO activity, (+ -) AB activity ⁴ / (+) AB activity	(-) fecundity / <i>ni</i>	[19]
<i>G. mellonella</i>	Bacteria	none ²	(+) Immune transcripts	<i>ni</i>	[20]
Orthoptera					
<i>T. oceanicus</i>	Bacteria (<i>S. marcescens</i>)	none	(+) Litic activity ⁵	(-) Sperm viability , ovary mass	[21]

AB: antibacterial, PO: phenoloxydase, HK: heat-killed, LPS: lipopolysaccharide (*E coli*), PGN: peptidoglycan, *ni*: not investigated. (+) increase, (-) decrease, (+ -) increases/decreases under different circumstances. ¹ Paternal immune priming ² Analysis make on eggs ³ AB activity increased in the F1 when the paternal (F0) generation injected with HK *B. thuringiensis*, decreased when F0 infected with *E coli*; ⁴ AB activity increased in larvae and pupae, decreased in adults; ⁵ Effect observed only in male

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Table S2 : Description of statistical models used to analyze the data. The response variable was not transformed unless otherwise stated (superscript indicates λ in boxcox transformation). N gives the number of mosquitoes included in each analysis. "Maximal model" gives the complete set of explanatory variables (and their interactions) included in the model. "Minimal model" gives the model containing only the significant variables and their interactions. Round brackets indicate variables fitted as random factors (1/Bird is the way to note that bird is a random variable). Square brackets indicate the error structure used (n: normal errors, b: binomial errors, qp: quasipoisson errors). inf F0: infection status of the maternal generation (infected/uninfected), infF1 : infection status of the offspring generation (infected/uninfected), hm: haematin excreted (blood meal size), size: wing length.

Variable of interest	Resp. variable	Model Nb	N	Maximal model	Minimal model	R subroutine [error struct]
<i>Plasmodium</i> infection						
Prob. infection	inf	1	165	inf F0* hm + (1/Bird)	1 + (1/Bird)	lmer [b]
Oocyst burden	Log (oob)	2	145	inf F0* hm + (1/Bird)	1 + (1/Bird)	lme [n]
Egg laying						
Egg laying probability	laid	3	1085	F0* F1*hm + (1/Bird)	F0*hm+ (1/Bird)	lmer [b]
Number of eggs	eggs	4	796	F0* F1 + (1/Bird)	F0*F1+ (1/Bird)	lme[n]
Number of eggs	eggs	5	796	F0* F1*hm + (1/Bird)	F0*F1+hm+ (1/Bird)	lme[n]
Hatching success	hatch	6	796	F0*F1 + (1/Bird)	F0* F1 + (1/Bird)	lmer [b]
Laying date	date	7	1085	F0* F1 + (1/Bird)	F1 + (1/Bird)	coxme
Haematin	hm ^{1/2}	8	1085	F1 + (1/Bird)	F1 + (1/Bird)	lme [n]
Haematin	hm ^{1/2}	9	1085	F0*F1 + (1/Bird)	F0*F1 + (1/Bird)	lme [n]
Wing size	size	10	353	F0*F1	1	glm [n]

Chapitre 4 :

Evolution de l'immunité transgénérationnelle chez les invertébrés

Chapitre 4 : Evolution de l'immunité transgénérationnelle chez les invertébrés

Article en préparation

Contexte

- Le transfert transgénérationnel de protection peut avoir des impacts importants à la fois sur la fitness des individus mais également sur la dynamique de transmission des agents pathogènes.
- Chez les invertébrés ce transfert de protection ne semble pas universel. Cependant, aucune étude n'a mis en évidence les paramètres responsables de l'évolution de ce mécanisme de défense.
- A l'aide de modèles théoriques et d'une revue de la littérature nous avons cherché à déterminer si le transfert parental de protection pouvait être gouverné par des facteurs écologiques propres aux espèces.

Résultats

- Les modèles théoriques ont mis en évidence que plusieurs paramètres écologiques pouvaient être responsables de l'évolution du transfert transgénérationnel de protection chez les invertébrés.
- L'analyse des données empiriques a confirmé en partie ces résultats. En effet, le transfert de protection est plus enclin à être observé chez les espèces philopatriques et ayant une espérance de vie relativement longue.

Conclusion

- L'évolution du transfert de protection semble donc en partie gouvernée par des facteurs écologiques. Cependant, davantage de données sur un plus grand nombre d'espèces sont nécessaires pour obtenir une vision plus globale de ce mécanisme de défense chez les invertébrés.

Evolution of transgenerational immunity in invertebrates

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Abstract: The transfer of immunity across generations has been found in several invertebrate species. Yet, the ecological factors driving the evolution of this trait remain poorly studied. Here we develop a theoretical host-parasite model and predict that long lifespan and low dispersal should promote the evolution of transgenerational immunity. We review experimental studies performed in 18 invertebrate species and study the association between host longevity and dispersal on the transfer of resistance and immunity to offspring. We found a positive correlation between these traits but the limited number of studies, the heterogeneity of the experimental procedures and the uneven distribution of host species used does not allow us to demonstrate a causative link between these ecological factors and transgenerational immunity. We discuss the implications of this work for the study of the evolution of immunity in invertebrate species.

Key words: transfer of protection, dispersal, longevity, drosophila, mosquitoes, eusocial insect

1. Introduction

The immunity of invertebrates is often assumed to be relatively simple and to lack the ability to develop adaptive immune responses (Hoffmann *et al.*, 1996). Yet, invertebrates have spectacularly plastic immune effectors that can generate true novelty and functional immune response changes in relation to previous expositions to pathogens (Ziauddin & Schneider, 2012; Armitage *et al.*, 2014). Experimental evidence of the existence of within-generation immune priming in invertebrates has grown considerably in the last decade (Schmid-Hempel, 2005; Best *et al.*, 2013). It has been documented in a range of invertebrate species including Decapoda (Witteveldt *et al.*, 2004), Brachiopoda (Little *et al.*, 2003), Lepidoptera (Tidbury *et al.*, 2011), Coleoptera (Roth *et al.*, 2009), Diptera (Rodrigues *et al.*, 2010) and Hymenoptera (Sadd & Schmid-Hempel, 2006). This immune priming seems to persist not only during the whole lifespan of the animal (Jacot *et al.*, 2005; Thomas & Rudolf, 2010) but also across generations.

Parental transfer of immunity, has been well documented in vertebrates (Hasselquist & Nilsson, 2009), and is increasingly being studied in invertebrates. It has been observed in a dozen invertebrate species (Huang & Song, 1999; Little *et al.*, 2003; Sadd *et al.*, 2005; Moret, 2006; Roth *et al.*, 2010; Yue *et al.*, 2013; Freitak *et al.*, 2014; López *et al.*, 2014; McNamara *et al.*, 2014; Fisher & Hajek, 2015; Norouzitallab *et al.*, 2015) but it is far from being widespread. Indeed, several studies have failed to detect any transgenerational transfer of immunity (Voordouw *et al.*, 2008; Linder & Promislow, 2009; Pigeault *et al.*, 2015). Others have even found a negative impact of maternal infection on offspring immunocompetence (Vantaux *et al.*, 2014). This variation among invertebrate species is puzzling. What factors may promote the evolution of transgenerational immunity?

In this study, we investigate whether the presence or absence of transgenerational immune protection can be explained by factors related to the biology of species. Following the theoretical approach developed by Garnier *et al.* (2012) we study the impact of various ecological factors on the evolution of invertebrate species. We explore the effects of host dispersal, host lifespan, immunity costs, force of infection and parasite virulence on the evolution of transgenerational immunity. We confront these predictions to the available experimental evidence obtained in invertebrate species. In particular, we focus on two of these predictions, namely that transgenerational immunity should evolve more readily in long-lived and philopatric species.

2. Materials and Methods

Theoretical analysis

The evolution of maternal transfer of immunity has been studied elsewhere in a single host population (see Garnier *et al.*, 2012). To study the effect of various factors on the evolution of maternal transfer of immunity in invertebrates we expand the previous theoretical model and consider two host populations (1 and 2) connected by migration (see supplementary information). Each population is assumed to be exposed to a different pathogen and the pathogen is not allowed to migrate between populations, which maximizes the heterogeneity of the environment. In population i (where $i = 1$ or 2) susceptible individuals, S_i , are exposed to a constant rate of infection h_i which yields infected individuals, I_i . We assume that infected individuals mount enough of an immune response to transmit transient immunity to some of their offspring with a probability θ . The ability to transfer immunity is further assumed to be associated with a fecundity cost c_θ . Infected individuals remain infected until death occurs, either naturally, with rate μ , or through parasite related mortality (i.e. virulence), with rate α . All individuals can produce offspring that can move to a different patch and η refers to the probability of dispersal. In the supplementary information we detail the derivation of the evolutionary stable investment in maternal transfer of immunity. We keep track of the origin of the maternally protected individuals using the notation M_{ij} for the density of maternally protected individuals produced in population i and currently in population j (where i and $j = 1$ or 2). Hence M_{ij} is immune to parasites from population i but only partially immune to pathogen from population j . The amount of cross immunity is governed by the parameter χ and the force of infection on M_{ij} is $(1 - \chi) h_j$, with $0 \leq \chi \leq 1$. Maternal protection is assumed to be transitory and it wanes at rate δ_M in all populations.

Empirical data: transgenerational effect scores

We carried out an extensive literature review (Table S1) to examine whether the different ecological factors identified in the theoretical model affect invertebrate immunity in the predicted way. For this purpose, we collected data from 27 published articles comprising a total of 18 invertebrate species. We identified two different types of transgenerational immune priming studies. First, a set of studies ($n=18$) investigate the impact of parental infection or immune stimulation on offspring immunity (henceforth **TEI**, for **Transgenerational Effect on Immunity**). These studies quantify and compare the

immunity of offspring issued from immune stimulated and naïve parents in different ways (melanisation, PO production, antibacterial peptide production, haemocyte number and immune transcripts) but the offspring remain uninfected. For simplicity, we scored these studies as either 1 (offspring of infected parents have an increased production of at least one of the immune effectors quantified) or 0 (offspring of infected parents have similar or lower production of a given immune effector). Second, we identified another set of studies ($n=16$) where both parents and offspring are exposed to living pathogens. These studies record the outcome of these infections in terms of parasite prevalence, parasite intensity or offspring survival (**TER for Transgenerational Effect on Resistance**), but no measurement of offspring immunity is obtained. As above, these studies were scored as either 1 (offspring from infected parents have lower prevalence, lower intensity or higher survival than offspring from naïve parents) or 0 (when the opposite or when no effect of parental infection is observed). In a limited number of cases ($n=7$) both TER and TEI studies have been carried out. Finally, we combined both types of studies to generate a synthetic measure of parental investment in offspring protection (**OTP for Overall Transgenerational Protection**). In the limited number of cases ($n=2$) where several studies were performed on the same invertebrate species with contradictory results (e.g. a TEI score of 1 in one of study and of 0 in another study) we adopted a "false negative" strategy and allocated the score of 1 to the entire species.

For each species, lifespan and dispersal data were obtained from the literature (Table S1). We define lifespan as the maximum estimated total longevity of a species, and dispersal, as the maximal estimated distances actively travelled by adults. It is, however, important to note that the protocols used to measure these parameters vary from one study to the next. In particular, whenever possible, we used estimates of lifespan and distance obtained under field conditions, but when this data was not available data obtained in the laboratory was used (Table S1).

Statistical analysis

All statistical analyses were performed using the software R (v.3.1.0, <http://www.cran.r-project.org/>). In order to compare TEI TER and OTP we first carried out a Fisher's exact test using longevity and dispersal as categorical variables. Species were classified as having a short (≤ 50 days) or long (> 50 days) lifespan, and those with a short (< 500 m) and long (> 500 m) dispersal range. This type of simplified analyses, however, obviates the fact that species are phylogenetically correlated and are therefore not statistically

independent units. We therefore carried a second analysis using a generalized least-square (gls) model that corrects for phylogenetic correlations in the model (packages 'nlme'). In these models the significance of the explanatory variables was established using a likelihood ratio test (which is approximately distributed as a Chi-square distribution, Bolker, 2008). Phylogenetic information (**Figure S1**) for the 18 species was obtained from the Interactive Tree of Life (<http://itol.embl.de>). Because the phylogenetic tree had no branch length, we used a Grafen correlation matrix (Grafen, 1989).

3. Results

Theory

We explored the effect of the different parameters of the model on the evolution of maternal transfer of immunity. As expected, we show that increasing the force of infection h or decreasing the cost c_θ associated with the transfer of immunity always selects for higher values of θ . As pointed out by Garnier et al. (2012) pathogen virulence has a non-monotonic effect on the evolution of θ . Both avirulent and very virulent pathogens select for low levels of maternal transfer of immunity. Indeed, when virulence becomes very high it is not worth investing in a resistant mechanism that will never be expressed as infected individuals have very little opportunity to reproduce before they die from the infection. High levels of investment in θ are only selected when pathogens induce an intermediate reduction in longevity. We also retrieve the effect of longevity discussed in Garnier et al. (2012). Short lived species do not invest in immunity because the survival benefit associated with immunity is overwhelmed by the intrinsic mortality rate, μ (**Figure 1A**). Our model allowed us to explore the effect of dispersal and cross immunity on the evolutionary outcome. When dispersal is high and cross immunity is low the investment of the mother is unlikely to protect the offspring because it will be exposed to a different pathogen. Consequently, higher investment in maternal transfer is only expected to evolve in philopatric species or in species with high levels of cross immunity (**Figure 1B**).

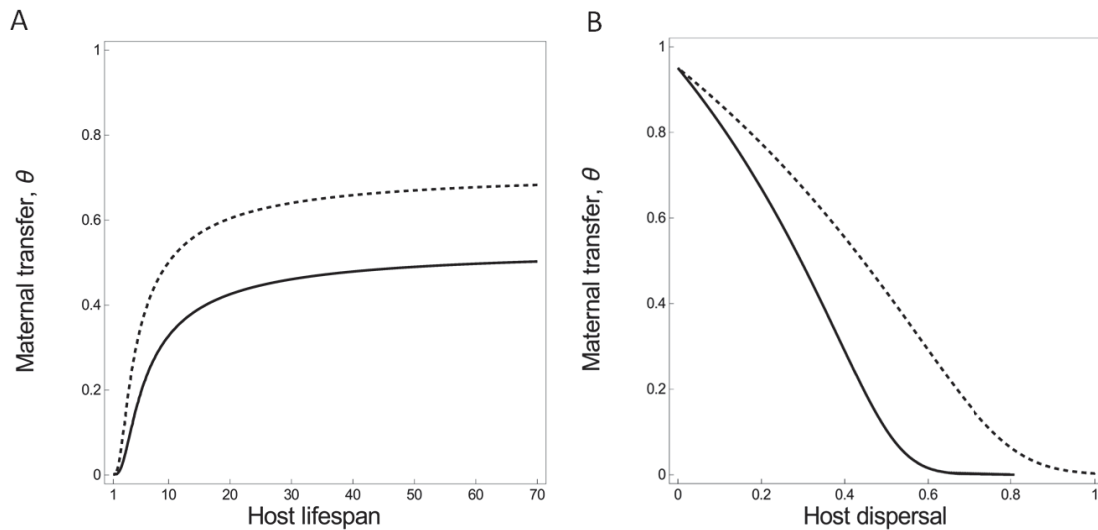


Figure 1: Evolutionary stable investment in the transfer of immunity θ with (dotted line, $\chi=0.5$) or without cross-immunity (full line, $\chi=0$) against (A) the longevity of the host and (B) the dispersal of the host. The underlying model we use is detailed in the supplementary information. Default parameter values used: $r_0 = 1.5$, $c_\theta = 0.1$, $k = 1.1$, $\delta_M = 1$, $K = 20$, $\mu = 0.02$, $h = 1$, $\eta = 0.3$.

Empirical data

We focused our attention on two key parameters of the theoretical models for which sufficient information is available in the literature: lifespan and dispersal. We investigated the impact of these two parameters in each of the scores identified above.

Long-lived (Figure S2A) and short-range dispersal (Figure S2B) species seem to have overall higher TER scores. However, the statistical analyses revealed no statistically significant effects of these two explanatory variables on the TER score (Fisher exact Test, lifespan: $p = 0.128$, dispersal: $p = 0.091$). A similar trend was observed for the TEI score (Figure S2C, S2D) but, again, no statistically significant effect of either dispersal (Fisher Exact Test, $p = 0.428$) or longevity ($p = 0.20$) was detected.

Interestingly, however, both dispersal and lifespan have a significant impact on the overall parental investment in offspring protection as quantified by the OTP score (**Figure 2A, 2B**). Species with long lifespans and short dispersal ranges have significantly higher OTP scores than their short-lived and highly dispersing counterparts (Fisher Exact Test, lifespan: $p=0.0122$, dispersal: $p=0.0149$). To verify whether these results hold when correcting for phylogenetic correlations, the analyses were repeated fitting a parametric generalized least-square model with and without phylogeny. In accordance with the results of the Fisher Exact Test, dispersal has a significant effect on the OTP score when phylogeny is not accounted for ($\chi^2_1 = 5.311$, $p = 0.021$). However, when phylogeny is included in the model the significant effect disappears ($\chi^2_1 = 1.765$, $p = 0.184$), indicating that the significant effect is driven by a

few related taxa. Lifespan, however, has no significant effect on the OTP score, regardless of whether phylogeny was included or not in the model (without: $\chi^2_1 = 1.921$, $p = 0.166$, with: $\chi^2_1 = 0.276$, $p = 0.599$).

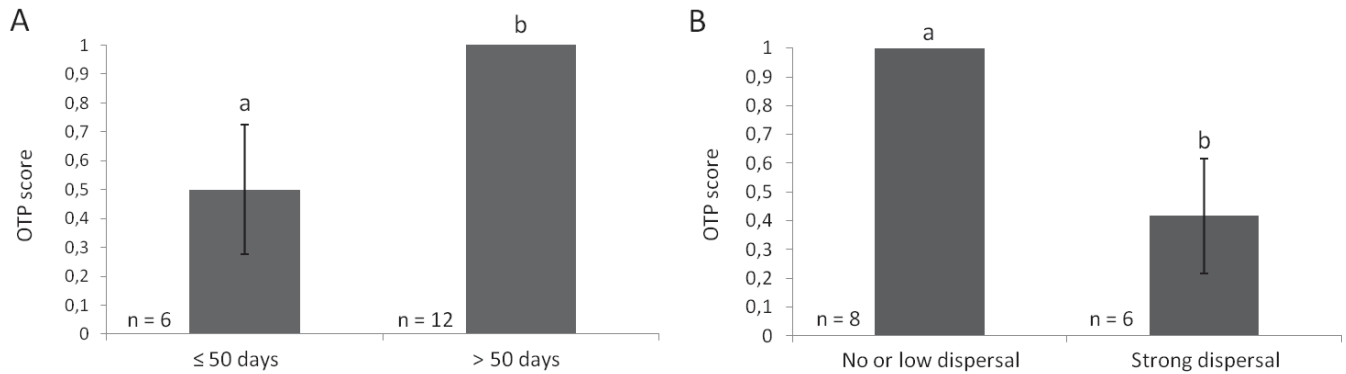


Figure 2: Overall Transgenerational Protection (OTP) score regarding species (A) longevity and (B) dispersal. Levels not connected by same letter are significantly different. Error bars represent $\pm$ SE (statistical test: Fisher's exact test).

4. Discussion

Life history evolution theory shows that various ecological factors may drive the evolution of transgenerational immunity (Garnier *et al.*, 2012; Metcalf & Jones, 2015). In particular, we predict that invertebrate species with a long lifespan and a short dispersal range are more likely to evolve this peculiar form of immunity. Indeed, the marginal gain in fitness obtained from transgenerational immunity is higher in long lived species where an infection can dramatically reduce life expectancy. Philopatry can also promote the evolution of transgenerational immunity because it increases the predictability of the offspring environment.

Here we tried to test the validity of these predictions in invertebrate species. Our analysis provides supporting evidence for the existence of an association between transgenerational immunity and both dispersal and longevity. Yet, it is unclear if this association is driven by the selective forces identified in our theoretical models or by a limited and biased sample size. Indeed, one of the problems for testing the predictions is due to the limited number of published studies on transgenerational immunity in invertebrate species. Besides, most of these studies are realised on species with similar life histories (eusocial, stored product pests, aquatic), where parents and offspring share the

same environment and/or have a long lifespan. This makes biological sense, but it produces a very biased view of what may be happening in insects as a whole. In our data set, only the four *Diptera* species and one *Lepidoptera* species are described as having both a short lifespan and a strong dispersal distance (TableS1). This sampling bias could explain why, when phylogeny is included in statistical analyses, the significant effect of dispersal rate on the transfer of protection disappears.

We currently lack an overall view of the incidence and impact of parental transfer of protection in invertebrates which limits our ability to predict the conditions under which this trait may have evolved. We suggest several types of studies which could be performed in order to test the validity of our predictions. Firstly, it would be interesting to investigate the parental transfer of protection in non-model organism exhibiting a range of life history traits. Terrestrial isopod species would be ideal candidates due to their limited dispersal potential (Beck & Price, 1981) and extended lifespans, which can range between one to more than five years, depending on species (Miller & Cameron, 1983; Alikhan, 1995; Quadros *et al.*, 2009). Secondly, it would be interesting to compare the parental protection between several phylogenetically close species but with very different life histories. The super-family of *Apoidea* is a very good model to perform these comparative studies as this taxon contains both eusocial bees and solitary ones. Finally, an alternative way to test some of these predictions could involve experimental evolution. It might be possible to carry out long term experiments on laboratory-friendly invertebrate species (*Daphnia*, *Drosophila*, *Artemia*, mosquitoes) with or without parasites and to manipulate various ecological factors (e.g. longevity, dispersal) to test their effects on the evolution of parental transfer of protection.

Our analysis points out a major distinction between studies that focus on either (i) the development of an immune response following a challenge (TEI) or on (ii) the outcome of the infection (TER). The overwhelming majority of studies have focused on the former (TEI) and very few studies combine both approaches (n=7). The transfer of a particular immune effector from parent to offspring (TEI) does not necessarily imply that the transfer is adaptive. The use of a few (typically one or two) immune assays as a proxy for parasite resistance has come under increased scrutiny, as evidence accumulates that they are not necessarily correlated with each other (Adamo, 2004). In other words, TEI may or may not be associated with increased protection. Yet, for transgenerational immune protection to evolve offspring have to be protected against parasites (TER). Conversely, however, higher offspring protection does not necessarily imply that the underlying mechanism is

immunological. Other transgenerational protective mechanisms, such as a higher nutritional provisioning of the eggs resulting in fitter offspring, could be at play. We therefore contend that an integrative view of the transgenerational immune memory requires both approaches.

In conclusion, we made predictions concerning the scenarios under which transgenerational immune protection is expected to evolve. Although currently available data seem to confirm an association between transgenerational immune protection and both decreased dispersal and increased lifespan, further experimental studies are needed that expand the range of taxa investigated and that quantify both the degree of protection accrued by the offspring of infected parents, and the underlying immune mechanism.

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Supplementary materials

Theoretical analysis

Following Garnier et al. (2012) we focus on the ability of a mutant host (with a strategy θ^m) to invade a resident host population (with a strategy θ) at equilibrium. The mutant may be present in different populations and in different host states. The densities of the different types of hosts are given in the vector $H^m = (M_{11}^m, M_{21}^m, S_1^m, I_1^m, M_{22}^m, M_{12}^m, S_2^m, I_2^m)^T$. The dynamics of the mutant can be fully described by the matrix F^m which accounts how many mutants are created in the 6 different host types and the matrix V^m which refers to transition between these types:

$$F^m = \begin{pmatrix} 0 & 0 & 0 & \theta^m(1-\eta)\lambda_1^m & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & \theta^m\eta\lambda_1^m \\ (1-\eta)\lambda_1^m & (1-\eta)\lambda_1^m & (1-\eta)\lambda_1^m & (1-\theta^m)(1-\eta)\lambda_1^m & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & \theta^m(1-\eta)\lambda_2^m \\ 0 & 0 & 0 & \theta^m\eta\lambda_2^m & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & (1-\eta)\lambda_2^m & (1-\eta)\lambda_2^m & (1-\eta)\lambda_2^m & (1-\theta^m)(1-\eta)\lambda_2^m \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \end{pmatrix} \quad (S1)$$

$$V^m = \begin{pmatrix} \delta_M + \mu & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & \delta_M + \mu + (1-\chi)h_1 & 0 & 0 & 0 & 0 & 0 & 0 \\ -\delta_M & -\delta_M & \mu + h_1 & 0 & 0 & 0 & 0 & 0 \\ 0 & -(1-\chi)h_1 & -h_1 & \mu + \alpha & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & \delta_M + \mu & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & \delta_M + \mu + (1-\chi)h_2 & 0 & 0 \\ 0 & 0 & 0 & 0 & -\delta_M & -\delta_M & \mu + h_2 & 0 \\ 0 & 0 & 0 & 0 & 0 & -(1-\chi)h_2 & -h_2 & \mu + \alpha \end{pmatrix} \quad (S2)$$

where, $\lambda_i^m = r_0(1 - c_\theta \theta^{m,k})(1 - N_i/K_i)$ is the fecundity of mutant hosts. As in Garnier et al. (2012) the maximal fecundity r_0 is reduced by the cost associated with the mutant strategy θ_m (where c_θ affects the magnitude of the cost and k the shape of the relationship between the investment in maternal transfer of immunity and the cost) and by the density dependence in each host population i .

We can further simplify this model under the additional assumption that the two populations are symmetric: $h_1 = h_2 = h$, $K_1 = K_2 = K$, $N_1 = N_2 = N$, $\lambda_1^m = \lambda_2^m = \lambda^m$. Because of the symmetry we can focus on a single population i and on the vector $H_i^m = (M_{ii}^m, M_{ji}^m, S_i^m, I_i^m)^T$. The dynamics of the mutant can be derived from the matrices F_i^m and V_i^m :

$$\mathbf{F}_i^m = \begin{pmatrix} 0 & 0 & 0 & \theta^m(1-\eta)\lambda^m \\ 0 & 0 & 0 & \theta^m\eta\lambda^m \\ \lambda^m + \delta_M & \lambda^m + \delta_M & \lambda^m & (1-\theta^m)\lambda^m \\ 0 & 0 & 0 & 0 \end{pmatrix} \quad (\text{S3})$$

$$\mathbf{V}_i^m = \begin{pmatrix} \delta_M + \mu & 0 & 0 & 0 \\ 0 & \delta_M + \mu + (1-\chi)h & 0 & 0 \\ 0 & 0 & \mu + h & 0 \\ 0 & -(1-\chi)h & -h & \mu + \alpha \end{pmatrix} \quad (\text{S4})$$

The per-generation growth rate of the mutant is given by the dominant eigenvalue of the matrix $\mathbf{B}_i^m = \mathbf{F}_i^m \cdot \mathbf{V}_i^{m-1}$ which is:

$$\mathbf{B}_i^m = \begin{pmatrix} 0 & \tau_{M_{ji} \rightarrow M_{ii}}^m & \tau_{S_i \rightarrow M_{ii}}^m & \tau_{I_i \rightarrow M_{ii}}^m \\ 0 & \tau_{M_{ji} \rightarrow M_{ji}}^m & \tau_{S_i \rightarrow M_{ji}}^m & \tau_{I_i \rightarrow M_{ji}}^m \\ \tau_{M_{ii} \rightarrow S_i}^m & \tau_{M_{ji} \rightarrow S_i}^m & \tau_{S_i \rightarrow S_i}^m & \tau_{I_i \rightarrow S_i}^m \\ 0 & 0 & 0 & 0 \end{pmatrix} \quad (\text{S5})$$

where $\tau_{X \rightarrow Y}^m$ is the per-generation transition between host type X and Y :

$$\tau_{M_{ji} \rightarrow M_{ii}}^m = \frac{(1-\eta)h\lambda^m(1-\chi)\theta^m}{(\alpha + \mu)(\delta_M + h(1-\chi) + \mu)}$$

$$\tau_{S_i \rightarrow M_{ii}}^m = \frac{(1-\eta)h\lambda^m\theta^m}{(h + \mu)(\alpha + \mu)}$$

$$\tau_{I_i \rightarrow M_{ii}}^m = \frac{(1-\eta)\lambda^m\theta^m}{\alpha + \mu}$$

$$\tau_{M_{ji} \rightarrow M_{ji}}^m = \frac{\eta}{(1-\eta)} \tau_{M_{ji} \rightarrow M_{ii}}^m$$

$$\tau_{S_i \rightarrow M_{ji}}^m = \frac{\eta}{(1-\eta)} \tau_{S_i \rightarrow M_{ii}}^m$$

$$\tau_{I_i \rightarrow M_{ji}}^m = \frac{\eta}{(1-\eta)} \tau_{I_i \rightarrow M_{ii}}^m$$

$$\tau_{M_{ii} \rightarrow S_i}^m = \frac{\lambda^m + \delta_M}{\delta_M + \mu}$$

$$\tau_{M_{ji} \rightarrow S_i}^m = \frac{\delta_M(\alpha + \mu) + \lambda^m(\alpha + h(1-\theta^m)(1-\chi) + \mu)}{(\alpha + \mu)(\delta_M + h(1-\chi) + \mu)}$$

$$\tau_{S_i \rightarrow S_i}^m = \frac{\lambda^m(h(1-\theta^m) + \alpha + \mu)}{(h + \mu)(\alpha + \mu)}$$

$$\tau_{I_i \rightarrow S_i}^m = \frac{(1 - \theta^m) \lambda^m}{\alpha + \mu}$$

The dominant eigenvalue of this matrix is of the form:

$$R_m = \frac{A + \sqrt{B}}{2} \quad (\text{S6})$$

with :

$$A = \tau_{S_i \rightarrow S_i}^m + \frac{\eta}{(1 - \eta)} \tau_{M_{ji} \rightarrow M_{ii}}^m$$

$$B = \left(\tau_{S_i \rightarrow S_i}^m - \frac{\eta}{(1 - \eta)} \tau_{M_{ji} \rightarrow M_{ii}}^m \right)^2 + 4 \tau_{S_i \rightarrow M_{ii}}^m \left(\tau_{M_{ii} \rightarrow S_i}^m + \frac{\eta}{(1 - \eta)} \tau_{M_{ji} \rightarrow S_i}^m \right)$$

As expected, in the absence of dispersal between population (i.e. $\eta = 0$), we recover the dominant eigenvalue (A9) derived in Garnier et al. (2012).

Table S1 : Summary transgenerational protection

	Refs	Immune stimulation (F0)	Immune stimulation (F1)	Increased resistance?	TER	Effects on immunity (F1)	TEI	Age	Active dispersal
Molusca									
<i>Chlamys farreri</i>	[1]	HK Bacteria	Bacteria	YES (survival)	1	YES (ab activity, Immune-related proteins)	1	> 50 days [28]	ni
Crustacea									
<i>Penaeus sp.</i>	[2]	Glucane	Virus	YES (survival)	1	ni	-	> 50 days [29]	ni
Brachiopoda									
<i>Daphnia magna</i>	[3]	Bacteria	same as F0	YES (prevalence)	1	ni	-	≤ 50 days [30]	No or low dispersal [46]
<i>Artemia</i>	[4]	Bacteria	same as F0	YES (survival)	1	ni	-	> 50 days [31]	No or low dispersal [47]
Hymenoptera									
<i>B. terrestris</i>	[5]	LPS	none	ni	-	YES (PO activity)	1	> 50 days [32]	No or low dispersal [48]
<i>B. terrestris</i>	[6]	HKbacteria	LPS	ni	-	YES (ab activity)	1	> 50 days [32]	No or low dispersal [48]
<i>B. terrestris</i>	[7]	HKbacteria	same as F0	ni	-	YES (ab activity)	1	> 50 days [32]	No or low dispersal [48]
<i>B. terrestris</i>	[8]	HKbacteria	HKbacteria/Tripanosoma	NO (burden*)	0	YES (ab activity)	1	> 50 days [32]	No or low dispersal [48]
<i>A. mellifera</i>	[9]	Bacteria	none / same as F0	YES (survival)	1	YES (haemocytes)	1	> 50 days [33]	No or low dispersal [33]
Diptera									
<i>D. melanogaster</i>	[10]	Bacteria	same as F0	NO (survival*)	0	ni	-	≤ 50 days [34]	Strong dispersal [49]
<i>Ae. aegypti</i>	[11]	Sephadex beads	same as F0	ni	-	NO (melanization)	0	≤ 50 days [35]	Strong dispersal [50]
<i>A. coluzzii</i>	[12]	Fungus	Apicomplexa	YES (prevalence)	1	ni	-	≤ 50 days [36]	Strong dispersal [51]
<i>A. coluzzii</i>	[13]	Apicomplexa	same as F0	NO (burden)	0	ni	-	≤ 50 days [36]	Strong dispersal [51]
<i>Cx. pipiens</i>	[14]	Apicomplexa	same as F0	NO (prevalence, burden)	0	ni	-	≤ 50 days [37]	Strong dispersal [52]
Coleoptera									
<i>T. mollitor</i>	[15]	LPS	same as F0	YES (survival)	1	YES (ab activity)	1	> 50 days [38]	No or low dispersal [18]
<i>T. mollitor</i>	[16]	LPS	same as F0	ni	-	YES (PO activity, haemocyte ¹)	1	> 50 days [38]	No or low dispersal [18]
<i>T. mollitor</i>	[17]	LPS	none	ni	-	YES (ab activity)	1	> 50 days [38]	No or low dispersal [18]
<i>T. mollitor</i>	[18]	LPS	none	ni	-	YES (ab activity)	1	> 50 days [38]	No or low dispersal [18]
<i>T. castaneum</i>	[19]	HK Bacteria	Bacteria	YES (prevalence ¹ , survie ¹)	1	YES (PO activity ¹ , ab activity ¹)	1	> 50 days [39]	No or low dispersal [53]
<i>T. castaneum</i>	[20]	HK Bacteria	Bacteria	YES (survie ¹)	1	YES (PO activity ¹ , Immune transcripts ¹)	1	> 50 days [39]	No or low dispersal [53]
<i>A. glabripennis</i>	[21]	HK Bacteria or HK Fungus or Fungus	Fungus	YES (survival)	1	ni	-	> 50 days [40]	No or low dispersal [54]
Lepidoptera									
<i>T. ni</i>	[22]	Bacteria	none	ni	-	YES (PO activity, Immune transcripts)	1	> 50 days [41]	Strong dispersal [55]
<i>P. interpunctella</i>	[23]	Virus	same as F0	YES (prevalence)	1	ni	-	> 50 days [42]	No or low dispersal [56]
<i>M. sexta</i>	[24]	PGN	none / PGN	ni	-	YES (PO activity, ab activity)	1	≤ 50 days [43]	Strong dispersal [57]
<i>M. sexta</i>	[25]	PGN	Parasitoid	YES (prevalence)	1	YES (ab activity)	1	≤ 50 days [43]	Strong dispersal [57]
<i>G. mellonella</i>	[26]	Bacteria	none	ni	-	YES (Immune transcripts)	1	> 50 days [44]	ni
Orthoptera									
<i>T. oceanicus</i>	[27]	Bacteria	none	ni	-	YES (Lytic activity)	1	> 50 days [45]	ni

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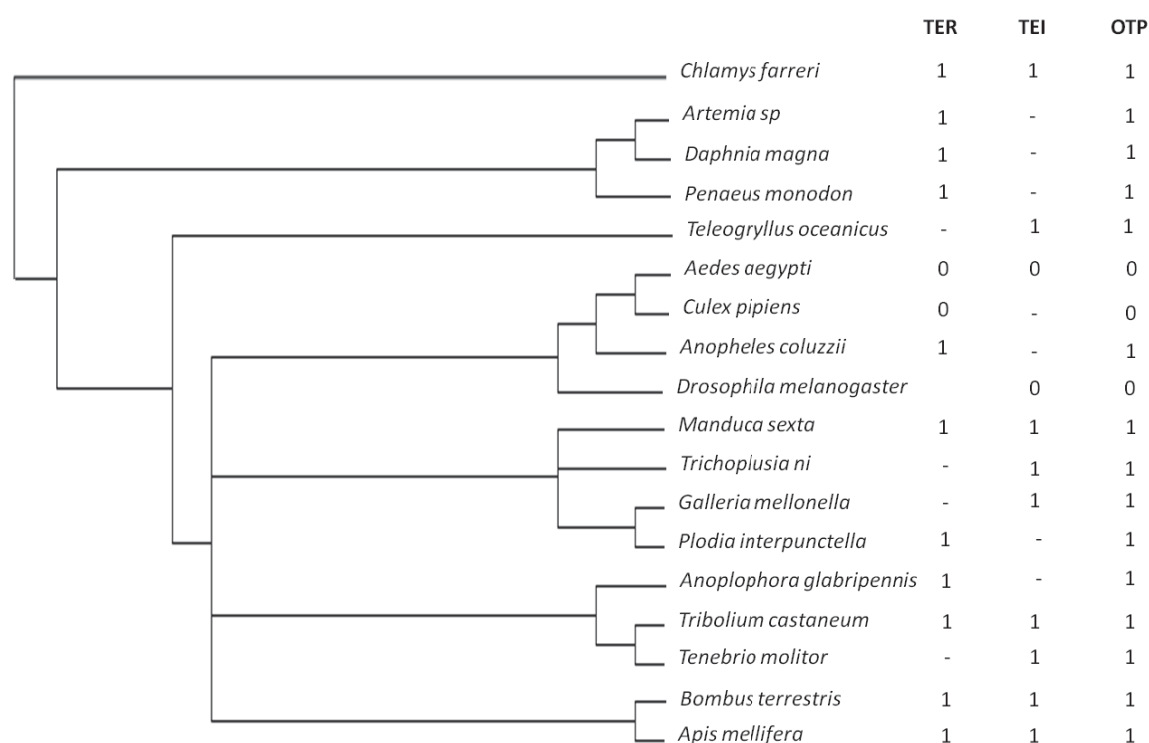


Figure S1: Phylogenetic associations between the 18 species of invertebrate included in the comparative analysis with their respective TER, TEI and OTP score.

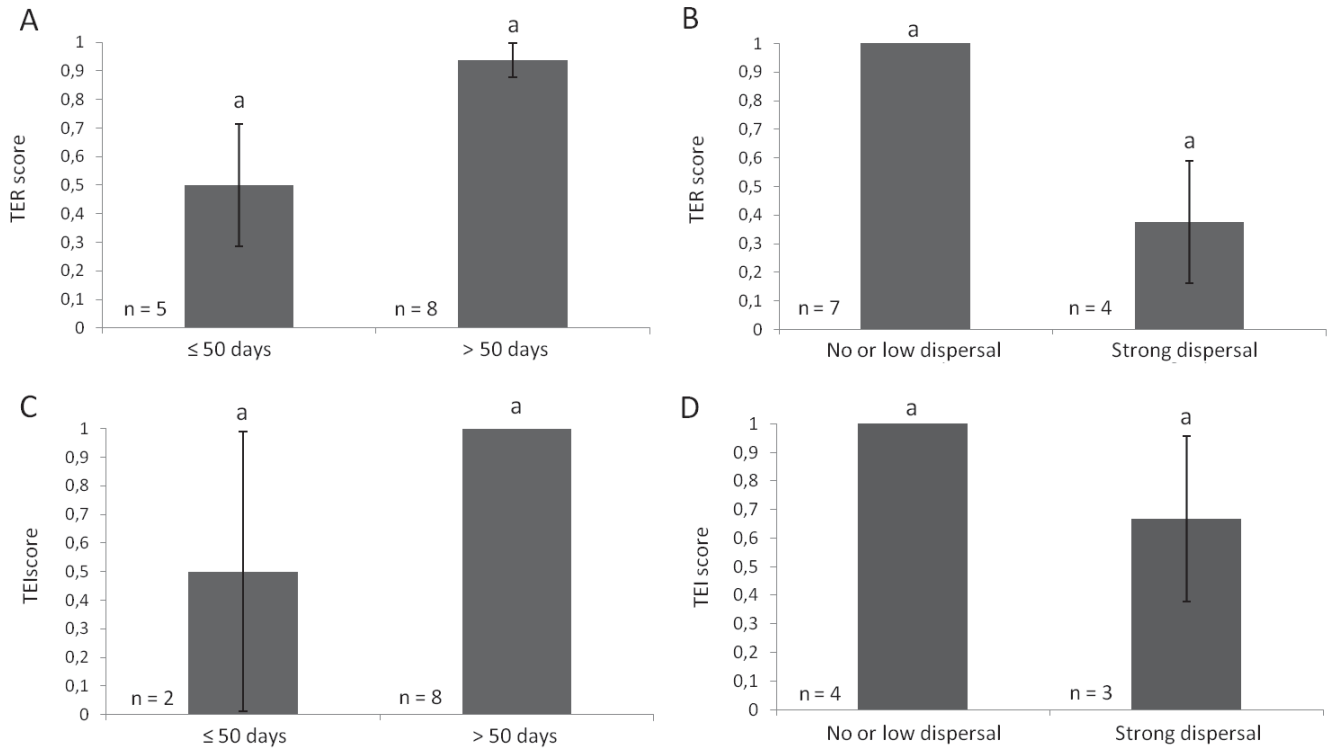


Figure S2: Transgenerational Effect on Resistance (TER) score regarding species (A) longevity and (B) dispersal and Transgenerational Effect on Immunity (TER) score regarding species (C) longevity and (D) dispersal. Levels not connected by same letter are significantly different. Error bars represent $\pm$ SE.

Chapitre 5:

Variations génétiques et héritabilités :
impact sur la qualité vectorielle des
moustiques

Chapitre 5: Variations génétiques et héritabilités : impact sur la qualité vectorielle des moustiques

Un deuxième block expérimental doit être réalisé prochainement

Contexte

- Au sein des populations naturelles de moustiques les variations phénotypiques observées au niveau de nombreux traits d'histoire de vie sont, en partie, expliquées par des variations génétiques. Déterminer la part de variance expliquée par la génétique additive au niveau de ces traits permet d'estimer leur héritabilité et donc de prédire, en partie, leur réponse à la sélection.
- À partir de moustiques capturés sur le terrain, nous avons cherché à déterminer la part de variation expliquée par la génétique additive au niveau de différents traits d'histoire de vie intervenant dans la dynamique de transmission du paludisme.

Résultats

- Alors que la taille des femelles et la taille de leur repas de sang ne semblent que très peu expliquées par une composante génétique, d'autres paramètres comme la fécondité et la résistance à *Plasmodium* ont montré des niveaux d'héritabilité élevés.

Conclusion

- Au sein de la population de moustiques sauvages échantillonnés, une forte hétérogénéité en termes de susceptibilité à *Plasmodium* a été observée. Elle semble être, en partie, expliquée génétiquement.
- Un deuxième bloc expérimental doit être réalisé afin de vérifier nos résultats mais également dans le but de déterminer si la résistance partielle à *Plasmodium* est associée à des coûts sur la fitness des moustiques.

Variations génétiques et héritabilités : impact sur la qualité vectorielle des moustiques

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Résumé

Les traits d'histoire de vie des moustiques sont connus pour être impactés par de nombreux paramètres environnementaux. Cependant une part non négligeable de la variation phénotypique observée au niveau de ces traits est sous-tendue par une base génétique. En utilisant des individus provenant d'une population naturelle de *Cx. pipiens*, vecteur principal de la malaria aviaire dans le sud de la France, nous avons cherché à déterminer la variance génétique et l'héritabilité d'un ensemble de paramètres essentiels à la transmission de *Plasmodium*. Nous avons notamment étudié la susceptibilité des vecteurs à ce parasite, leur comportement de piquûre, la taille de leur repas de sang ainsi que leur fécondité. Nous avons pu mettre en évidence que les variations observées au niveau de plusieurs traits, tels que la fécondité ou la résistance à *Plasmodium*, étaient expliquées par une composante génétique non négligeable.

Mots clés : malaria aviaire, *Culex pipiens*, héritabilité, susceptibilité, fécondité, génétique quantitative

Introduction

Le maintien et la transmission des agents pathogènes responsables de la malaria sont dépendants de la capacité vectorielle de leurs vecteurs (Beerntsen *et al.*, 2000; Rivero *et al.*, 2010) elle même fortement liée aux traits d'histoire de vie de ses derniers. Ces traits d'histoire de vie sont connus pour être impactés par de nombreux paramètres environnementaux. Il a en effet été montré que la taille des moustiques (Ciota *et al.*, 2014), leur comportement de piqûre (Nasci & Mitchell, 1994; Araujo *et al.*, 2012; Ciota *et al.*, 2014; Costanzo *et al.*, 2015), leur probabilité de survie (Rueda *et al.*, 1990; Costa *et al.*, 2010; Costanzo *et al.*, 2015) ou encore leur résistance à *Plasmodium* (Noden *et al.*, 1995; Okech *et al.*, 2004; Mordecai *et al.*, 2013; Zélé *et al.*, 2014a) sont influencés par un grand nombre de paramètres tels que la température, l'humidité ou l'alimentation. Cependant une part non négligeable de la variation phénotypique observée au niveau de ces traits est sous-tendue par une base génétique (Sorci *et al.*, 1997; Beerntsen *et al.*, 2000; Mpho *et al.*, 2002; Schneider *et al.*, 2011). Concernant la résistance au parasite de la malaria, Clay Huff a démontré il y a plus de 80 ans que la susceptibilité de *Culex pipiens* à une souche de *Plasmodium* pouvait être augmentée grâce à des stratégies sélectives d'accouplement (Huff C. G., 1929). En 1986, Collins et collaborateurs ont réussi, grâce à une expérience de sélection artificielle, à créer une lignée de moustiques résistante à plusieurs espèces de *Plasmodium* (Collins *et al.*, 1986). Des études plus récentes ont également mis en évidence l'existence de variations génétiques naturelles induisant une résistance partielle à la malaria dans certaines populations de moustiques d'Afrique de l'ouest (Niaré *et al.*, 2002). Le maintien, en milieu naturel, d'une certaine variabilité en terme de résistance à l'infection par *Plasmodium* est expliqué en partie par la relation existante entre les effets négatifs de l'infection sur la fitness de son hôte (Hogg & Hurd, 1995; Vézilier *et al.*, 2012) et les coûts de la résistance au parasite (Yan *et al.*, 1997, Hurd *et al.*, 2005).

Les traits phénotypiques complexes, tels que la résistance à un agent pathogène, sont souvent sous le contrôle de nombreux loci (Severson *et al.*, 1995) et sont, la plupart du temps, également influencés par l'environnement (Lefèvre *et al.*, 2013).

La génétique quantitative permet d'estimer dans quelle mesure la variation d'un trait (variance phénotypique) est conditionnée par l'environnement (variance environnementale) et par la génétique (variance génétique). Un des paramètres de génétique quantitative couramment mesuré est l'héritabilité d'un trait. Ce paramètre correspond à la proportion de

variation d'un paramètre attribuée à l'effet de la génétique additive, il permet notamment de prédire la réponse à la sélection (Bosio *et al.*, 1998).

Dans ce chapitre, afin d'étudier la composante génétique et l'héritabilité de plusieurs traits d'histoire de vie des moustiques, comprenant la susceptibilité à *Plasmodium*, nous avons mis en place une expérience de génétique quantitative en utilisant un protocole « Full-sib » (Falconer, D.S. & Mackay 1996). Nous nous sommes servis de cet outil pour essayer de déterminer la part génétique responsable de la variabilité phénotypique en termes de susceptibilité à *Plasmodium* observée au sein d'une population de moustiques sauvages appartenant à l'espèce *Culex pipiens*. En effet, suite à une session de capture (Triadou, France, été 2013) il a été mis en évidence une grande variabilité de susceptibilité de ces vecteurs à l'infection par *Plasmodium* (**Figure S1**). De plus un phénotype de résistance intéressant a été observé chez deux femelles où des oocystes mélanisés ont été observés au niveau de la paroi de leur tube digestif. Nous avons alors cherché à déterminer la part génétique responsable de la variabilité de résistance observée au sein de cette population mais nous nous sommes également intéressés à un ensemble de traits d'histoire de vie essentiels à la transmission de *Plasmodium* tels que, le comportement de piquûre, la taille du repas de sang et la fécondité des moustiques.

Matériels et Méthodes

Souche de Plasmodium et infection des hôtes vertébrés

Nous utilisons une souche de *Plasmodium relictum* SGS1 isolée en 2009 à partir d'un moineau sauvage (Pigeault *et al.*, 2015). Le parasite a été depuis cette date maintenu au laboratoire par des passages réguliers entre hôtes infectés et hôtes sains (Pigeault *et al.*, 2015). Les deux oiseaux (*Serinus canaria*) utilisés lors de l'expérience ont été infectés par une injection intra-péritonéale de 80µL de sang provenant de notre stock d'oiseaux infectés. Durant l'expérience, les moustiques ont pris leur repas de sang 10 jours après l'infection des oiseaux ce qui correspond à la phase aigue de la parasitémie (Vézilier *et al.*, 2010).

Etablissement des familles

L'étude a été réalisée sur des moustiques sauvages appartenant à l'espèce *Cx. pipiens*. Cette espèce de moustique a été décrite comme étant le vecteur principal de *Plasmodium relictum* en Europe (Valkiunas, 2004; Zélé *et al.*, 2014b). Le fait que *Cx. pipiens* pond des œufs en forme de raft (regroupement de plusieurs centaines d'œufs) et que cette

espèce soit décrite comme étant principalement monoandre (Bullini *et al.*, 1976; Vinogradova, 2000) permet facilement de former des familles « full sib ». En septembre 2014, huit pontes de *Culex pipiens*, qui constitueront les 8 familles de l'étude (nommées de A à H), ont été récupérées sur le terrain dans une petite parcelle d'eau peu profonde (Le Triadou, France).

Les pontes ont été rapportées au laboratoire et chacune a été placée dans un bac en plastique contenant 1L d'eau minérale (Eau minérale Carrefour). Les huit pontes ont éclos le lendemain de leur capture. Afin de contrôler la densité de larves par bac, 100 larves par ponte (famille) ont été prises au hasard et placées dans un nouveau bac contenant 1L d'eau minérale. Les larves ont été nourries tous les deux jours avec 400 mg de mélange composé de Tetramin, de nourriture pour lapin et de poudre de foie de porc (rapport 1 :1 :1). L'eau des bacs a été changée tous les deux jours. Nous avons cherché à minimiser les variations environnementales entre familles en les plaçant toutes dans une même enceinte climatique (25°C, 80% d'humidité, cycle jour nuit 12/12).

Le huitième jour après la mise en bac de larves, les bacs ont été placés dans des cages d'émergence. L'émergence, synchrone pour les huit familles, a eu lieu 11 jours (± 2 jours) après l'éclosion des œufs. Mâles et femelles (issus d'une même famille) ont été laissés ensemble durant 6 jours afin de leur permettre de s'accoupler. Puis 40 femelles de chaque famille ont été capturées de manière aléatoire et ont été marquées avec un point de peinture acrylique colorée sur leur dos (Liquitew, Heavy body acrylicTM). Des tests préliminaires ont mis en évidence que la peinture acrylique n'avait aucun impact sur la survie, la capacité de se nourrir ou la fécondité des femelles. Chacune des familles a été marquée d'une couleur différente afin de pouvoir être identifiée par la suite. Chaque famille a ensuite été divisée en deux groupes égaux et placés dans deux nouvelles cages (cages de gorgement). Au final deux cages de gorgement contenant chacune 160 femelles (20 par famille) ont été produites (**FigureS2**).

Trois jours après avoir constitué les deux cages de gorgement, un oiseau infecté par *Plasmodium relictum* a été placé dans chacune d'elles (Jour 0). Les oiseaux ont été laissés toute la nuit dans les cages afin de permettre à un maximum de moustiques de se nourrir. Suite à cette nuit de gorgement, les femelles non nourries ont été dénombrées et identifiées afin de calculer la proportion de femelles gorgées par famille. Puis, toutes les femelles nourries ont été capturées, observées sous loupe binoculaire, afin de déterminer à quelle famille elles appartenaient, et ont été isolées dans des tubes (tube d'haematine). Des cotons

imbibés de solution sucrée (10 %) ont été placés sur chaque tube afin de permettre aux femelles de se nourrir. Quatre jours plus tard (Jour 5), toutes les femelles ont été transférées dans un nouveau tube contenant 4mL d'eau minérale et 1mg de spiruline afin de permettre aux femelles de pondre (tube de ponte). Un coton imbibé de solution sucrée (10 %) a été à nouveau placé sur chacun des tubes. Trois jours plus tard, soit 8 jours après le repas de sang infecté, le nombre d'œufs dans chaque ponte a été compté (voir ci dessous) et toutes les femelles ont été disséquées sous le microscope (X40) afin de déterminer la prévalence et l'intensité d'infection par *Plasmodium* (quantification d'oocystes dans le tube digestif). Une aile a également été prélevée sur chacune des femelles afin d'être mesurée (Schneider *et al.*, 2007). La taille du repas de sang a été estimée en quantifiant le produit de dégradation de l'hémoglobine sécrété par les femelles au fond des tubes d'haematine (Vézilier et al 2012). Chaque ponte a été photographiée à l'aide d'une loupe binoculaire équipée d'une caméra numérique. Le nombre d'œufs a ensuite été quantifié à l'aide du logiciel Mesurim Pro (Académie d'Amiens, France).

En résumé, au total 8 paramètres ont été mesurés: la taille des ailes (proxy de la taille des femelles), la proportion de femelles nourries par famille, la taille du repas de sang, la proportion de femelles par famille ayant pondu au moins un œuf, le nombre d'œufs pondus et, enfin, la prévalence et l'intensité d'infection par *Plasmodium* huit jours après la prise du repas de sang.

Effet génétique sur les traits d'histoire de vie

L'effet génétique sur les 8 traits des moustiques cités ci-dessus a été testé en utilisant des modèles mixtes d'analyses de variance (méthode : REML) avec l'effet *famille* placé en aléatoire. Sachant que les variables réponse peuvent dépendre de l'oiseau sur lequel les moustiques se sont nourris, la variable *oiseau* a été placée en aléatoire. La *taille des femelles* ainsi que la *taille du repas de sang* ont été ajoutées en facteurs fixes lorsque cela était pertinent (**TableS1**). Dans le cas où un effet fixe a été ajouté au modèle, l'interaction entre l'effet fixe et l'effet *famille* (placé en aléatoire) a également été testée. Les traits: taille des femelles, taille du repas de sang, nombre d'œufs pondus et l'intensité d'infection ont été testés à l'aide d'un *lmer* (package : lme4) avec une erreur normale. L'intensité d'infection et la taille du repas sanguin, ont été préalablement normalisées avec une transformation racine carrée. Le taux de gorgement et le taux de ponte ont été étudiés en utilisant un *lmer* (package : lme4) avec une erreur binomiale. L'ensemble des modèles utilisés dans cette étude sont présentés dans la **TableS1**.

Estimation de l'héritabilité (h^2)

Les modèles mixtes présentés précédemment ont été utilisés afin d'estimer la part de variance expliquée par l'effet famille. A partir de cette donnée ainsi que de la variance phénotypique totale (variance totale du modèle) nous avons pu calculer l'héritabilité au sens strict (Falconer & Mackay, 1996) de chacun des traits mesurés : $h^2 = 2 \times (\sigma^2_A) / \sigma^2_p$. Avec σ^2_A = la variance expliquée par l'effet famille et σ^2_p = la variance phénotypique totale observée. Notre estimation de l'héritabilité au sens strict assume le fait que les effets maternels, la variance de dominance et la variance résultant d'interactions épistatiques entre gènes sont négligeables. L'ensemble des analyses a été réalisé avec le logiciel R (.3.1.0, <http://www.cran.r-project.org/>).

Box 1 :

- La variance phénotypique (VP) d'un trait peut être exprimée en tant que $VP = VA + VD + VI + VE$: où VA est la variance génétique additive; VD, la variance de dominance; VI, la variance résultant d'interactions épistatiques entre les gènes; et VE, la variance liée à l'environnement. VD et VI sont généralement considérées comme négligeables (mais voir Crnokrak & Roff 1995).

Les variations de l'héritabilité au sens strict ($h^2 = VA / VP$) peuvent être causées par des changements dans les composants génétiques ou dans les composants environnementaux de la variance.

- Par consensus, il y a trois principales catégories de caractères selon la valeur de leur héritabilité.

Les caractères à variation peu héritable ($h^2 < 0.2$) : ces caractères sont généralement très sensibles aux conditions environnementales et à des phénomènes génétiques non additifs, notamment des interactions de dominance. Il est également supposé que les traits présentant une faible héritabilité sont des paramètres qui, en milieu naturel, sont soumis à une forte sélection ce qui explique en partie pourquoi ils présentent moins de variation génétique.

Les caractères à variation moyennement héritable ($0.2 < h^2 < 0.4$)

Les caractères à variation fortement héritable ($h^2 > 0.4$) : caractères beaucoup moins sensibles aux variations environnementales. Les caractères présentant une forte variation génétique sont des caractères qui répondront plus facilement à la sélection.

Sources : Falconer & Mackay 1996

Résultats

Taille des femelles

Concernant l'effet famille, aucun impact de ce paramètre n'a été observé (model1 : $\chi^2_1 = 2.719$ p = 0.099). En effet, il n'explique que 2.14 % de la variance phénotypique totale mesurée ce qui aboutit à une estimation de l'héritabilité très faible ($h^2 = 0.1$).

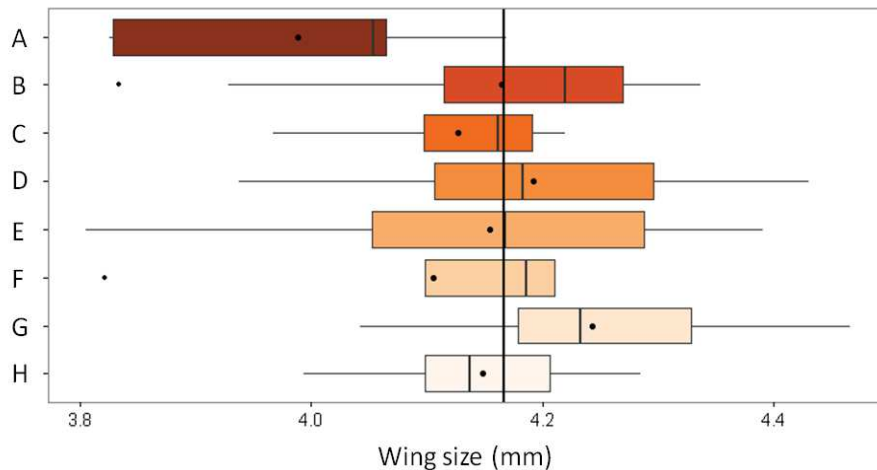


Figure 1 : Variation de la taille des ailes (mm) des femelles au sein de chacune des 8 familles (de A à H). Le trait vertical noir représente la moyenne de la population. Au niveau des boîtes à moustache, le point correspond à la moyenne de la famille et la ligne correspond à la médiane. Les boîtes au dessus et en dessous des médianes montrent respectivement les premier et troisième quartiles. Les lignes horizontales délimitent 1,5 fois l'écart inter-quartile. Au-dessus de ces valeurs les individus sont considérés comme des valeurs aberrantes et sont marqués par des petits points noirs.

Comportement de piqure et taille du repas de sang

Il existe une forte variance entre familles en terme de proportion de femelles ayant pris un repas de sang. En effet, le pourcentage de femelles nourries est multiplié par quatre entre les familles où peu de femelles se sont nourries comparées à celles présentant les plus hauts taux de gorgement (**Figure 2A**). On a donc un effet famille très significatif (model 2 : $\chi^2_1 = 77.53$ p < 0.0001) qui explique près de 14% de la variance observée. L'estimation de l'héritabilité de ce paramètre donne un $h^2 = 0.29$. Cependant, parmi les femelles qui ont pris un repas de sang, on peut constater que la taille de ce dernier n'est pas influencée par l'effet famille (model3 : $\chi^2_1 = 2.01$, p = 0.075, **Figure 2B**). Moins de 1% de la variance total observée est expliqué par ce paramètre ($h^2 = 0.01$). Il faut noter que la taille du repas de sang est cependant influencé par la taille des individus (model 4 : $\chi^2_1 = 4.54$, p = 0.033).

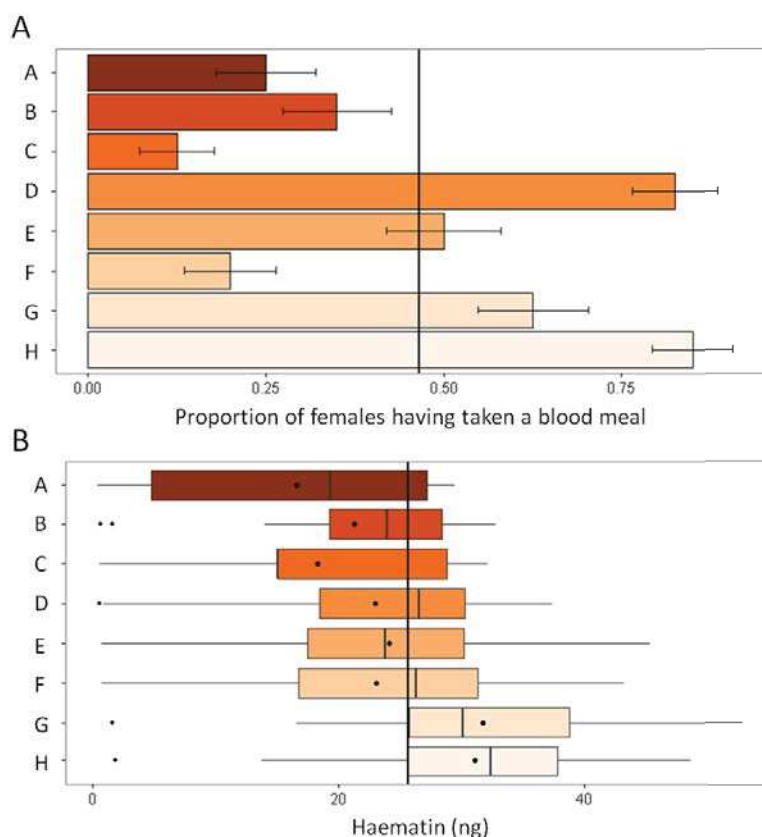


Figure 2: Variation de (A) la proportion de femelles ayant pris un repas de sang par famille ainsi que (B) de la quantité d'haematine excrétée (ng) par femelle. Le trait noir représente la moyenne de la population. Voir le légende de la Figure 1 pour l'explication des boîtes à moustache.

Probabilité de ponte et fécondité

La variation entre les familles en termes de proportion de femelles infectées qui vont pondre au moins un œuf est faible (**Figure 3A**). En effet, moins de 1% de la variance phénotypique est expliquée par l'effet famille (model 5 : $\chi^2_1=3.819$ $p=0.0502$). Il faut également noter qu'aucun effet de la taille du repas sanguin sur ce paramètre n'a été observé (model 6 : $\chi^2_1=2.927$ $p=0.0871$). Cependant, pour ce qui est de la fécondité, à savoir le nombre d'œufs pondus, l'effet famille est cette fois ci fortement significatif (model 7 : $\chi^2_1=12.03$, $p<0.0001$). Il explique plus de 13% de la variance phénotypique observée, ce qui donne une héritabilité de $h^2 = 0.27$. La taille du repas de sang a également un fort effet sur la fécondité (model 8 : $\chi^2_1=18.94$ $p<0.0001$). En effet le nombre d'œufs pondus est positivement corrélé à la quantité de sang ingéré ($R\text{-squared} = 0.296$, $F_{1,99}=30.32$, $p < 0.0001$). Cependant, même après avoir ajouté ce paramètre dans le modèle l'effet famille reste significatif (model 8 : $\chi^2_1=6.1159$ $p = 0.0067$).

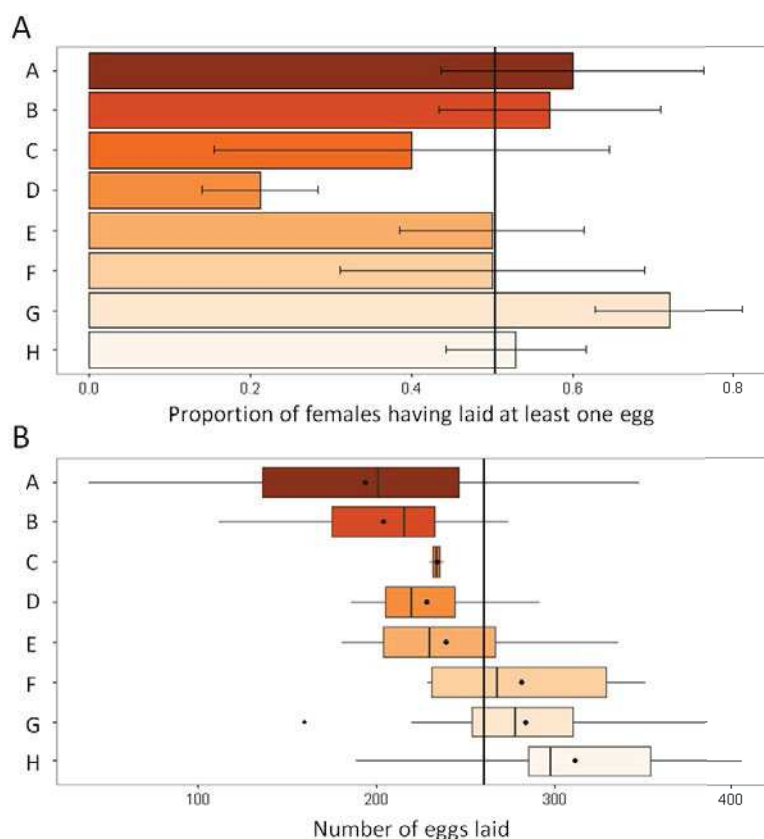


Figure 3 : Variation de (A) la proportion, pour chaque famille, de femelles ayant pondu au moins un œuf (B) du nombre totaux d'œufs pondus par femelle. Le trait noir représente la moyenne de la population. Voir le légende de la Figure 1 pour l'explication des boîtes à moustache.

Infection par *Plasmodium*

La probabilité d'infection par *Plasmodium* suite à la prise d'un repas de sang infecté était de 100% pour toutes les familles. Aucune variation n'a donc été observée. Cependant concernant l'intensité d'infection, à savoir le nombre moyen d'oocystes par estomac, une très forte variation entre famille a été détectée. Alors que la charge en oocystes moyenne au sein de la population était de 421 ± 27 oocystes par estomac, un rapport de 4.2 a été observé entre la famille la moins infectée et celle qui présentait la plus grande intensité d'infection (respectivement 154 ± 73 , 644 ± 84 , **Figure 4**). L'effet famille explique près de 20% de la variance totale observée et est donc très significatif (model 9 : $\chi^2_1 = 16.48$, $p < 0.0001$). L'estimation du taux d'hérédité de la successibilité à *Plasmodium* est de $h^2 = 0.38$. La taille du repas de sang impacte également l'intensité d'infection des femelles (model 10 : $\chi^2_1 = 18.05$, $p < 0.0001$). En effet, une corrélation positive est observée entre ces deux paramètres. Mais malgré l'ajout de la taille du repas de sang dans le modèle, l'effet famille reste toujours très significatif (model 10 : $\chi^2_1 = 18.19$, $p < 0.0001$).

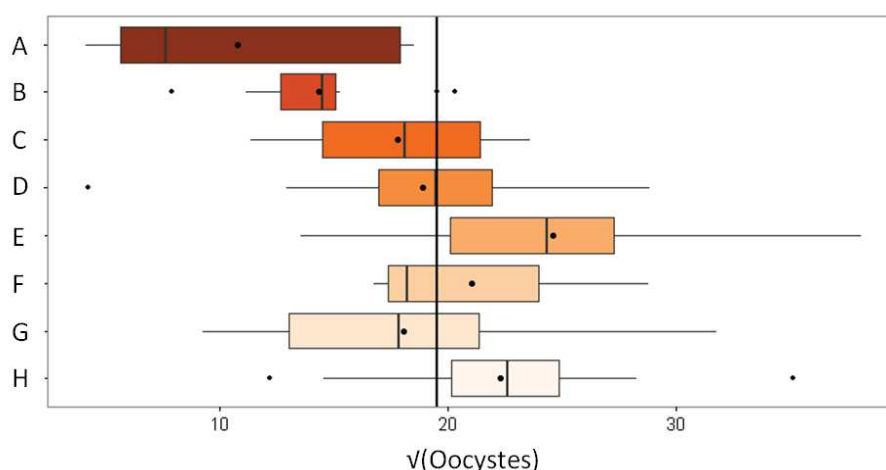


Figure 4: Variation du nombre d’oocystes par femelle de chacune des 8 familles. Le trait noir représente la moyenne de la population. Voir le légende de la Figure 1 pour l’explication des boîtes à moustache.

Discussion

Au cours de notre étude nous avons essayé de déterminer la part génétique responsable de la variabilité phénotypique observée au niveau de plusieurs traits d’histoire de vie de *Cx. pipiens*, le vecteur principal de la malaria aviaire en Europe (Zélé *et al.*, 2014b). Nous avons notamment mis en évidence que la génétique additive expliquait une part importante de la variabilité mesurée en termes de susceptibilité à *Plasmodium*.

Pour un grand nombre de paramètres morphologiques une base génétique sous-jacente a été démontrée (Gockel *et al.*, 2002). Ces caractères sont décrits comme fortement héréditaires chez les espèces endothermes, cependant il semblerait que ces traits le soient moins chez les espèces ectothermes (Mousseau & Roff, 1987). Ceci est notamment expliqué par le fait que, contrairement aux endothermes, les traits morphologique des ectothermes sont très souvent positivement corrélés à leur fitness (Mousseau & Roff, 1987). L’un des traits morphologiques les plus couramment étudiés est la taille des individus. En effet, la taille du corps est l’une des caractéristiques les plus importantes de l’organisme qui est décrite comme affectant à la fois le succès d’accouplement et la fécondité (Reeve *et al.*, 2000). L’un des moyens les plus utilisés pour approximer la taille des arthropodes ailés est de mesurer la longueur de leurs ailes. Dans la littérature il n’y a pas de consensus concernant la part génétique responsable de la variation phénotypique observée au niveau de ce paramètre morphologique (Mpho *et al.*, 2002). En effet, certaines études ont mesuré des niveaux d’héritabilités relativement faibles (Woods *et al.*, 1998; Mpho *et al.*, 2002) alors que

d'autres au contraire ont observé un résultat inverse avec des niveaux d'héritabilités très hauts (Bjorksten *et al.*, 2000). Chez les moustiques le taux d'héritabilité de ce trait semble varier d'une espèce à l'autre. En effet, chez des *A. gambiae* sauvages le h^2 est égale à 0.35 (Lehmann *et al.*, 2006). Cependant chez cette même espèce, mais avec une population maintenue en laboratoire un taux d'héritabilité plus haut a été observé ($h^2 = 0.46$, Voordouw *et al.*, 2008). Cette différence peut s'expliquer par le fait qu'en conditions de laboratoire le taux d'héritabilité est la plupart du temps surestimé (Simons & Roff, 1994). En effet, les populations d'organismes élevés dans des environnements homogènes présentent généralement des niveaux de variance phénotypique totale inférieure aux populations sauvages (Simons & Roff, 1994). Cependant des taux d'héritabilité élevés sont également observés en milieu naturel, notamment chez *Ae. aegypti* où le h^2 est supérieur à 0.5 (Schneider *et al.*, 2011). Chez *Culex pipiens*, à l'inverse, le h^2 mesuré est beaucoup plus faible avec une valeur inférieure à 0.2 (Mpho *et al.*, 2002). Notre résultat issu de la population de *Cx. pipiens* originaire du sud de la France va dans le même sens puisque nous mesurons un h^2 de 0.1. La part génétique responsable de la variabilité phénotypique observée au niveau de la taille des moustiques semble donc varier fortement d'une espèce à l'autre. Ce trait morphologique est également décrit comme fortement influencé par des paramètres abiotiques tels que la température ou l'accès aux ressources nutritives (Lyimo *et al.*, 1992; Nasci & Mitchell, 1994; Araujo *et al.*, 2012; Hidalgo *et al.*, 2015). Cela laisse supposer qu'en conditions naturelles une part non négligeable des variations observées au niveau de la taille des individus est liée à la variance environnementale.

Dans un second temps nous nous sommes intéressés à la variation du comportement de piqûre et de la taille du repas observés au sein de la population sauvage de *Culex pipiens*. Ces deux paramètres sont importants dans la dynamique de transmission de *Plasmodium*. En effet, la probabilité de piqûre ainsi que la taille du repas de sang vont fortement influencer la probabilité et l'intensité d'infection des vecteurs (Pigeault *et al.*, 2015). Ce que nous montrons dans notre étude, c'est que la part génétique responsable des variations en termes de taille de repas de sang est quasiment nulle. Ceci peut s'expliquer par le fait que la taille du repas de sang est un paramètre qui est très dépendant de la taille des individus (Takken *et al.*, 1998; Pigeault *et al.*, 2015). Pour ce qui est du comportement de piqûre, peu de données sont disponibles concernant les bases génétiques de la variation de ce comportement chez les moustiques. Au sein de la population sauvage de *Cx. pipiens* une part importante de la variance phénotypique totale dans ce trait est expliquée par la

génétique additive, ce qui se traduit par une estimation de l'héritabilité de ce trait relativement élevée ($h = 0.29$). Cependant cette mesure de probabilité de gorgement regroupe différents paramètres. Le fait qu'une femelle se nourrisse ou non va notamment dépendre de sa capacité de vol au sein de notre système expérimental, de sa capacité d'adaptation à se nourrir sur un nouveau système (oiseaux entravé) mais, également, de sa réponse comportementale liée à l'attraction de l'hôte vertébré. En effet, l'attraction des femelles pour un hôte donné peu varier entre famille, un article récent a mis en évidence que l'attraction relative d'*Aedes aegypti* pour l'homme était variable et présentait une forte héritabilité ($h^2 = 0,62$, Fernández-Grandon *et al.*, 2015). Ils ont également montré que l'activité de vol des femelles était également très héritable ($h^2=0.67$, Fernández-Grandon *et al.*, 2015). Il semble donc que ces paramètres, potentiellement responsables de variations quant à la proportion de femelles nourries, soient en partie contrôlés par une composante génétique additive ce qui pourrait expliquer nos résultats.

Contrairement aux paramètres morphologiques, les traits d'histoires de vie, tels que longévité et fécondité, sont des paramètres qui contribuent directement à la fitness des individus. Par conséquent on pourrait s'attendre à ce qu'ils soient maximisés par la sélection naturelle et donc présentent peu de variation génétique (Fisher, 1930; Mousseau & Roff, 1987). Malgré ces prévisions, en milieu naturel un fort niveau de variation en terme de génétique additive est observé au niveau de ces traits (Mousseau & Roff, 1987; Houle, 1992; Bradshaw & Holzapfel, 1996). Les facteurs pouvant expliquer le maintien de cette variance génétique sont multiples. En effet, les taux de mutation, l'avantage de l'hétérozygotie, la migration ou les compromis existant entre différents traits d'histoire de vie sont proposés comme des mécanismes pouvant, en partie, expliquer ces variations génétiques au sein des populations sauvages (Mousseau & Roff, 1987). Au cours de notre étude nous nous sommes intéressés à la fécondité en mesurant à la fois la probabilité de pondre au moins un œuf suite à la prise d'un repas de sang ainsi qu'au nombre totaux d'œufs pondus. Pour ce qui est de la probabilité de pondre au moins un œuf nous n'avons pas mis en évidence de variation en termes de génétique additive au sein de notre modèle biologique. En effet le taux de ponte moyen semble relativement stable entre les familles (en moyenne, 50% des femelles ont pondu au moins un œuf après avoir pris un repas sanguin). Aucune étude à notre connaissance ne s'est intéressée à la part génétique responsable des variations observées quant à ce paramètre. Cependant cela a été étudié chez plusieurs espèces de moustiques dans le cas de l'autogénie, c'est à dire la capacité de pondre un œuf sans avoir pris de repas

de sang. Chez *Aedes albopictus* et *Aedes aegypti*, il a notamment été montré que ce trait était fortement influencé par des paramètres environnementaux (Mori *et al.*, 2008; Ariani *et al.*, 2015), mais également par une composante génétique non négligeable (Mori *et al.*, 2008; mais voir Ariani *et al.*, 2015). En revanche, concernant la taille de ponte, nous avons observé une très forte variation entre familles engendrant une estimation d'héritabilité relativement élevée ($h=0.27$). Bien que ceci puisse paraître surprenant en raison de l'action supposée de la sélection naturelle sur la fitness (Fisher, 1930; McCleery *et al.*, 2004), des résultats similaires ont récemment été décrits chez la drosophile et chez une espèce de Lépidoptère (respectivement Long *et al.*, 2009; Quezada-García & Bauce, 2014). Il ne faut pas oublier non plus que la fécondité des femelles est également liée au succès reproducteur des mâles. Or, Voordouw & Koella (2007) ont observé des variations génétiques quant au succès de reproduction de ces derniers.

Enfin, concernant la variation en termes de susceptibilité à *Plasmodium*, il est bien établi que la résistance des moustiques aux parasites est en partie déterminée génétiquement. Des variations de successibilité à *Plasmodium* ont été observées dans des populations naturelles de moustiques (Kilama, 1973; Niaré *et al.*, 2002) et des lignées résistantes ont été créées en laboratoire (Ward 1963, Collins *et al.*, 1986; Yan *et al.*, 1997). Au cours de notre étude, nous n'avons pas identifié d'effet famille sur la prévalence d'infection. Cependant de forts effets sur la charge en oocystes ont été observés. Le fait que l'intensité d'infection et la prévalence ne soient pas impactés de la même manière a également été observé lors de l'infection de différentes lignées isofemelles d'*A. stephensis* par *P. yoelii* (Lambrechts *et al.*, 2006). Ce résultat suggère que les bases génétiques responsables de ces deux traits liés à l'infection sont différentes. Le maintien de la variation génétique en termes de résistance aux agents pathogènes au sein des populations sauvages est expliqué en partie par la relation existante entre les effets négatifs du parasite sur la fitness de son hôte et les coûts de la résistance à ce même parasite (Yan *et al.*, 1997). Ceci se vérifie au sein du modèle moustique/*Plasmodium*. En effet, *Plasmodium* est connue pour induire un coût sur la fécondité de son hôte (Hogg & Hurd, 1995; Vézilier *et al.*, 2012) et au sein des lignées de laboratoire sélectionnées pour être résistantes au parasite de la malaria un coût important de la résistance a été observé sur la fitness des femelles (Yan *et al.*, 1997; Hurd *et al.*, 2005). Elles ont en effet été décrites comme pondant moins d'œufs et vivant moins longtemps (Yan *et al.*, 1997; Hurd *et al.*, 2005). Au sein de notre modèle biologique nous savons que l'infection par *Plasmodium* induit un coût non négligeable sur la fécondité

des moustiques (Vézilier *et al.*, 2012; Pigeault *et al.*, 2015). Il aurait été intéressant de déterminer si les familles présentant les niveaux de susceptibilités les plus bas sont également celle avec la fécondité la moins bonne. Cependant, de part le faible nombre de familles utilisées au cours de l'expérience, nous n'avons pas pu analyser et tester ces corrélations.

Afin de tester ces prédictions, nous avons essayé au cours de cette thèse de créer des lignées résistantes de *Cx pipiens*. En effet la forte variation de génétique additive observée au niveau de l'intensité d'infection laissait penser que ce caractère répondrait à la sélection expérimentale. Cependant au bout de trois générations de sélection nous avons perdu l'ensemble de nos lignées. Dans le but de répondre à cette question nous avons le projet de répéter l'expérience présentée au sein de cette étude mais avec un nombre de familles plus important. De plus nous allons également modifier en partie notre protocole afin de s'assurer que les variances phénotypiques observés entre familles ne sont pas liés au fait que chacune des familles a été élevés dans un bac différent. Pour cela nous allons réaliser un élevage individuel des larves.

Contributions des auteurs

RP, AR et SG ont conçu l'expérience, RP et FG ont réalisé les expériences, RP et PD ont analysé les données, RP, AR et SG ont écrit l'article.

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Matériels supplémentaires

Tableau S1 : Liste des modèles statistiques utilisés

Variable of interest	Response variable	Model Nb.	N	Maximal model	Minimal model	Rsubroutine [err struct.]
Wing size	size	1	102	1 + (1 famille) + (1 cage)	1 + (1 cage)	lmer[n]
Feeding behaviour	BM	2	320	1 + (1 famille) + (1 cage)	1 + (1 famille) + (1 cage)	lmer[b]
Blood meal size	v(hae)	3	90	1 + (1 famille) + (1 cage)	1 + (1 cage)	lmer[n]
Blood meal size	v(hae)	4	90	wing + (1 + wing famille) + (1 cage)	wing + (1 cage)	lmer[n]
Probability of laying	laid	5	139	1 + (1 famille) + (1 cage)	1 + (1 cage)	lmer[b]
Probability of laying	laid	6	139	hae + (1 + hae famille) + (1 cage)	1 + (1 cage)	lmer[b]
Number of eggs laid	eggs	7	74	1 + (1 famille) + (1 cage)	1 + (1 famille) + (1 cage)	lmer[n]
Number of eggs laid	eggs	8	74	hae + (1 + hae famille) + (1 cage)	hae + (1 famille) + (1 cage)	lmer[n]
Oocyst burden	V(Oocyst)	9	101	1 + (1 famille) + (1 cage)	1 + (1 famille) + (1 cage)	lmer[n]
Oocyst burden	V(Oocyst)	10	101	hae + (1 + hae famille) + (1 cage)	hae + (1 famille) + (1 cage)	lmer[n]

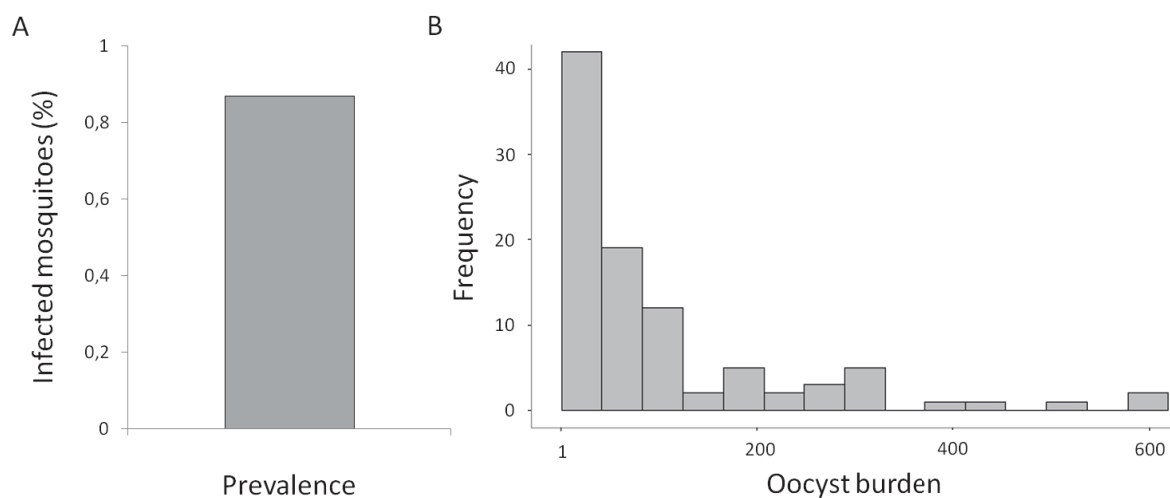


Figure S1 : (A) Prévalence et (B) intensité d'infection par *Plasmodium* observés au sein de la population sauvage de *Cx pipiens* capturé en juin 2013 (Triadou, France).

Chapitre 6:

Paramètres intra- et inter-hôte
susceptibles d'impacter la transmission du
paludisme

Chapitre 6: Paramètres intra- et inter-hôte susceptibles d'impacter la transmission du paludisme

Article publié dans « *Philosophical transactions of the royal society B* »

Contexte

- Peu de données sont disponibles quant à l'effet « hôte vertébré » sur l'infection des vecteurs par *Plasmodium*.
- À partir des données collectées par mon équipe depuis plus de cinq ans, nous nous sommes intéressés à cet effet en étudiant, notamment, l'impact de l'hétérogénéité d'infection de l'hôte sur le taux d'infection et la fitness des vecteurs.

Résultats

- Nous avons identifié de nombreuses relations entre différents paramètres liés aux infections telles que la parasitémie, la gamétocytémie, la morbidité de l'hôte, le taux de transmission aux vecteurs et la fitness des moustiques.
- Il a également été mis en évidence que le mode de maintien de notre souche de *Plasmodium* au laboratoire a induit une évolution du parasite avec notamment une augmentation de son intensité d'infection chez l'oiseau.

Conclusion

- Cette étude a permis d'avoir une vision plus complète des paramètres intra- et inter-hôte susceptibles d'impacter la transmission du paludisme.
- Elle a également permis de mettre en évidence que le maintien d'une lignée de parasite au laboratoire pouvait induire une évolution rapide de ce dernier.

Research



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Avian malaria: a new lease of life for an old experimental model to study the evolutionary ecology of *Plasmodium*

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Avian malaria has historically played an important role as a model in the study of human malaria, being a stimulus for the development of medical parasitology. Avian malaria has recently come back to the research scene as a unique animal model to understand the ecology and evolution of the disease, both in the field and in the laboratory. Avian malaria is highly prevalent in birds and mosquitoes around the world and is amenable to laboratory experimentation at each stage of the parasite's life cycle. Here, we take stock of 5 years of experimental laboratory research carried out using *Plasmodium relictum* SGS1, the most prevalent avian malaria lineage in Europe, and its natural vector, the mosquito *Culex pipiens*. For this purpose, we compile and analyse data obtained in our laboratory in 14 different experiments. We provide statistical relationships between different infection-related parameters, including parasitaemia, gametocytaemia, host morbidity (anaemia) and transmission rates to mosquitoes. This analysis provides a wide-ranging picture of the within-host and between-host parameters that may bear on malaria transmission and epidemiology.

1. Introduction

Avian malaria is the oldest experimental system for investigating the biology and transmission of *Plasmodium* parasites. In 1898, Ronald Ross, an army surgeon working in India, carried out a series of carefully controlled experiments using *Plasmodium*-infected sparrows and *Culex* mosquitoes to demonstrate that the disease was transmitted through mosquito bites, thereby solving a centuries-old puzzle and preparing the ground for the first epidemiological models of malaria transmission and the first successful attempts at malaria control [1]. For the following 50 years, avian malaria became the experimental system of choice for malaria research. Avian malaria was used for elucidating key aspects of the biology and transmission of malaria parasites [2,3], as well as for the routine testing and development of the first antimalarial drugs [4]. The discovery of *Plasmodium berghei* in thicket rats in Central Africa in 1949, however, marked a switch to rodent malaria research and the decline of experimental studies on avian malaria.

In the past 15 years, however, work on avian malaria has seen a drastic surge, largely spurred on by the routine blood screening of wild-caught birds around the world (electronic supplementary material, figure S1). Molecular studies have revealed an unexpected level of diversity in avian malaria parasites, which rivals anything that has been found in other vertebrate hosts. There are currently around 600 mitochondrial cytochrome *b*-based lineages of avian *Plasmodium* available (MalAvi Database [5]), although disagreement continues about the relative relevance of morphological characteristics and sequence differences for establishing species boundaries in avian malaria [6]. Avian malaria is much more prevalent (in some areas up to 80% of birds are infected [7]) and

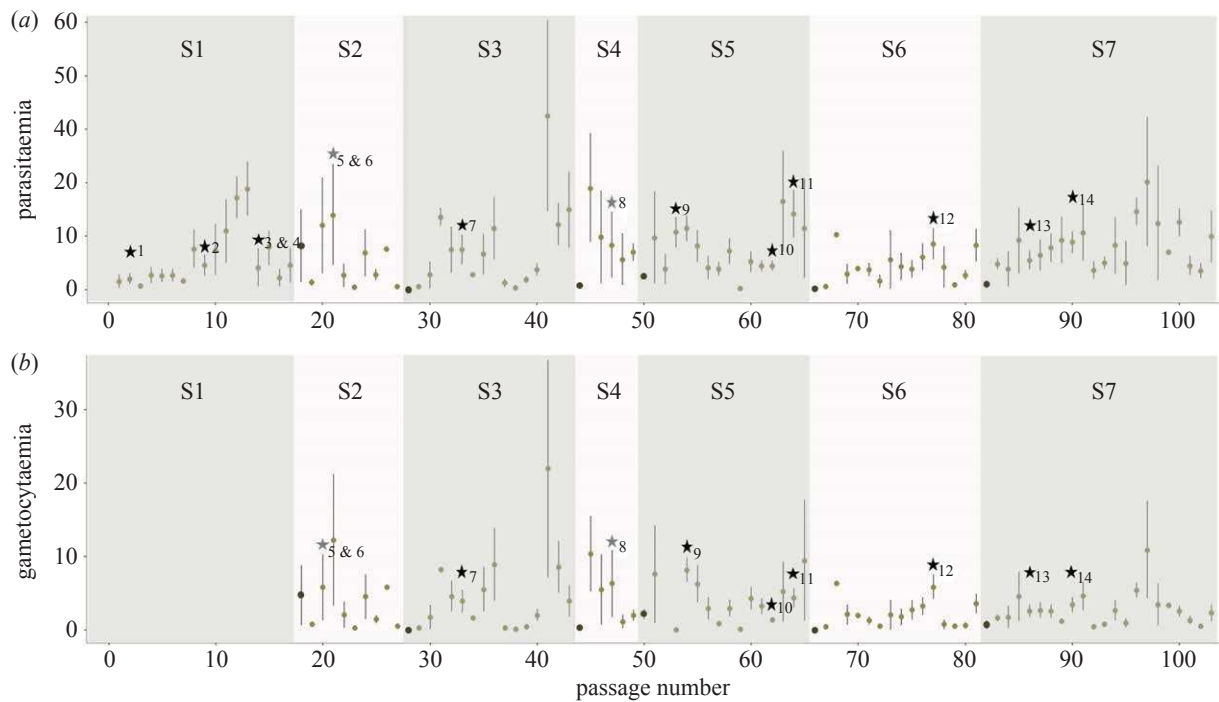


Figure 1. Parasitaemias (a) and gametocytaemias (b) of *P. relictum*-infected birds across all the passages since the isolation of the parasite strain from wild sparrows in 2009. Parasitaemia is quantified as the total % of red blood cells infected; gametocytaemia as the % of red blood cells infected by gametocytes. Dots indicate the mean; bars on either side indicate the standard errors. The horizontal axis indicates the total passage number comprising both 'standard' (bird-to-bird) passages (pale grey dots) and 'mosquito' (bird-to-mosquito-to-bird) passages (dark grey dots). The graph is divided into seven different segments (labelled S1–S7). Each segment starts with a mosquito passage followed by a (varying) number of standard passages. Stars indicate the times at which the 14 different experiments analysed in this paper took place (black stars, acute infection experiments; grey stars, chronic infection experiments). In the passages that took place during the first segment (S1), the gametocytaemia was not quantified, hence the missing values. The number of birds that were used in each passage is given in the electronic supplementary material, figure S2. (Online version in colour.)

widespread (it is present in all continents except Antarctica) than any other vertebrate malaria. Compared to the exponentially growing body of information on the interactions between *Plasmodium* and its bird hosts, however, we still know comparatively very little about avian malaria vectors in the wild [8,9]. Several dozen different vector species are currently listed on the MalAvi database, although for *Plasmodium*, *Culex* mosquitoes are by far the most common vectoring genus [9].

In addition to work carried out in the wild, avian malaria has recently come back to the research scene as an experimental laboratory model for investigating the evolutionary ecology of malaria transmission. For the past 5 years, we have been working in the laboratory on the interactions between the most common avian malaria lineage in Europe, *Plasmodium relictum* SGS1, and its natural vector, the mosquito *Culex pipiens*. This research has been spurred on by the attractive possibility of using an experimental model that bypasses both the ethical and technical constraints associated with experimentation on human malaria, and the two main drawbacks of using rodent malaria: the use of strains that have been kept in the laboratory since their isolation from the wild in the 1950s, and the fact that their natural vector is either unknown or cannot be kept in the laboratory, constraining transmission experiments to convenient, but unnatural, mosquito–parasite combinations.

SGS1 is the most generalist of all the currently described avian malaria lineages: it has thus far been found infecting 95 different species in 10 different orders (MalAvi database, [5]). Its prevalence depends on the host species and geographical region, but it can attain extremely high levels,

particularly in passerine birds: in the South of France 70–80% of sparrows and up to 4% of *Cx pipiens* mosquitoes are infected with this lineage [7,8]. Our SGS1 strain was isolated from wild sparrows caught 5 years ago and has since been kept in the laboratory through serial intraperitoneal passages between domestic canaries (*Serinus canaria*) with the occasional passage via *Cx pipiens* mosquitoes (figure 1). Although we ignore whether wild canaries are infected with SGS1 in their natural range, in France domestic canaries kept outdoors become naturally infected by the parasite (R. Pigeault, A. Nicot, S. Gandon, A. Rivero 2012, personal observation), suggesting they are suitable hosts for this generalist *Plasmodium* lineage outside the laboratory context.

Our experiments on avian malaria have shed light on several aspects of mosquito–*Plasmodium* interaction, including: the role of mosquito genetic diversity [10–14] and bacterial co-infections [8,15] on the outcome of the infection, and the ability of *Plasmodium* both to manipulate mosquito behaviour [16] and adjust its within-bird transmission strategies in response to mosquito availability [17] to maximize its own transmission. These experiments have, however, also generated a great deal of ancillary data on the role of host variability in shaping *Plasmodium* transmission, a crucial piece of the transmission puzzle about which there is still insufficient information in the malaria literature. Indeed, the overwhelming majority of mosquito infection experiments are aimed at comparing the traits of infected and uninfected mosquitoes under different experimental conditions and therefore logically use mosquito as the replication unit. For practical as well as ethical reasons, most of these experiments typically involve up to five vertebrate hosts, and

between-host variability is seen as a cumbersome source of statistical noise that needs to be controlled for.

Here we compile and analyse data obtained in our laboratory in 14 different experiments involving around 120 birds and over 5000 mosquito hosts. The aim is to take stock of 5 years of experimental avian malaria research and provide a wider picture of the within-host and between-host parameters that may bear on *Plasmodium* transmission and, ultimately, epidemiology [18]. For this purpose, we provide statistical relationships between different infection-related parameters, including parasite multiplication, gametocyte production, host morbidity (anaemia) and transmission rates to mosquitoes. We begin by analysing the data arising from the serial passage of our strain through both intraperitoneal injections and mosquito bites during the past 5 years. We then analyse data arising from the experiments to elucidate: (i) the relationships between different traits within the bird, such as parasite multiplication (parasitaemia), investment in transmission (gametocytaemia and gametocyte conversion ratio) and virulence (anaemia); (ii) which of the variables measured in the bird and in the mosquitoes are best at predicting the probability and intensity of *Plasmodium* infection in mosquitoes and, finally, (iii) which variables are best at predicting the fitness of infected mosquitoes. We discuss our results and the limitations of our system with respect, most notably, to the rodent malaria system, the laboratory model that has for many years been the reference for addressing evolutionary questions in malaria [19,20].

2. Material and methods

(a) Malaria parasite

The data originate from 14 different studies spanning 5 years (electronic supplementary material, table S1). *Plasmodium relictum* SGS1 is the aetiological agent of the most prevalent form of avian malaria in Europe [21]. The lineage used in our experiments was isolated by Gabriele Sorci (CNRS Dijon) from sparrows caught in the region of Dijon (France) in 2009. The lineage was passaged to naive canaries (*Serinus canaria*) by intraperitoneal injection. Since then it has been maintained by carrying out regular passages between our stock canaries through intraperitoneal injections (henceforth 'standard passages') around every three weeks. Roughly, every 30 weeks the line was passaged through a mosquito ('mosquito passages'; figure 1).

Recipient canaries were infected by injecting them with ca 80–100 µl of blood from the infected canary stock. All canaries within an experiment were infected using the same pool of blood. For this purpose, blood from between two and five donor canaries was collected using heparinized tubes to avoid coagulation. The blood was then pooled in an Eppendorf tube and diluted in an equal volume of phosphate-buffered saline (PBS). This pool of blood was then injected intraperitoneally to several recipient birds (the number of birds used varied between experiments, see electronic supplementary material, table S1). In most of the experiments, mosquito feeding took place using recipient birds in the acute phase of the infection (days 10–12 post infection), except for three experiments which were carried out during the chronic phase of the infection (days 30–304 post infection, depending on the experiments, electronic supplementary material, table S1). In all experiments, parasite load was monitored at the putative peak of the acute infection (days 10–12 post infection, [17]). In chronic infections, the parasite load was also checked immediately before the mosquito feed. In both cases, blood was sampled by puncturing the wing vein.

In acute infections, parasite load was quantified using blood smears as described by Valkiunas *et al.* [21] and two different parameters were measured: the parasitaemia (total proportion of red blood cells infected) and the gametocytaemia (proportion of red blood cells infected by gametocytes, the sexual stages of the parasite that are transmitted to mosquitoes). Packed cell volume, a standard proxy for anaemia [22], was calculated by taking 50 µl of blood from the brachial vein using a heparinized glass microcapillaries. Samples were immediately centrifuged (5 min, 8000 r.p.m.) and the proportion of red blood cells in the blood was estimated using a micro-haematocrit reader [23].

(b) Mosquito experimental infections and dissections

The large majority of our experiments were carried out using a laboratory strain of *Cx pipiens* (SLab). Three of the experiments were carried out with *Cx pipiens* collected from the field (see the electronic supplementary material, table S1 for details). The experimental protocol was identical irrespective of mosquito origin. Mosquito feeding took place either 10–12 days (acute phase) or 30–300 days (chronic phase) after the onset of the infection (electronic supplementary material, table S1). In those experiments in which we were able to quantify blood meal size we did so by placing engorged females individually in numbered plastic tubes until all haematin (a product of the degradation of haemoglobin) was excreted. When fecundity was studied 3–5 days later, females were transferred to a new tube containing 4–5 ml of mineral water to allow them to lay their eggs. The egg rafts were photographed and the eggs were counted using the Mesurim Pro freeware (<http://svt.ac-amiens.fr/spip.php?article40&lang=fr>). Female size was estimated by measuring the length of its wing along its longest axis. Seven to nine days after the infected blood meal, females were sampled and dissected to count the oocysts in their midguts [10]. This allowed us to estimate infection prevalence (oocyst presence/absence) and burden (number of oocysts per female). Measurement of lifespan was incompatible with the quantification of oocysts. In those experiments in which lifespan was measured, mosquitoes were transferred from the oviposition tube to a new tube which was checked daily to record mortality.

(c) Statistical analysis

Analyses were carried out using the R statistical package (v.3.1.0, <http://www.cran.r-project.org/>). The different statistical models built to analyse the data (numbered models 1–33) are described in the electronic supplementary material, table S2. Of the 14 experiments reported here, 10 were carried out using acute stage infections and SLab mosquitoes, two using chronic infection and wild mosquitoes and one using acute infections and wild mosquitoes (electronic supplementary material, table S1). As there were not enough data from chronic infections or from wild mosquito experiments to do any in-depth analyses, unless specifically stated the statistical analyses were restricted to the 11 experiments that used acute infections and SLab mosquitoes. The serial passage analyses were carried out using our full parasitaemia and gametocytaemia records since the isolation of the parasite strain 5 years ago. This dataset comprises 103 passages (including standard passages and mosquito passages) and over 415 birds. The general procedure for building the statistical models was as follows. Maximal models were built by including all biologically appropriate main effects and higher order interactions. In some of the models, we included random effects to correct for two potential sources of pseudoreplication: the experiment effect (experiments were separated by several months, or years, and it is inevitable that environmental conditions change slightly from one experiment to the next) and the bird effect (mosquitoes feeding on the same bird are not independent from each other). Whether the model contained random effects or not determined the type of R subroutine we used (electronic

supplementary material, table S2). Models containing exclusively fixed effects were analysed using standard glm procedures and a normal error structure. Some of the response variables had to be transformed to correct for non-normal errors. Models containing both fixed and random effects, on the other hand, were analysed using mixed effects models with a normal distribution (lme, nlme package, <http://www.cran.r-project.org/>), except when the response variable was a proportion (e.g. oocyst prevalence), in which case the data were analysed using a binomial error distribution (lmer, lme4 package, <http://www.cran.r-project.org/>). The serial passage analyses were corrected for temporal autocorrelation by including a lag 1 term (i.e. parasitaemia or gametocytaemia at time t as a function of parasitaemia or gametocytaemia at $t - 1$). The maximal model was simplified by sequentially eliminating non-significant terms and interactions to establish a minimal model [24]. The significance of explanatory variables was established using a likelihood ratio test (LRT) which is approximately distributed as a χ^2 distribution [25]. The significant χ^2 -values given in the text are for the minimal model, while non-significant values correspond to those obtained before deletion of the variable from the model.

3. Results

(a) Serial passage analyses

Since its isolation from the wild, our strain of *P. relictum* SGS1 was maintained through a combination of standard and mosquito passages. Figure 1*a,b* shows the variation in parasitaemia and gametocytaemia across the different passages. Parasitaemia increased slightly but significantly across the whole passage sequence (model 1: $\chi^2_1 = 4.860$, $p = 0.0230$). The mean parasitaemia over the first 10 passages was $3.5 \pm 0.74\%$ ($\pm$ s.e.), while over the last 10 passages the mean parasitaemia had more than doubled to $8.21 \pm 1.24\%$. To test whether this was a global trend or whether passage through mosquitoes bore any effect on the observed trend, we divided the dataset into seven consecutive segments, each starting with a mosquito passage and followed by a series of standard passages (figure 1*a*). This trend for an increase in parasitaemia was observed after each mosquito passage (model 2: slope = 0.032, $\chi^2_1 = 9.926$, $p = 0.001$), irrespective of the segment (model 2: $\chi^2_1 = 4.139$, $p = 0.0614$). Interestingly, there was a significant decrease in parasitaemia immediately following a mosquito passage (electronic supplementary material, figure S3). Such a decrease could simply be due to differences in the inoculum size: there are likely to be significantly fewer parasites injected by the mosquito than by an intraperitoneal injection. If this is the case, we would expect parasitaemia to first decrease following a mosquito passage, then recover following the subsequent standard passage. When the first infection following a mosquito passage was removed from the analyses, the slope of the trend decreased slightly (slope = 0.022), but the trend remained significant, albeit marginally (model 3: $\chi^2_1 = 4.232$, $p = 0.031$). However, when both passages were removed from the analyses, the resulting trend within each segment was lost (model 4: slope = 0.009, $\chi^2_1 = 3.772$, $p = 0.4125$), indicating that both points were influential in the observed increase in parasitaemia within each segment. This was confirmed with a second analysis, where the parasitaemia within each segment was fitted as a categorical variable with three values: first passage, second passage and the rest. As expected, this new variable was highly significant (model 5: $\chi^2_1 = 12.343$, $p = 0.0012$). Contrast analyses

revealed that the estimated average parasitaemia for the first (mosquito) passage was marginally but significantly lower than that of the second (standard) passage (model 5: $t = 2.30$, $p = 0.021$). These two parasitaemias were, however, much lower than the mean parasitaemia in the rest of the passages (model 5: $t = 3.64$, $p = 0.0003$).

By contrast, gametocytaemia did not show any change across the whole passage sequence (model 6: $\chi^2_1 = 0.024$, $p = 0.8501$). The mean gametocytaemia over the first 10 passages was $2.8 \pm 0.75\%$ ($\pm$ s.e.), while over the last 10 passages the mean gametocytaemia had barely changed $2.3 \pm 0.50\%$. To test whether passage through mosquitoes bore any effect on the observed trend, we analysed each segment separately, as above. No trend in gametocytaemia was observed in any of the segments (model 7: $\chi^2_1 = 0.529$, $p = 0.3753$).

(b) Trait relationships within the bird

Each of the 11 acute stage infection experiments reported here is represented in figure 1*a,b* by a black star. These experiments showed a large variance in both the bird parasitaemias and gametocytaemias (electronic supplementary material, figure S4) both among experiments (parasitaemia, model 8: $F_{10,61} = 3.03$ $p = 0.0044$; gametocytaemia, model 9: $F_{8,53} = 5.01$ $p = 0.0002$) and within experiments, even though the birds were infected concomitantly with the same pool of blood (electronic supplementary material, figure S4).

Within each bird, there was a strong association between peak parasitaemia and gametocytaemia (model 10: $\chi^2_1 = 100.37$, $p < 0.0001$; figure 2). This was partially expected, as our definition of total parasitaemia encompasses all *Plasmodium* stages, including gametocytes, so both axes were not independent. The results were, however, identical when we analysed separately asexual and sexual (gametocyte) stages of *Plasmodium* (model 11: $\chi^2_1 = 56.089$, $p < 0.0001$). We calculated the reproductive effort of malaria parasites as the density of gametocytes relative to the total number of parasites at the peak of the infection. For simplicity, we term this 'conversion ratio', to distinguish it from 'conversion rate' which refers to the investment into gametocytes across parasite cohorts [26]. In our system, gametocytes constituted on average a third (mean $\pm$ s.e., $33 \pm 2.4\%$) of the intracellular parasite population in the blood. There was, however, a great degree of variation around this mean, much of which was explained by differences between experiments (model 12: $F_{8,53} = 9.88$ $p < 0.001$).

As expected, infection by *P. relictum* led to a marked loss of red blood cells (anaemia). Infected birds showed a 28.5% decrease in the packed cell volume with respect to uninfected birds (model 13: $\chi^2_1 = 1052$, $p < 0.0001$). This decrease was, however, independent of the density of parasites: highly parasitaemic birds had similar anaemia to weakly parasitaemic birds (model 14: $\chi^2_1 = 0.0765$, $p = 0.782$). We found no association between conversion ratio and anaemia (model 15: $\chi^2_1 = 1.928$, $p = 0.165$).

(c) Predictors of mosquito infection

(i) Bird-related factors

We analysed the effect of four bird-related factors (infection stage, parasitaemia, gametocytaemia, anaemia) on the probability and intensity of *Plasmodium* transmission to mosquitoes. One of the strongest predictors of whether mosquitoes became infected by *Plasmodium* (oocyst prevalence), and of the intensity

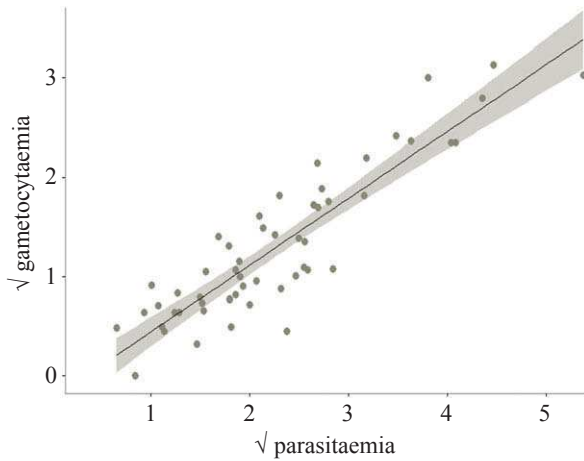


Figure 2. Regression between the parasitaemia and gametocytaemia (expressed as the square root of the proportion of red blood cells infected with a particular stage) in each bird infection. (Online version in colour.)

of the subsequent infection (oocyst burden) was whether they fed on an acutely or chronically infected bird. Acute stage infections resulted in four times higher prevalences (model 16: $\chi^2_1 = 95.513$, $p < 0.0001$) and more than 10 times higher oocyst burdens (model 20: $\chi^2_1 = 26.664$, $p < 0.0001$) than chronic stage infections (figure 3).

As there were not enough data from chronic infections to do any in-depth analyses (electronic supplementary material, table S1), all subsequent results come from acute infections. When mosquitoes fed on acutely infected birds, there was a very strong positive association between the probability that they would become infected and the intensity of the ensuing infection: birds that rendered higher oocyst prevalences also rendered higher oocyst burdens (model 23: $\chi^2_1 = 17.176$, $p < 0.0001$; electronic supplementary material, figure S5). Despite this, however, oocyst prevalence and oocyst burden were not always explained by the same explanatory variables.

Bird parasitaemia was not correlated with oocyst prevalence (model 17: $\chi^2_1 = 0.324$, $p = 0.3240$; figure 4a) but was a good predictor of oocyst burden (model 21: $\chi^2_1 = 5.6413$, $p = 0.0175$; figure 4c). Fitting the quadratic term (parasitaemia²) significantly improved the model fit (model 21: $\chi^2_1 = 4.386$, $p = 0.0362$), suggesting that oocyst burden is a decelerating polynomial function of bird parasitaemia (figure 4b). The relationship between higher bird parasitaemia and higher oocyst burden in mosquitoes may be mediated by blood intake. Interestingly, we found a positive association between the density of parasites in the blood and the volume of blood ingested by the mosquitoes (model 25: $\chi^2_1 = 4.947$, $p = 0.019$; figure 5).

The patterns for gametocytaemia were, however, different. Although there was a trend for higher oocyst burdens as gametocytaemia increased (figure 4d), it was not statistically significant (model 22: $\chi^2_1 = 1.359$, $p = 0.2436$). Also, unexpectedly, we found a weak but statistically significant negative relationship between bird gametocytaemia and the probability of mosquito infection (model 18: $\chi^2_1 = 5.102$, $p = 0.0239$; figure 4b). Anaemia, on the other hand, did not explain either parasite prevalence (model 19: $\chi^2_1 = 0.154$, $p = 0.695$) or burden (model 24: $\chi^2_1 = 3.781$, $p = 0.052$) in mosquitoes.

(ii) Mosquito-related factors

We then analysed the mosquito-related factors that may determine transmission. Previous work has shown that one

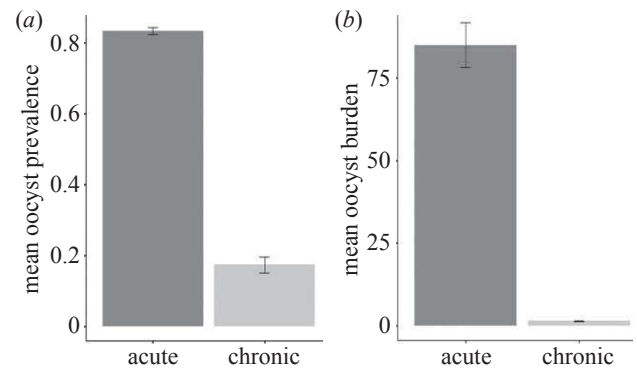


Figure 3. Mean infection prevalence (a) and oocyst burden (b) in mosquitoes fed on acute or chronic bird infections. Bars represent standard errors around the mean.

of the main predictors of oocyst prevalence and burden is the amount of blood meal ingested by the mosquito [10]. Our results confirmed the existence of very strong relationships between haematin (a standard proxy for blood meal quantification) and oocyst prevalence (model 26: $\chi^2_1 = 122.4$, $p < 0.0001$; figure 6a) and between haematin and oocyst burden (model 27: $\chi^2_1 = 123.88$, $p < 0.0001$; figure 6b).

Haematin itself was dependent on several factors, foremost of which were the anaemic status of the bird and the size of the mosquito. Unsurprisingly, larger mosquitoes took larger blood meals (electronic supplementary material, figure S6). The increase in haematin production with mosquito size was slightly greater for mosquitoes feeding on uninfected than on infected birds, as evidenced by a significant interaction between size and infection status (model 29: $\chi^2_1 = 43.448$, $p < 0.0001$). Bird anaemia was also important: there was a highly significant relationship between the packed cell volume in the bird and the amount of haematin excreted by mosquitoes feeding on that bird (model 28: $\chi^2_1 = 28.221$, $p < 0.0001$; electronic supplementary material, figure S7). It follows that mosquitoes feeding on infected birds which were highly anaemic produced less haematin than mosquitoes feeding on uninfected birds (on average 34% less).

(d) Predictors of mosquito fitness

We determined which bird or mosquito-related factors predicted the fitness (fecundity, longevity) of mosquitoes. One of the key predictors of mosquito fecundity was the infection status of the bird on which they fed: females feeding on an infected bird laid significantly fewer eggs (11% fewer, on average) than those feeding on uninfected ones (model 30: $\chi^2_1 = 21.719$, $p < 0.0001$; figure 7a). This could be due to either a direct effect of the parasite on fecundity (on average 80–90% of mosquitoes that fed on an infected bird became infected) or the indirect result of feeding on blood with a lower protein content (anaemic blood). To test for this, we added haematin, a proxy for the amount of haemoglobin ingested, into the model. As expected, both haematin and its quadratic term (haematin²) were key predictors of mosquito fecundity, indicating that egg production was a saturating function of the amount of blood ingested. However, the presence of haematin in the model did not reduce the significance of the infection effect (model 31: $\chi^2_1 = 36.148$, $p < 0.0001$; figure 7b) suggesting that the reduction in egg production was not entirely explained by the decrease in blood quality.

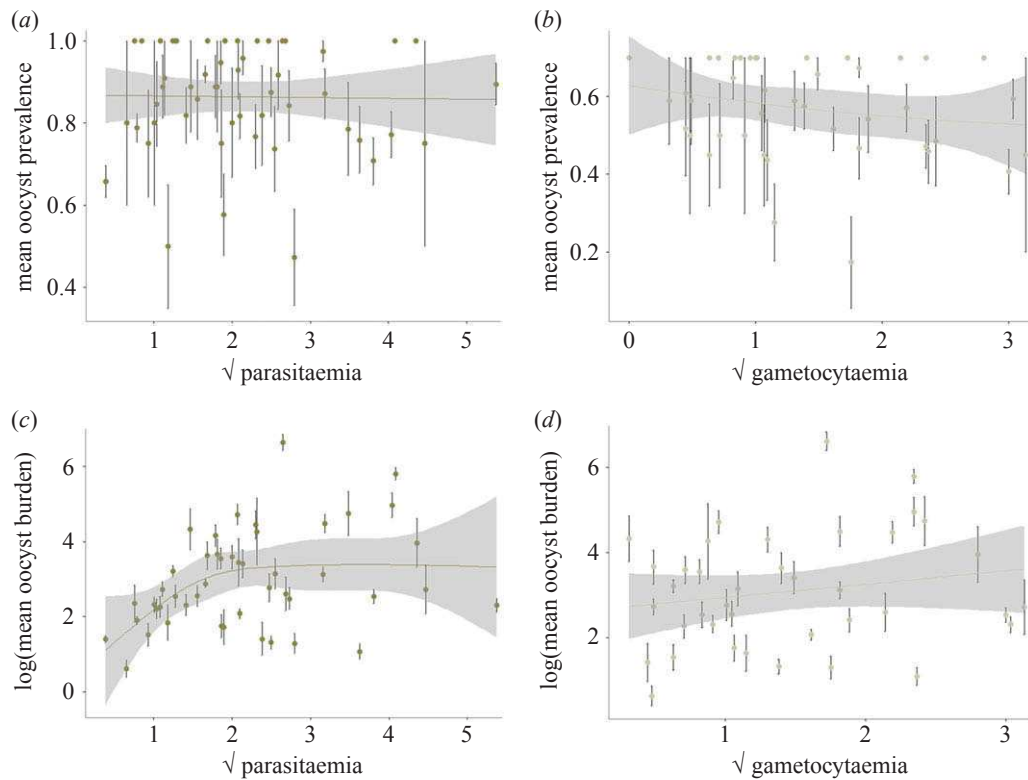


Figure 4. Mean infection prevalence and oocyst burden in mosquitoes as a function of (square root transformed) parasitaemia (*a,c*) and gametocytaemia (*b,d*) in the bird. Bars represent standard errors around the mean. Shaded areas on either side of the regression line represent the 95% CI. (Online version in colour.)

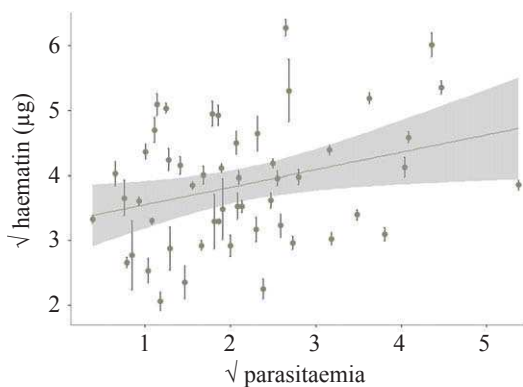


Figure 5. Mean haematin excreted by mosquitoes as a function of bird parasitaemia (total % of red blood cells infected). Bars represent standard errors around the mean. Both variables have been square root transformed to improve the fit of the model. (Online version in colour.)

In contrast to fecundity, variations in mosquito lifespan were not explained either by the infection status of the bird (model 32: $\chi^2_1 = 0.2531$, $p = 0.6309$), or by the size of the blood meal ingested (model 32: $\chi^2_1 = 1.306$, $p = 0.2531$). Interestingly, lifespan was positively correlated with bird parasitaemia: mosquitoes fed on birds with higher parasitaemias were longer lived (model 33: $\chi^2_1 = 5.740$, $p = 0.0143$; figure 8).

4. Discussion

(a) Serial passages

Previous work has shown that when parasites of various *Plasmodium* species are serially passed, they often generate higher parasite densities [20]. These results fit in with the general prediction that, in the absence of mosquitoes, serial passages should select for higher rates of asexual reproduction and thus

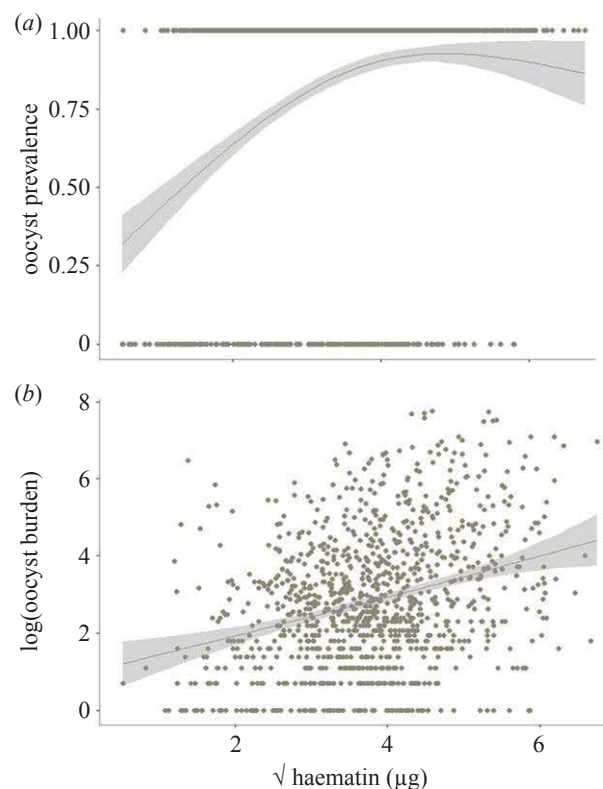


Figure 6. Oocyst prevalence (*a*) and burden (*b*) as a function of haematin. Haematin has been square root transformed to improve the fit of the model. (Online version in colour.)

for higher parasitaemia [20,27]. In some cases, this increase in parasitaemia has been accompanied by an increase in transmission potential, measured as gametocyte production [19], whereas in others gametocyte production has been reduced [28].

Our results show that, despite regular passages through the mosquitoes, parasitaemia increased slightly but significantly

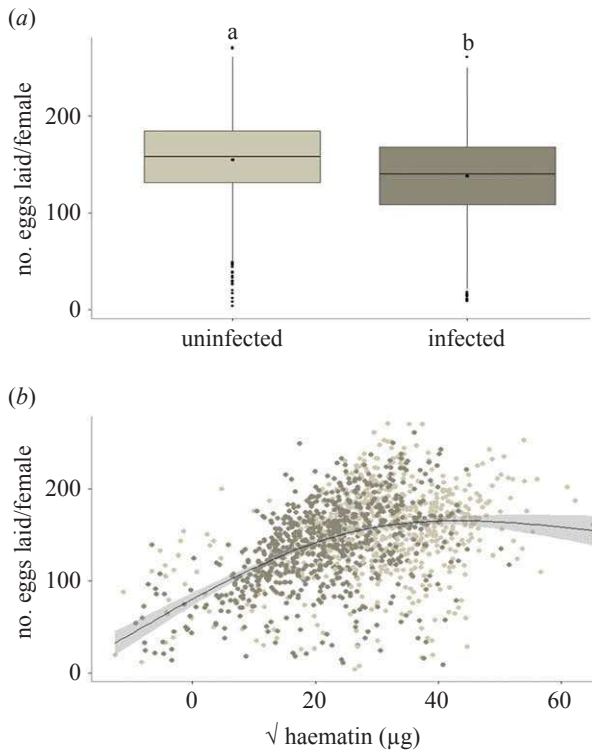


Figure 7. Number of eggs laid by females as a function of their infection status (a) and the amount of blood meal ingested (b). Light grey box plot and dots represent uninfected females, dark grey box plot and dots represent infected females. In (b), haematin has been square root transformed to improve the fit of the model. (Online version in colour.)

over the course of the passages, yet no significant change in gametocytaemia with time was detected. A more detailed analysis of our serial passage time series indicated, however, that passage through a mosquito did indeed result in a significant, but short lived, decrease in parasitaemia. Transmission through mosquitoes is very different from intraperitoneal injections in many ways, the most obvious of which is inoculum size. This alone could explain the differences in parasitaemia observed [29]. Another, and perhaps more important difference, is that while in intraperitoneal injections the blood-stage parasites are injected more or less directly into the bloodstream, mosquitoes inject sporozoites which need to go through the whole exoerythrocytic merogony in the reticuloendothelial cells of several organs (liver, spleen) before reaching the blood [21]. The decrease in parasitaemia we observed after a mosquito passage may simply be due to a delay in the arrival of parasites in the bloodstream. However, we also detected a negative carry-over effect of the mosquito passage in the subsequent standard (intraperitoneal) passage immediately following it. This suggests that the mosquito effect goes beyond a mere dose or delay issue, and that passage through mosquitoes could durably alter the outcome of evolution through passage experiments. Similar results have recently been obtained in the rodent malaria model, where passage through mosquitoes reduced parasitaemia for five passages before increasing again [30]. In addition, lower parasitaemia was associated with a reduced virulence and a modification of the expression of genes involved in antigenic variation [30]. Clearly, more detailed experiments could be carried out with our system to decipher the impact of mosquito passages on the experimental evolution of *P. relictum*.

Further experiments will also be needed to address two important issues that hinder the interpretation of our results

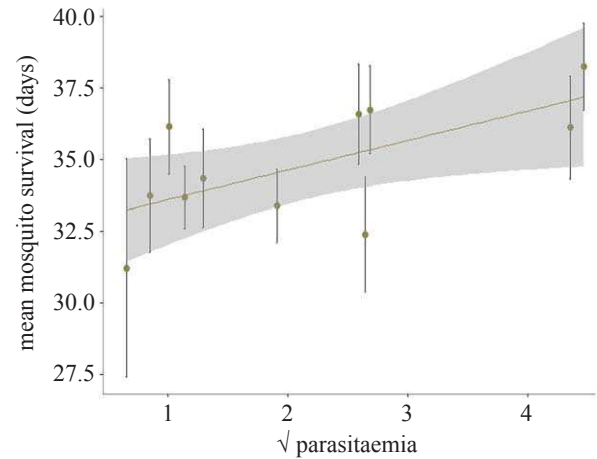


Figure 8. Mean mosquito survival as a function of bird parasitaemia (total % of red blood cells infected). Bars represent standard errors around the mean. (Online version in colour.)

and make it difficult to compare them with those obtained in rodent malaria [20]. First, rodent malaria experiments have been carried out using single genotype isolates [31], while our strain is a field isolate containing a single *P. relictum* lineage (as defined by its *cyt-b* sequence [5]) but with an unknown amount of genetic variability. It is therefore not impossible that the genetic diversity of our strain may have changed through the serial passages, an interesting possibility that will be worth exploring when the molecular tools become available with the upcoming the full genome sequence of the SGS1 strain. Second, for bird welfare reasons (wing vein punctures result in scabbing that takes a few days to heal), parasitaemia was measured only once during the acute phase of the infection. Based on preliminary (unpublished) results showing that in our system peak parasitaemia is attained on average 10–12 days post infection, all our acute parasitaemia measurements were therefore taken within this time window. This has three obvious shortcomings. First, any between-bird variability in peak parasitaemia cannot be accounted for. Second, as mentioned above, the timing of peak parasitaemia may be different in mosquito-triggered infections (our time estimates were based on intraperitoneal infections). Finally, the peak parasitaemia may have shifted with time as a by-product of the serial passages. All of these issues are worthy of study and we are currently developing new blood sampling methods that will allow us to monitor parasitaemia daily to explore them further.

(b) Bird infection traits

One of the most troubling facts when working with avian malaria is the large variance in bird parasitaemias we obtain, much of which can be explained by unavoidable variations in conditions between experiments. However, even within a single experiment, birds infected concomitantly from the same pool of blood show a large variance in both their parasitaemia and gametocytaemia (electronic supplementary material, figure S4). The reasons for this variation are not known but may be numerous. Birds vary widely in their susceptibility to infection according to, among other things, their age [7,32,33], body condition [34,35] and any previous or concurrent infections [36,37]. Canaries are not a standard experimental animal; hence we lack information about many of these key parameters, and their potential role in the observed

variance in parasitaemias and gametocytaemias. Further work is required to explore the influence of these factors on susceptibility to malaria infections.

In our experiments, gametocyte conversion ratios also showed a great degree of variation between birds. At the acute stage of the infection, gametocytes constituted, on average, a third of the total *P. relictum* parasite population, which is much higher than the values obtained in other *Plasmodium* species. The difference is particularly striking with the rodent malaria species *P. chabaudi*, where gametocytes represent at most 3% of the parasites present in an infection [38,39]. Conversion ratios in human malaria, on the other hand, vary between 0.3 and 21% depending on host age [40] and genotype [41]. The largest conversion ratios recorded are observed in wild lizards, where gametocytes represent between 50 and 85% of the total parasite population, depending on the season [42]. Whether the high conversion ratios we observed are a distinctive feature of our experimental system or whether they are representative of other avian malaria parasites is not known. We know of only one other study that has quantified conversion ratios in an avian malaria species [43]. In *P. juxtanucleare*, gametocytes were found to represent around 3% of the total parasite population, but the particular experimental protocol employed (old chronic infections and several doses of corticosteroids to impair the birds' immunity) make this result difficult to generalize [43]. We clearly need more studies that quantify the plasticity in gametocyte conversion ratios in avian malaria in response to different environmental conditions. The possibility of doing this both in the wild and in controlled experimental infections in the laboratory is unparalleled among currently available malaria systems.

(c) Virulence in birds and mosquito transmission

It is widely accepted that avian malaria parasites impose fitness costs to their hosts, although the nature of these costs vary depending on the bird and *Plasmodium* species. A well-known example of the potentially devastating effects of avian malaria on host fitness is the decline and extinction of naive populations of honeycreepers after the accidental introduction of the parasite to the Hawaiian Islands in the early twentieth century [44]. Detecting fitness costs on endemic infections in the wild has, however, proved more challenging. Long-term monitoring of a wild population of blue tits has revealed strong associations between malaria infection on bird survival and recapture rates [45]. More recently, malaria infection has been found to accelerate the senescence of great reed warblers through telomere degradation [46]. Experimental infections, on the other hand, have consistently shown anaemia to be one of the primary virulence determinants in avian malaria infections [23,36,42]. Likewise, in this analysis, *P. relictum* infections resulted, on average, in a 30% reduction in the red blood cell count during the acute phase of the infection (10–12 days after inoculation). However, in contrast with a report on rodent malaria [19], we found no phenotypic association between parasitaemia and virulence. Unlike this earlier study, however, we did not monitor the temporal dynamics of the infection: our estimates of parasitaemia are based on single measurements 10–12 days after the infection. The relationship between parasitaemia and virulence deserves further studies that should also consider other measures of virulence (e.g. weight loss [35]).

Parasitaemia was found to be a good predictor of transmission to mosquitoes. This is most apparent through the striking differences in oocyst prevalence and burden in mosquito infections issued from acutely and chronically infected birds (figure 3). This raises the important question of the relative contribution of acute versus chronic infections to transmission in the field, given that acute *Plasmodium* infections are very short lived, and the vast majority of wild-caught infected birds harbour chronic infections [33]. Additionally, there is a marked seasonal variation in avian malaria prevalence in birds [47,48] and the proportion of birds with an acute infection in a population may fluctuate with the number of infected vectors throughout the year.

Within the range of parasitaemias observed in acute infections, we also found a clear association between parasite burden in the host and in the mosquito, even though the number of oocysts saturates beyond 4% of red blood cells infected (figure 4b). We failed, however, to detect a clear relationship between parasitaemia and the probability of infection of mosquitoes (figure 4c). As has been found in other systems, and most notably in rodent and human malaria [20,39], parasitaemia and gametocytaemia during the acute stage of the infection were strongly correlated (figure 2). It is therefore surprising that we failed to detect a positive association between gametocytaemia and the prevalence or intensity of *Plasmodium* infection in mosquitoes (figure 4b,d). This result echoes those from other malaria studies, which also reported a lack of a clear relationship between gametocytes and mosquito infection in *Plasmodium* [49,50] (but see [51,52] for two studies where such a relationship was found). This problem may be due in large part to the estimation of gametocyte densities by microscopy, which misses a significant proportion of the gametocytes present [49,50]. Molecular tools based on the amplification of gametocyte-specific RNA have been developed for rodent and human malaria and allow the quantification (and sexing) of gametocytes with greater accuracy (reviewed in [53]). Similar tools in avian malaria are urgently needed, as they will offer new opportunities to study phenotypic plasticity in gametocyte density and sex ratio in response to different ecological and evolutionary scenarios, both in the field and in the laboratory.

The question remains as to why parasitaemia is a better predictor of mosquito infection rates than gametocytaemia (see also [17]). It is unlikely that parasitaemia bears directly on mosquito infectivity. Parasitaemia, however, is correlated with several in-host factors, including host immunity and metabolic profiles which may affect the likelihood of mosquito infection [54]. Interestingly, we found a significant positive relationship between blood parasitaemia and the volume of blood ingested by mosquitoes (figure 5). The mechanisms underlying this positive relationship are not known. One possibility is that parasitaemia may correlate negatively with blood quality, and that mosquitoes compensate for poorly nutritious blood by feeding more, which would result in higher infection levels. In human malaria, it is well established that peak parasitaemia is associated with decreased concentrations of several blood metabolites, including lipid and sugar levels [55]. In our experiments, the only measure of blood quality we recorded was the number of red blood cells, which are known to be an important source of protein for mosquitoes. This variable did not, however, explain the observed variation in parasitaemia.

An alternative explanation for the observed increase in blood meal size with parasitaemia could be a dose-dependent manipulation of mosquito behaviour by the parasite. *Plasmodium* parasites have been shown to manipulate their hosts at different stages of the parasite's life cycle ([16,56], but see [57]). In particular, we previously reported that infected birds attract significantly more mosquitoes than uninfected ones, possibly through the emission of distinct olfactory signals [16]. None of these reported cases of parasite manipulation has, however, been shown to be dependent on parasite numbers.

(d) Mosquito fitness

Malaria transmission depends critically on the fitness of its infected vectors. For this reason, the effects of *Plasmodium* infection on mosquito life-history traits such as fecundity and lifespan have received a lot of attention [58,59]. The largest effect of *P. relictum* parasites on *Culex* mosquitoes is a drastic reduction in their fecundity, which can result in a loss of up to 40% of eggs laid [12]. Our findings are consistent with these previous results qualitatively, if not quantitatively: infected females laid on average 11.5% fewer eggs than their uninfected counterparts. This reduction in egg production could be due to two non-mutually exclusive factors. First, haemoglobin is the main source of protein for egg production in mosquitoes [60], and infected birds are anaemic. The reduction in fecundity may therefore simply be an indirect by-product of anaemia. Second, there is evidence from rodent malaria suggesting that *Plasmodium* induces a direct cost on female fecundity. *Plasmodium yoelii* induces apoptosis in mosquito ovaries [61] and increases egg resorption [62]. Although no molecule of *Plasmodium* origin has been identified that would justify talking about parasite manipulation, it has been widely assumed that reproductive curtailment is an adaptive strategy of the parasite to increase mosquito survival through a trade-off in energy allocation between reproduction and survival [63,64].

The effect of *Plasmodium* on mosquito survival is still highly contentious and seems to be extremely sensitive to the particular mosquito–*Plasmodium* combination used and to the specific laboratory conditions under which it is measured [12,59]. Despite earlier results showing that, under certain experimental conditions, *P. relictum* infections are associated with an increase in mosquito longevity [12], here we found no clear association between *Plasmodium* infection and mosquito longevity, although slight differences in the experimental protocols may have blurred the picture. We did, however, find an intriguing positive relationship between bird parasitaemia and mosquito survival (figure 8). Given that parasitaemia is a good predictor of oocystaemia (figure 4b), one possibility is that this positive relationship is the result of mosquitoes with higher oocyst burdens living longer. It is, unfortunately, impossible to verify this with the current dataset as longevity and oocyst burden cannot be quantified in the same individual (mosquitoes need to be killed to quantify oocysts).

5. Conclusion and perspectives

This study represents a comprehensive review of the knowledge we have accumulated regarding between-host trait variability and trait associations in malaria parameters and how they affect transmission. Some of the statistical

relationships obtained, such as the relationship between parasitaemia and gametocyte production, or that between blood meal size and oocyst number, are consistent with findings in other experimental systems [20,65]. Others, however, such as the relationship between gametocytaemia and mosquito infection rates, were expected but not found. We need to take a leaf out of the rodent malaria studies and develop markers that will allow us to quantify gametocyte numbers and sex ratios more accurately. This will open the way for novel studies looking at the genetic and environmental determinants underpinning gametocyte conversion rates and sex ratios in avian malaria both in the laboratory [66] and, crucially, in the field. Finally, we also obtained results that were unexpected and that require further study. This is the case for the positive relationship between parasitaemia in the bird and both the haematin excreted by mosquitoes, an imperfect proxy for blood meal size (figure 5), and their subsequent longevity (figure 8). Further experiments are needed to disentangle the potential mechanisms underlying these relationships before any conclusions can be drawn. Finding a satisfactory method to quantify blood meal size that is independent of the amount of haemoglobin in the blood, the main drawback of haematin, would go a long way towards disentangling the effects of anaemia and clarifying the central role seemingly played by blood meal size in these interactions.

The analysis of our serial passage experiments also revealed very interesting patterns. In particular, as shown in earlier studies [30] we show that the mode of transmission (intraperitoneal or via mosquitoes) affects subsequent within-bird dynamics and deserves further investigation. In addition, the number of serial passages had an effect on parasitaemia but not on gametocytaemia. Could this pattern result from the genetic or epigenetic modifications of the gametocyte conversion ratio? The *P. relictum* genome will provide the framework for tracking mutations that may be involved in the control of within-host dynamics. Unlike previous studies based on multiple clones of *P. chabaudi* [19,20], we are currently unable to unravel phenotypic from genetic correlations, and hence we cannot examine the genetic constraints moulding avian malaria evolution. It is time to expand our work to assess new malaria lineages and explore the life-history variation associated with the large genetic diversity of avian malaria in the field [5]. Ultimately, the ability to combine empirical observations in the field and in the laboratory on different avian malaria genotypes in both the bird and the mosquito vector will, we believe, generate major contributions to the study of the evolutionary ecology of *Plasmodium*.

Ethics. Experiments were approved by the Ethical Committee for Animal Experimentation established by the authors' institution (permit no. CEEA-LR-1051).

Data accessibility. Dryad website (<http://dx.doi.org/10.5061/dryad.35hs3>).

Authors' contributions. R.P., J.V., S.C., F.Z., S.G. and A.R. conceived and designed the experiments cited in this paper. R.P., J.V., S.C., F.Z. and A.N. performed the experiments. R.P. compiled and analysed the data. P.P. was instrumental in developing the experimental canary infections. R.P., S.G. and A.R. wrote the paper. All authors read and commented on earlier drafts of the paper and approved the final version of the manuscript.

Competing interests. We declare we have no competing interests.

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Avian malaria: a new lease of life for an old experimental model to study the evolutionary ecology of *Plasmodium*

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Supplementary materials

Includes Supplementary Tables S1 and S2, and Supplementary Figures S1-S7

Table S1 - Summary of the experiments included in the paper. The table indicates: the year in which they were carried out, the number of birds used and whether they were infected or uninfected, the average number of mosquitoes per bird, whether the birds were at the chronic or acute stage of the infection, and the traits measured in both the bird and mosquito hosts. *par*: parasitaemia, *gam*: gametocytaemia, *conv*: conversion rate, *pcv*: packed cell volume (anaemia), *prev*: oocyst prevalence, *burd*: oocyst burden, *hm*: haematin, *size*: mosquito size, *surv*: survival, *fec*: fecundity

Exp nb.	Year	Nb birds		Mosquitoes/ bird (mean)	Bird infection stage	Mosquito strain	Traits measured		REF
		Uninf	Inf				In the bird	In the mosquito	
1	2009	0	9	24	Chronic	Wild	<i>par</i>	<i>prev, burd</i>	[1]
2	2009	0	13	15	Chronic	Wild	<i>par</i>	<i>prev, burd</i>	[1]
3	2009	5	5	55	Acute	SLab	<i>par, gam, conv</i>	<i>prev, burd, hm, size, surv</i>	[2]
4	2010	6	6	44	Acute	SLab	<i>par, gam, conv</i>	<i>prev, burd, hm, size, surv</i>	[2]
5	2010	0	3	150	Acute	SLab	<i>par</i>	<i>prev, burd, hm</i>	[3]
6	2010	0	5	10	Acute	SLab	<i>par, gam, conv</i>	<i>prev, burd, hm, size, fec</i>	[4]
7	2011	5	4	54	Acute	SLab	<i>par, gam, conv, pcv</i>	<i>prev, burd, hm, size, fec, surv</i>	[5]
8	2012	0	14	21	Chronic	SLab	<i>par, pcv</i>	<i>prev, burd</i>	[6]
9	2012	0	4	15	Acute	SLab	<i>par</i>	<i>prev, burd, hm,</i>	[4]
10	2013	0	5	47	Acute	SLab	<i>par, gam, conv, pcv</i>	<i>prev, burd, hm, size</i>	[7]
11	2013	0	6	50	Acute	SLab	<i>par, gam, conv, pcv</i>	<i>prev, burd, hm, size, fec</i>	[5]
12	2013	10	9	63	Acute	SLab	<i>par, gam, conv, pcv</i>	<i>prev, burd, hm, size, fec</i>	[8]
13	2014	0	5	23	Acute	SLab	<i>par, gam, conv, pcv</i>	<i>prev, burd, hm, size</i>	[7]
14	2014	0	10	31	Acute	Wild	<i>par, gam, conv</i>	<i>prev, burd, hm</i>	[9]

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- [9] Pigeault, R., Nicot, A., Gandon, S., Rivero, A. unpublished data

Table S2: Description of statistical models used. The response variable was not transformed unless otherwise stated. N gives the number of mosquitoes included in each analysis. "Maximal model" gives the complete set of explanatory variables included in the model. "Minimal model" gives the model containing only the significant variables and their interactions. Square brackets indicate variables fitted as random factors (nested variables are indicated with a backslash). Curly brackets indicate the error structure used (n: normal errors, b: binomial errors). Please see Table S1 for the acronyms of the variables fitted in the models. Other acronyms: *exp* = experiment, *asex* = proportion of asexuals, *para_{t-1}* = parasitaemia at the previous time point, *pass1* = all passages (starting at 1 finishing at 103), *pass2* = passages within each segment (each segment starts with a mosquito passage followed by a variable number of standard passages), *pass3* = passages within each segment but excluding the mosquito passage, *pass4* = passages within each segment but excluding the mosquito passage and the first standard passage immediately following it, *pass5* = passages within each segment as a categorical variable with 3 levels (mosquito p fitted as a categorical variable with three values: first passage (mosquito), second passage (standard), and the rest (standard)), *S* = segment number.

Variable of interest	Resp. variable	Model nb	N (mosquit)	N (birds)	Maximal model	Minimal model	R subroutine {error struct}
Serial passages							
Parasitemia	log (<i>par</i>)	1	-	414	<i>par_{t-1}</i> + <i>pass1</i>	<i>par_{t-1}</i> + <i>pass1</i>	glm {n}
	log (<i>par</i>)	2	-	414	<i>par_{t-1}</i> + <i>pass2</i> * <i>S</i>	<i>par_{t-1}</i> + <i>pass2</i> + <i>S</i>	glm {n}
	log (<i>par</i>)	3	-	414	<i>par_{t-1}</i> + <i>pass3</i> * <i>S</i>	<i>par_{t-1}</i> + <i>pass3</i> + <i>S</i>	glm {n}
	log (<i>par</i>)	4	-	414	<i>par_{t-1}</i> + <i>pass4</i> * <i>S</i>	<i>par_{t-1}</i>	glm {n}
	log (<i>par</i>)	5	-	414	<i>par_{t-1}</i> + <i>pass5</i> * <i>S</i>	<i>par_{t-1}</i> + <i>pass5</i>	glm {n}
Gametocytemia	log (<i>gam</i>)	6	-	338	<i>gam_{t-1}</i> + <i>pass1</i>	<i>gam_{t-1}</i>	glm {n}
	log (<i>gam</i>)	7	-	338	<i>gam_{t-1}</i> + <i>pass2</i> * <i>S</i>	<i>gam_{t-1}</i>	glm {n}
Trait correlations within the bird							
Parasitemia	<i>par</i>	8	-	62	<i>exp</i>	<i>exp</i>	glm {n}
Gametocytaemia	<i>gam</i>	9	-	54	<i>exp</i>	<i>exp</i>	glm {n}
	sqrt (<i>gam</i>)	10	-	54	sqrt (<i>par</i>) + [1 <i>exp</i>]	sqrt (<i>par</i>) + [1 <i>exp</i>]	lme {n}
	sqrt (<i>gam</i>)	11	-	54	sqrt (<i>asex</i>) + [1 <i>exp</i>]	sqrt (<i>asex</i>) + [1 <i>exp</i>]	lme {n}
	<i>conv</i>	12	-	54	<i>exp</i>	<i>exp</i>	glm {n}
Conversion rate	<i>conv</i>	12	-	54	<i>exp</i>	<i>exp</i>	glm {n}
Anaemia (packed cell volume)	<i>pcv</i>	13	-	42	<i>inf</i> + [1 <i>exp</i>]	<i>inf</i> + [1 <i>exp</i>]	lme {n}
	<i>pcv</i>	14	-	27	sqrt (<i>par</i>) + [1 <i>exp</i>]	1 + [1 <i>exp</i>]	lme {n}
	<i>pcv</i>	15	-	54	<i>conv</i> + [1 <i>exp</i>]	1 + [1 <i>exp</i>]	lme {n}

Table S2: Description of statistical models used (cont'd)

Predictors of mosquito infection							
<i>Bird-related factors</i>							
Oocyst prevalence	prev	16	1681	-	stage + [1 bird]	stage + [1 bird]	lmer {b}
	prev	17	-	52	sqrt (par) + sqrt (par ²) + [1 exp]	1 + [1 exp]	lmer {b}
	prev	18	-	44	sqrt (gam)+ [1 exp]	sqrt (gam)+ [1 exp]	lmer {b}
	prev	19	-	27	pcv + [1 exp]	1 + [1 exp]	lmer {b}
Oocyst burden	burd ^{-0.12}	20	1209	-	stage + [1 exp]	stage + [1 exp]	lme {n}
	log (burd)	21	-	47	sqrt (par) + sqrt (par ²) + [1 exp]	sqrt (par) + sqrt (par ²) + [1 exp]	lme {n}
	log (burd)	22	-	39	sqrt (gam) + [1/exp]	1 + [1 exp]	lme {n}
	log (burd)	23	-	47	prev + [1 exp/bird]	prev + [1 exp/bird]	lme {n}
	log (burd)	24	-	27	pcv + [1 exp]	1 + [1 exp]	lme {n}
Blood meal size (haematin)	sqrt (hm)	25	-	52	sqrt (par) + [1 exp]	sqrt (par) + [1 exp]	lme {n}
<i>Mosquito-related factors</i>							
Oocyst prevalence	prev	26	1110	-	sqrt (hm) + sqrt (hm ²) + [1 exp/bird]	sqrt (hm) + [1 exp/bird]	lmer {b}
Oocyst burden	log (burd)	27	1110	-	sqrt (hm) + sqrt (hm ²) + [1 exp/bird]	sqrt (hm) + [1 exp/bird]	lme {n}
Blood meal size (haematin)	sqrt (hm)	28	-	42	pcv + [1 exp]	pcv + [1 exp]	lme {n}
	sqrt (hm)	29	1596		size*inf + [1 exp/bird]	size*inf + [1 exp/bird]	lme {n}
Predictors of mosquito fitness							
Fecundity	fec	30	1480		inf + [1 exp/bird]	inf + [1 exp/bird]	lme {n}
	fec	31	1480		hm*inf + hm ² + [1 exp/bird]	hm*inf + hm ² + [1 exp/bird]	lme {n}
Survival	surv	32	442		hm*inf + [1 exp/bird]	1+ [1 exp/bird]	lme {n}
	surv	33	-	11	sqrt (par) + [1 exp]	sqrt (par) + [1 exp]	lme {n}

Supplementary Figures

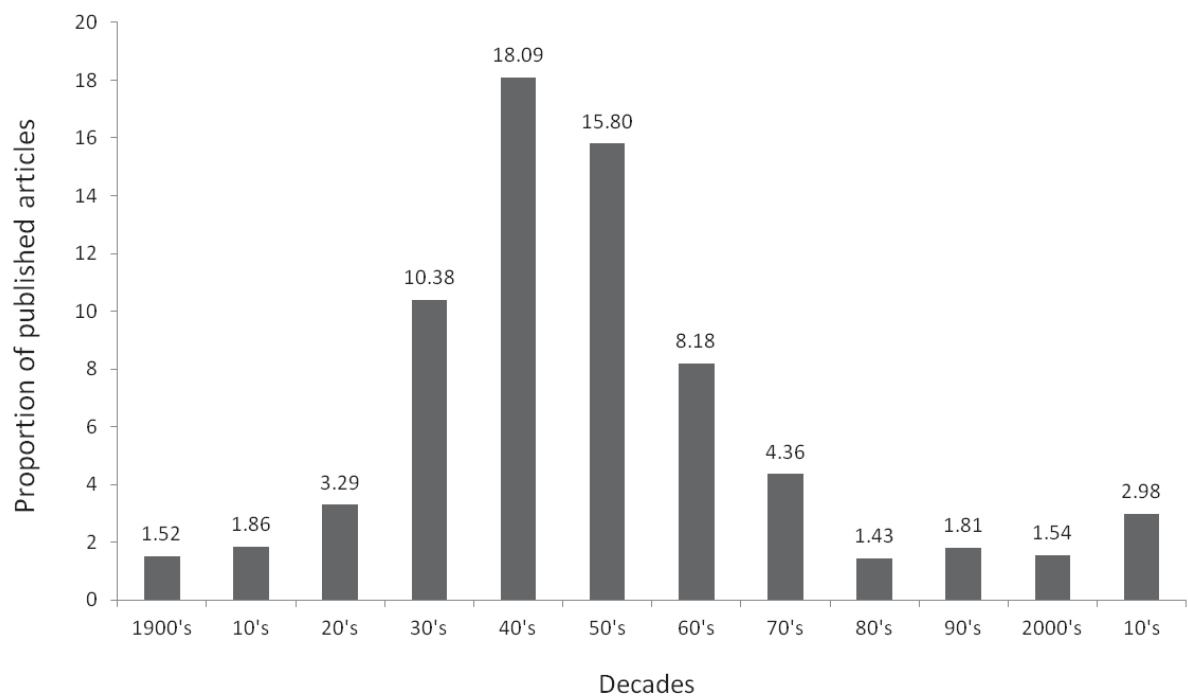


Figure S1. Proportion of total malaria papers published between 1900 and 2014 that deal with avian malaria. Search carried out on the Web of Science using the search terms "*Plasmodium* or malaria" (for total malaria papers) and "((plasmodium or malaria*) and (avian or bird*)) or (plasmodium and (gallinaceum or relictum or elongatum or cathemerium or lophurae or fallax))" (for avian malaria papers).

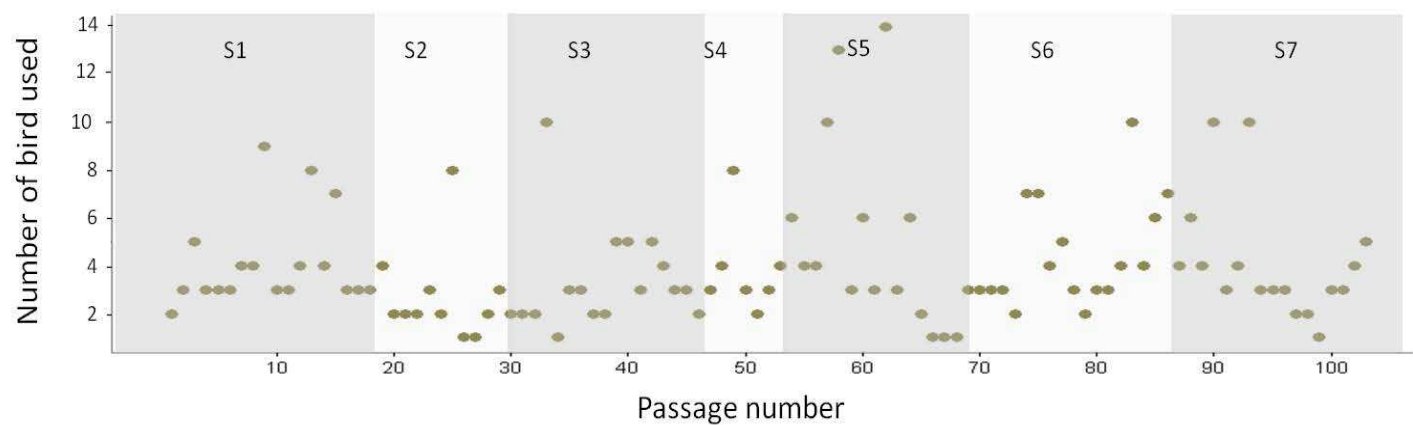


Figure S2. Number of bird used in the serial passage analysis.

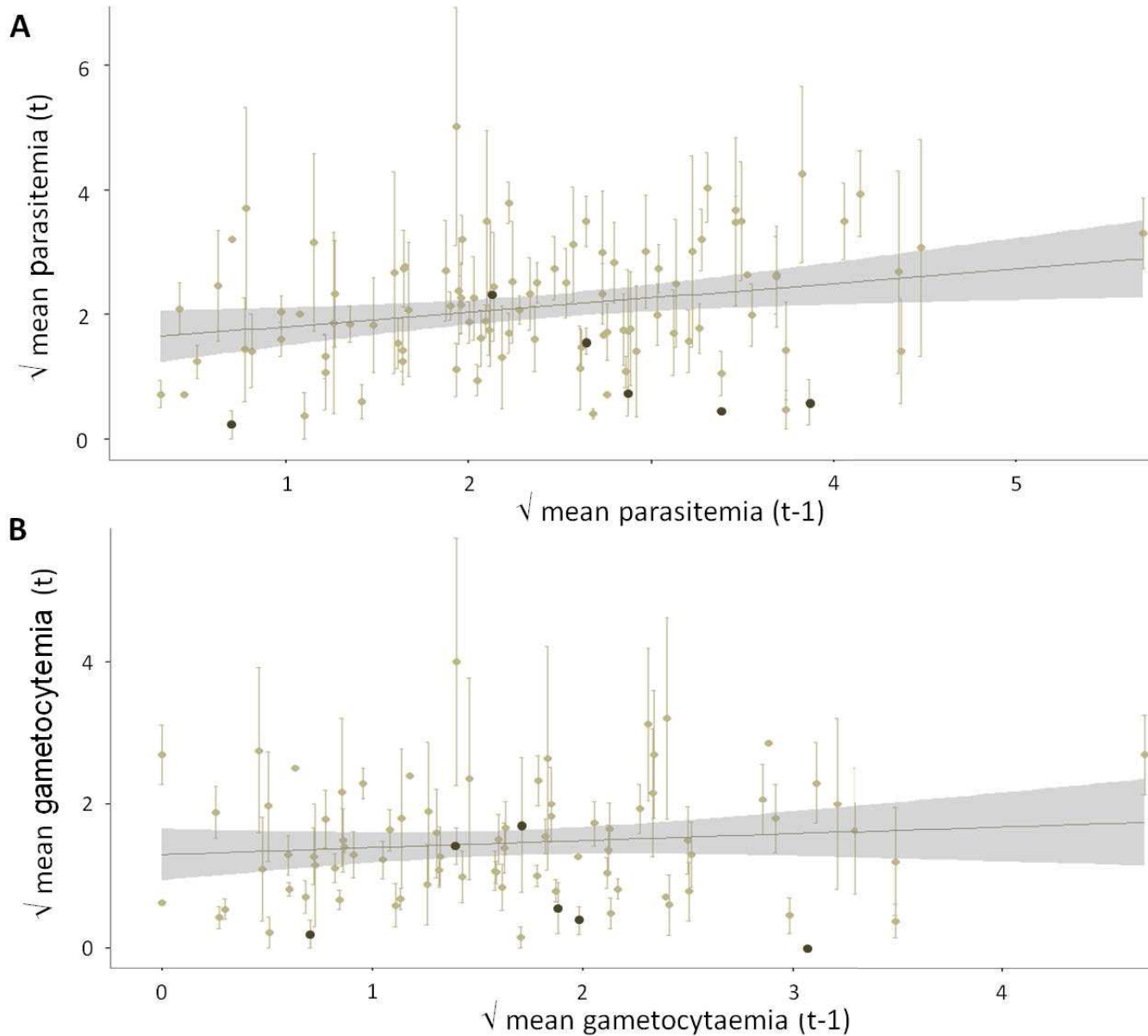


Figure S3. Correlation between mean parasitaemia (A) or gametocytaemia (B) at time (t) and mean parasitaemia or gametocytaemia at time (t-1) over the whole passage sequence since the isolation of the parasite strain from wild sparrows in 2009. Bars represent standard errors around the mean. Both variables have been square root transformed to improve the fit of the model. Dark grey dots represent the parasitaemias and gametocytaemias immediately following a mosquito passages (see Figure 1)

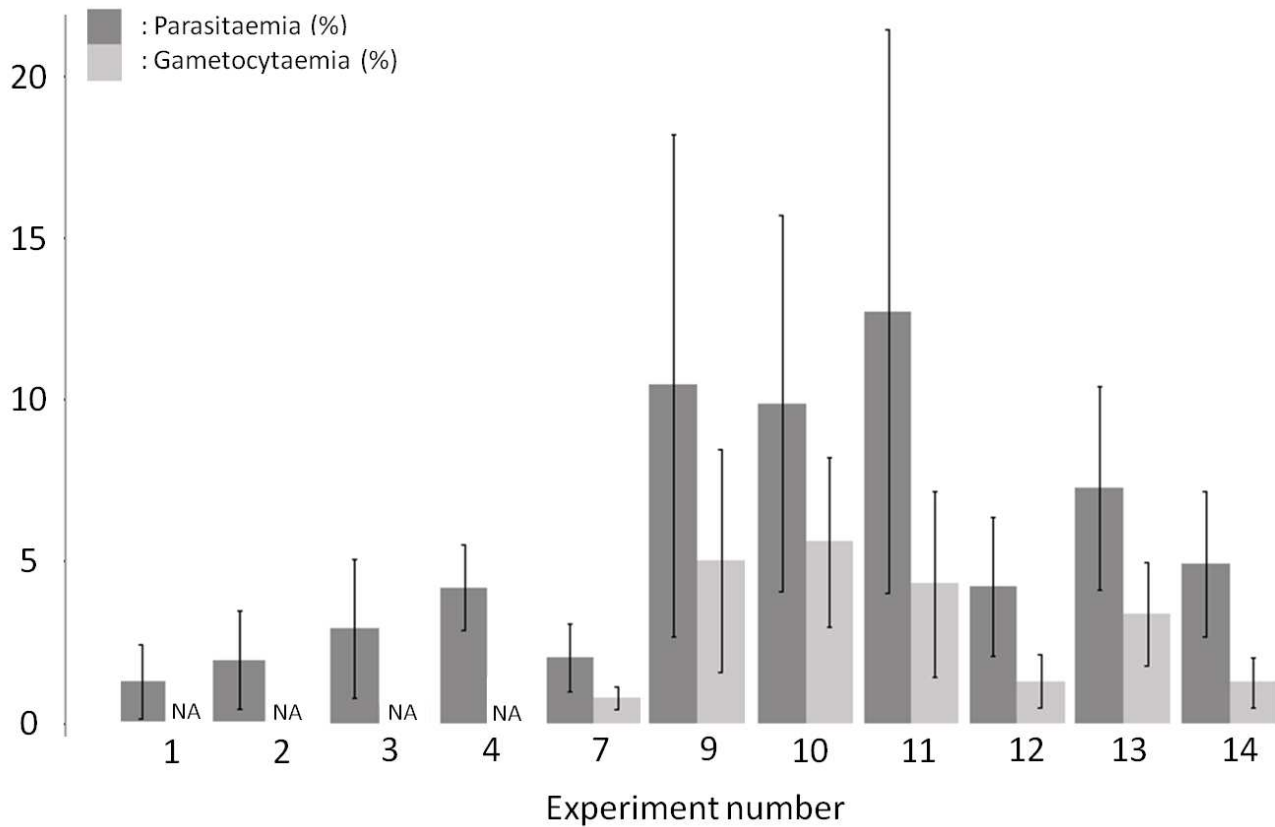


Figure S4. Mean parasitaemia (dark grey bars) and gametocytaemia (pale grey bars) in birds used in each of the 11 experiments reported in this paper. Bars represent standard errors around the means.

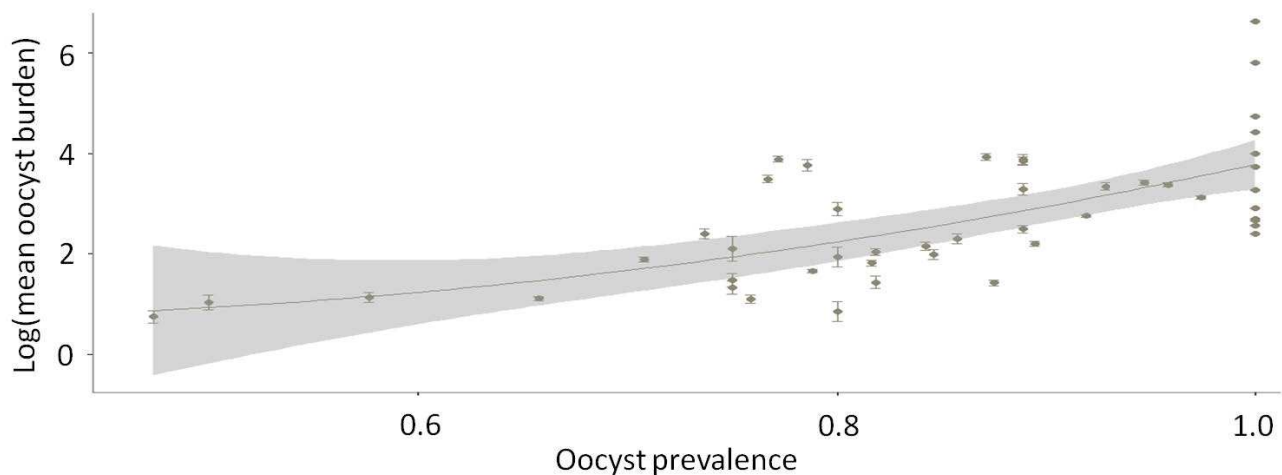


Figure S5. Relationship between oocyst prevalence and mean oocyst burden. Each point represents a different bird. Shaded areas on either side of the regression line represent 95% confidence intervals.

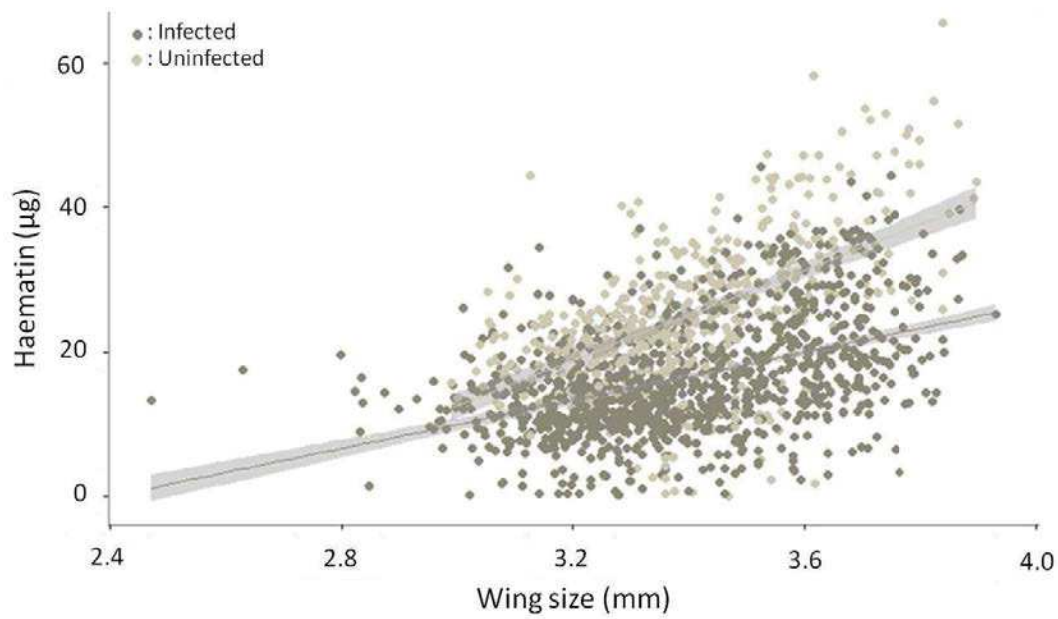


Figure S6. Relationship mosquito size (wing length) and blood meal size quantified as the amount of haematin excreted. Light grey dots: uninfected mosquitoes, dark grey dots: infected mosquitoes. Shaded areas on either side of the regression line represent 95% confidence intervals.

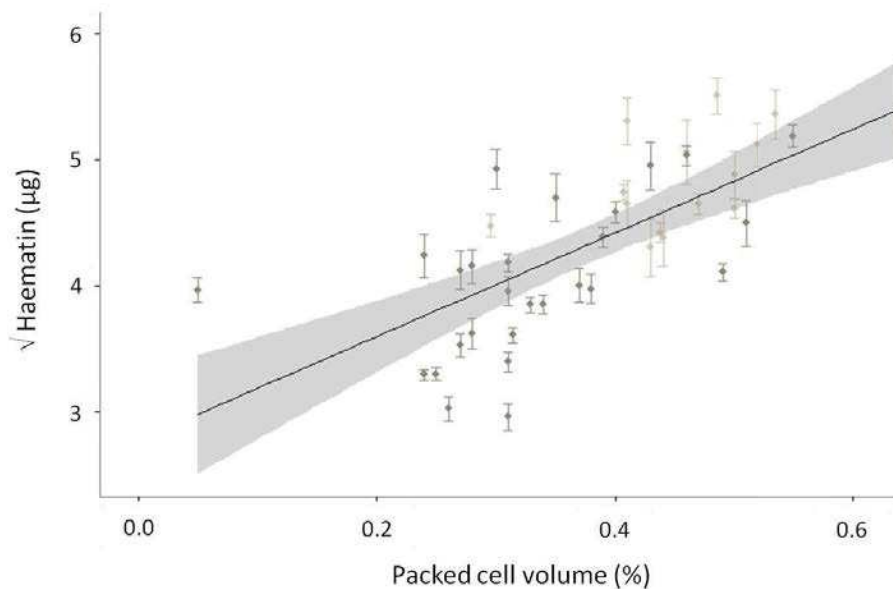


Figure S7. Relationship mosquito anaemia (quantified as packed cell volume) and blood meal size (quantified as the amount of haematin excreted). Light grey dots: uninfected mosquitoes, dark grey dots: infected mosquitoes. Shaded areas on either side of the regression line represent 95% confidence intervals.

Chapitre 7:

Malaria aviaire et « hypothèse de Cendrillon »

Chapitre 7: Malaria aviaire et « hypothèse de Cendrillon »

Ce chapitre ne présente qu'une partie des travaux réalisés au cours de cette étude

Contexte

- La parasitémie d'un hôte vertébré infecté par *Plasmodium* va varier au cours du temps. Ces fluctuations vont pouvoir influencer de manière importante le taux de transmission du parasite au moustique.
- Au sein de ce chapitre, nous avons essayé de déterminer si, comme proposé par Frank Hawking il y a plus de cinquante ans, il existait une augmentation périodique de la parasitémie des hôtes en lien avec la période d'activité des vecteurs (« hypothèse de Cendrillon »).

Résultats

- Nous avons pu mettre en évidence que *Plasmodium relictum* est plus infectieux au cours des périodes d'activité de son vecteur.
- Cette augmentation d'infectiosité semble, en partie, associée à une augmentation de la parasitémie au sein des hôtes vertébrés.

Conclusion

- Bien que notre étude mette en évidence une variation de l'infectiosité de *Plasmodium* en lien avec la période d'activité des moustiques, l'« hypothèse de Cendrillon » ne semble pas communément admise au sein de la communauté scientifique.
- D'autres explications peuvent, en effet, être proposées quant à l'évolution de cette périodicité. Il a notamment été montré que des variations circadiennes de l'immunité de l'hôte pouvaient conduire à des fluctuations périodiques de la charge en parasite.

The Cinderella hypothesis in avian malaria

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Abstract

The efficacy of malaria transmission from its vertebrate host to its mosquito vector can be altered by multiple biotic and abiotic factors. Here we explore the effects of between- and within-individual variations on the probability and the intensity of transmission to mosquitoes in avian malaria. In particular, we monitor the within-host dynamics of malaria in artificially infected birds and we detect a periodic increase of parasitemia in the evening that coincides with an increase in mosquito biting activity. These results support the Cinderella hypothesis claiming that within-host malaria dynamics has evolved to match the rhythm imposed by the behavior of its main mosquito vector. In addition, we discuss other potential factors that may explain the wide distribution of intensities of infection among mosquitoes that blood-fed on vertebrate hosts infected by malaria parasites.

Key words: Cinderella hypothesis, Hawking phenomenon, circadian rhythm, periodicity, malaria

Introduction

The probability and the intensity of mosquito infection is a key component of *Plasmodium* transmission. However, a great deal of variation of infection levels is often observed among mosquitoes from a single population: some mosquitoes are not infected, others are only weakly infected, and yet others have high infection intensities (Kilama, 1973; Niaré *et al.*, 2002; Pruck-Ngern *et al.*, 2014). Part of this variation may be due to genetic differences in mosquito susceptibility to *Plasmodium* infections (Ward, 1963; Collins *et al.*, 1986; Yan *et al.*, 1997; Lambrechts *et al.*, 2006) but also to environmental factors such as temperature or nutrition (Lefèvre *et al.*, 2013; Ciota *et al.*, 2014).

Another source of variation may involve the vertebrate host. Several studies have demonstrated a positive correlation between parasite burden (especially sexual stage) within the host and vector infection intensity (Huff & Marchbank, 1955; Hogg & Hurd, 1995; Schneider *et al.*, 2007; Churcher *et al.*, 2013; Da *et al.*, 2015). Hence, variation in parasitemia among different vertebrate individuals is likely to affect transmission to mosquitoes. But this variation may also occur within the same individual host during the course of the infection. There is a strong variation of parasitaemia between acute and chronic stage of parasite infection (Cornet *et al.*, 2014; Pigeault *et al.*, 2015). Chronic stage is characterized as having a lower parasitaemia, and so a lower probability of parasite transmission, than acute one (Cornet *et al.*, 2014; Pigeault *et al.*, 2015). But in several species of malaria, the parasitemia fluctuates at a much shorter time scale during the course of a single day associated to the parasite cell cycle duration (Gautret & Motard, 1999; Mideo *et al.*, 2013). These periodic fluctuations in parasitemia and gametocytemia have the potential to have dramatic effects on malaria transmission. The adaptive nature of this periodicity remains controversial (Mideo *et al.*, 2013) but one hypothesis is particularly fascinating: Hawking *et al.*, (1966) postulated that malaria parasites may evolve periodic within-host dynamics to match the periodicity of vector's activity. Indeed, daily rhythms have been observed in a wide variety of behavioral, physiological and metabolic processes in several insect vectors (review in Barrozo *et al.*, 2004) and in mosquitoes, many studies have shown the existence of circadian rhythms underlying several physiological processes (Chiba *et al.*, 1993; Barrozo *et al.*, 2004; Rund *et al.*, 2011; Fritz *et al.*, 2014). For example, in *Culex pipiens*, fly activity is clearly bimodal with two peaks occurring near dawn and dusk (Chiba *et al.*, 1993; Barrozo *et al.*, 2004) and the highest rate of blood feeding occurs during the late photophase and early

scotophase (Fritz *et al.*, 2014). Hence, malaria parasites may time dramatic developmental changes around midnight (like Cinderella, Mideo *et al.*, 2013) to match the increased biting activity of mosquitoes. It is possible to demonstrate theoretically that such periodic activity may generate selection for a matching periodicity in malaria growth rates within vertebrate hosts (see **Box 1**). Yet the experimental demonstration of the importance of mosquito behavior on the evolution of rhythmic within-host growth is difficult. One key element of this hypothesis relies on the synchronization between within-host growth in vertebrates, infectivity and biting behavior of mosquitoes (see **Box 1**). Several studies have confirmed this covariation between these different variables in rodent and primate *Plasmodium* species (Hawking *et al.*, 1968; Gautret *et al.*, 1996; Yang, 1996 but see Bray *et al.*, 1976).

Here we explore the validity of Hawking proposition in *P. relictum*, the most prevalent avian malaria parasite in Europe. For this propose (i) we followed the dynamics of infection and the daily rhythmicity of hosts' infectivity during both the acute and the chronic phases of the infection, (ii) we have fed *Cx pipiens* on the blood of bird containing *P. relictum* in acute but also in chronic infection stage at different times of the day (6h, 12h, 18, 00h). At each time point, we quantified the parasitemia in the bird, in the blood ingested by mosquito and, seven days after the blood meal, we measured the infection prevalence and intensity.

Box 1: Evolution of adaptive rhythmicity

To model the evolution of rhythmic transmission strategies we first need to model the epidemiological dynamics of malaria. For the sake of simplicity the vertebrate host population is assumed to be constant and equal to $N = S(t) + I(t)$, where $S(t)$ and $I(t)$ are the densities of uninfected and infected hosts, respectively. Similarly, the mosquito vector population is also assumed to be constant and equal to $N_V = V(t) + V_I(t)$, where $V(t)$ and $V_I(t)$ are the densities of uninfected and infected vectors, respectively. The activity of the vector $a(t)$ is assumed to fluctuate with a period $T = 1$ day. Lower mosquito activity is assumed to lower the biting rate and the transmission and, consequently, the epidemiological dynamics is also going to fluctuate periodically. The following set of differential equations describes the temporal dynamics of the different types of hosts (the dot notation indicates differential over time):

$$\dot{I}(t) = S(t)V_I(t)a(t)\beta_2 - (d + \alpha(t))I(t)$$

$$\dot{V}_I(t) = I(t)V(t)a(t)\beta_1(t) - m_I V_I(t)$$

Where d is the natural mortality rate of the vertebrate host and α is the virulence of malaria (the extra mortality induced by the infection); m_I is the mortality rates of infected vectors; β_1 is the transmission rate from the vertebrate host to the vector; β_2 is the transmission rate from the vector to the vertebrate host.

To study the selection acting on the evolution of the timing of within-host dynamics in the vertebrate host we allow $\alpha(t)$ and $\beta_1(t)$ to vary with time in the above equation. To study this question one can consider the fate of a mutant malaria strategy M that would alter its replication in the vertebrate host. As in classical models of virulence evolution, more replication is costly because it may induce the death of the vertebrate host (i.e. higher $\alpha(t)$) but it allows the parasite to transmit more efficiently (i.e. higher $\beta_1(t)$). The parameters a and b govern the specific shape of the virulence-transmission trade-off: $\beta_1(t) = a [\alpha(t)]^b$. Because the frequency of the fluctuation in mosquito activity is much higher than other perturbations of the system we may assume that the density of infected hosts remains approximately stable which allows us to reduce the above system to:

$$\dot{V}_I(t) \approx \left(\frac{\tilde{a}N\beta_2}{(d + \tilde{\alpha} + \tilde{a}\tilde{V}_I\beta_2)} V(t)a(t)\beta_1(t) - m_I \right) V_I(t)$$

Where the tilde refers to the average over one period (1 day) of the fluctuation. To study parasite evolution we can monitor the dynamics of a rare mutant parasite M that may have different within-host exploitation strategy:

$$\dot{p}_M(t) \approx N\beta_2 V(t)(r_M(t) - \bar{r}(t)) p_M(t)$$

With:

$$r_M(t) = \frac{\tilde{a}}{(d + \tilde{\alpha}_M + \tilde{a}\tilde{V}_{IM}\beta_2)} a(t)\beta_{1M}(t)$$

Integrating the change in frequency of the mutant parasite genotype M over one day allows deriving a condition for the invasion of the mutant:

$$s_M = \frac{1}{T} \int_0^T (\dot{p}_M(t)/p_M(t)) dt$$

$$s_M \approx \tilde{\alpha}^2 N \beta_2 \tilde{V} \left(\underbrace{\frac{\tilde{\alpha} \tilde{\beta}_{1M}}{(d + \tilde{\alpha}_M + \tilde{\alpha} \tilde{V}_{IM} \beta_2)} - \frac{\tilde{\alpha} \tilde{\beta}_1}{(d + \tilde{\alpha} + \tilde{\alpha} \tilde{V}_I \beta_2)}}_{\text{Classical transmission-virulence trade off}} + \underbrace{\frac{COV_t(a, \beta_{1M})}{(d + \tilde{\alpha}_M + \tilde{\alpha} \tilde{V}_{IM} \beta_2)} - \frac{COV_t(a, \beta_1)}{(d + \tilde{\alpha} + \tilde{\alpha} \tilde{V}_I \beta_2)}}_{\text{Match between mosquito behavior and within-host growth}} \right)$$

The first term in the above equation for s_M is the classical trade-off between transmission and virulence. The second term measures the benefit associated with a closer match between parasite dynamics in the vertebrate host and the rhythmicity in mosquito behavior.

Material and methods

Plasmodium relictum (lineage SGS1) is the aetiological agent of the most prevalent form of avian malaria in Europe (Valkiunas, 2004). Our lineage was isolated from an infected sparrow caught in the region of Saintes Maries-de-la-Mer (France) in May 2015 and transferred to naïve canaries (*Serinus canaria*). Since then it has been maintained by carrying out regular passages between our stock canaries through intraperitoneal injections (Pigeault et al 2015).

Experimental canaries ($n = 8$) were infected by injecting them with ca. 80µL of blood from the infected canary stock. Parasitaemia was thereafter monitored daily at 12h00 (12:00 PM) for seventy days (**Figure 1**). For this purpose, between 5-10µL of blood were collected from the medial metatarsal vein. A drop of this blood sample was smeared onto a slide for the visual quantification of the parasitaemia and gametocytaemia (as described in Valkiunas 2004), and the rest was frozen for the molecular quantification of the parasitaemia (see below). In order to determine whether there is a circadian rhythm in the parasite dynamics, for 3 days during the acute phase of the parasite's infection (days 12, 13 and 14) the blood sampling was carried out every 6 hours (6h, 12h, 18h and 00h).

Experiments were conducted with a laboratory isogenic strain of *Cx. pipiens* mosquitoes. Mosquitoes were reared as described by Vézilier *et al.*, 2010. In order to determine whether the circadian rhythm of the parasite in the bird has an impact on mosquito infection rates the eight infected birds were haphazardly assigned to two

treatments: “exposed” (n=3) or “unexposed” (n=5) to mosquito bites. For this purpose, on day 13 straight after the blood sample (see above), the three birds in the “exposed” treatment were placed with 70, 7-day old, mosquitoes at 6-hour intervals (6h, 12h, 18h and 00h). Each time a different batch of mosquito was used and each batch was kept in the cage for 135 minutes. During this blood meal period, the cages were visited every 45 minutes and all blood fed females were removed and counted. Blood fed mosquitoes were divided in two groups. One half was frozen individually in order to quantify the parasites ingested in the blood meal using molecular techniques (see below). The other half was placed individually in numbered plastic tubes (30 ml) covered with a mesh. Food was provided in the form of a cotton pad soaked in a 10% glucose solution. Seven days later (day 7 post blood meal) the females were taken out of the tubes and the amount of haematin excreted at the bottom of each tube was quantified as an estimate of the blood meal size (Vézilier *et al.*, 2010). Females were then dissected and the number of *Plasmodium* oocysts in their midguts counted with the aid of a binocular microscope (Vézilier *et al.*, 2010). The wings of females were removed prior to the midgut dissection and measured along its longest axis as an index of body size. The whole protocol was repeated when the parasite infection reached its chronic stage, on days 62, 63, 64 and 68 post infection.

The molecular quantification of parasites in the mosquito blood meal was carried out using a quantitative PCR (qPCR) protocol adapted from Cornet *et al.*, (2013). Briefly, DNA of bloodfed females was extracted using standard protocols (Qiagen DNeasy 96 blood and tissue kit). For each individual, we conducted two qPCRs in the same run: one targeting the nuclear 18s rDNA gene of *Plasmodium* (Primers: 18sPlasm7 5'-AGCCTGAGAAATAGCTACC-ACATCTA-3', and 18sPlasm8 5'-TGTTATTTCTTGTCACCTCTC- TTCTTT-3'), and the other targeting the 18s rDNA gene of the bird (18sAv7 5' GAAACTCGCAATGGCTCATTAAATC- 3', and 18sAv8 5'-TATTAGCTCTAGAATTACCACAGTTATCCA-3'). All qPCRs were run on an ABI 7900HT real-time PCR system (Applied Biosystems). Parasite intensities were calculated as relative quantification values (RQ). RQ can be interpreted as the fold-amount of target gene (*Plasmodium* 18s rDNA) with respect to the amount of the reference gene (Bird18s rDNA) and are calculated as $2^{-(Ct_{18s\ Plasmodium} - Ct_{18s\ Bird})}$. For this purpose, all samples were run in triplicate and their mean was used to calculate the threshold Ct value (the number of PCR cycles at which fluorescence is first detected, which is inversely correlated with the initial amount of DNA in a sample) using the software Light Cycler 480 (Roche). For convenience, RQ values were standardised by $\times 10^4$ factor and log-transformed (Cornet et al 2014).

Statistical analysis

Analyses were carried out using the R statistical package (v. 3.1.1). The different statistical models built to analyse the data are described in the Supplementary Materials (**Table S1**). The analyses of response variables which may depend on which bird mosquitoes fed on, such oocyst prevalence and oocyst burden were carried out using mixed model procedures: fitting bird as a random factor in the models and treatment (time of day or stage of infection) and blood meal size (haematin) as fixed factors. Oocyst prevalence (presence/absence of oocysts) and the proportion of females having taken a blood meal were analysed using the *lmer* mixed model procedure and binomial errors. Oocyst burden was normalised using a logarithmic transformation and analysed with the *lme* procedure with normal errors. Differences in blood meal size between the treatments were analysed using the *lme* procedure. The proportion of females having taken a blood meal at each time of day was analyzed independently for each capture session (45min, 90min and 135min) using glm procedures with quasibinomial errors in order to correct for over dispersion. When necessary the calculation of the variance percentage explain by fixed and random factors were performed using the package MumMin (Nakagawa & Schielzeth, 2013).

Maximal models, including all higher-order interactions, were simplified by sequentially eliminating non-significant terms and interactions to establish a minimal model (Crawley, 2012). The significance of the explanatory variables was established using either a likelihood ratio test (which is approximately distributed as a Chi-square distribution, (Bolker, 2008) or an F test. The significant Chi-square or F values given in the text are for the minimal model, whereas non-significant values correspond to those obtained before the deletion of the variable from the model. *A posteriori* contrasts were carried out by aggregating factor levels together and by testing the fit of the simplified model using an LRT (Crawley, 2012).

The distributions of the amount of parasite ingested by mosquito at each time of day were compared with the oocyst number observed in mosquito seven days after the blood meal. For this we have used the two-sample Kolmogorov–Smirnov test.

At the time of submitting this thesis manuscript, only the analyses of the acute phase of the infection had been completed. Besides, we focus mainly on the within-host dynamics on day 13. A more elaborate analysis (spectral analysis) of the within-host dynamics over several days is currently developed to detect periodicity.

Results

Dynamics of infection

The acute stage peak parasitaemia was reached between the day 10 and 14 post-infection, depending on the birds (**Figure1A**). The mean peak parasitaemia was $7.41 \pm 1.89\%$ with a minimum value of 1.32% and maximum of 16%. Birds with the highest peak parasitaemia reached this peak later than birds with lower peak parasitaemia, although the statistical correlation was not significant ($S = 35.22$ $p=0.093$, $\rho = 0.58$, **Figure S1**). The dynamics of infection were, however, relatively consistent across birds. Parasitaemia increases rapidly between day 8 and days 10-14 post infection and then decreases drastically from day 16 onwards. Starting from day 23 the intensity of infection was very low and that until the end of the experiment (between 0 and 0.2%). The same pattern is observed in the gametocytaemia (**Figure1B**): a strong heterogeneity between birds, with a maximum peak that is attained between days 10 and 14 and that ranges between 0.25% and 3.38% (mean $1.68 \pm 0.42\%$).

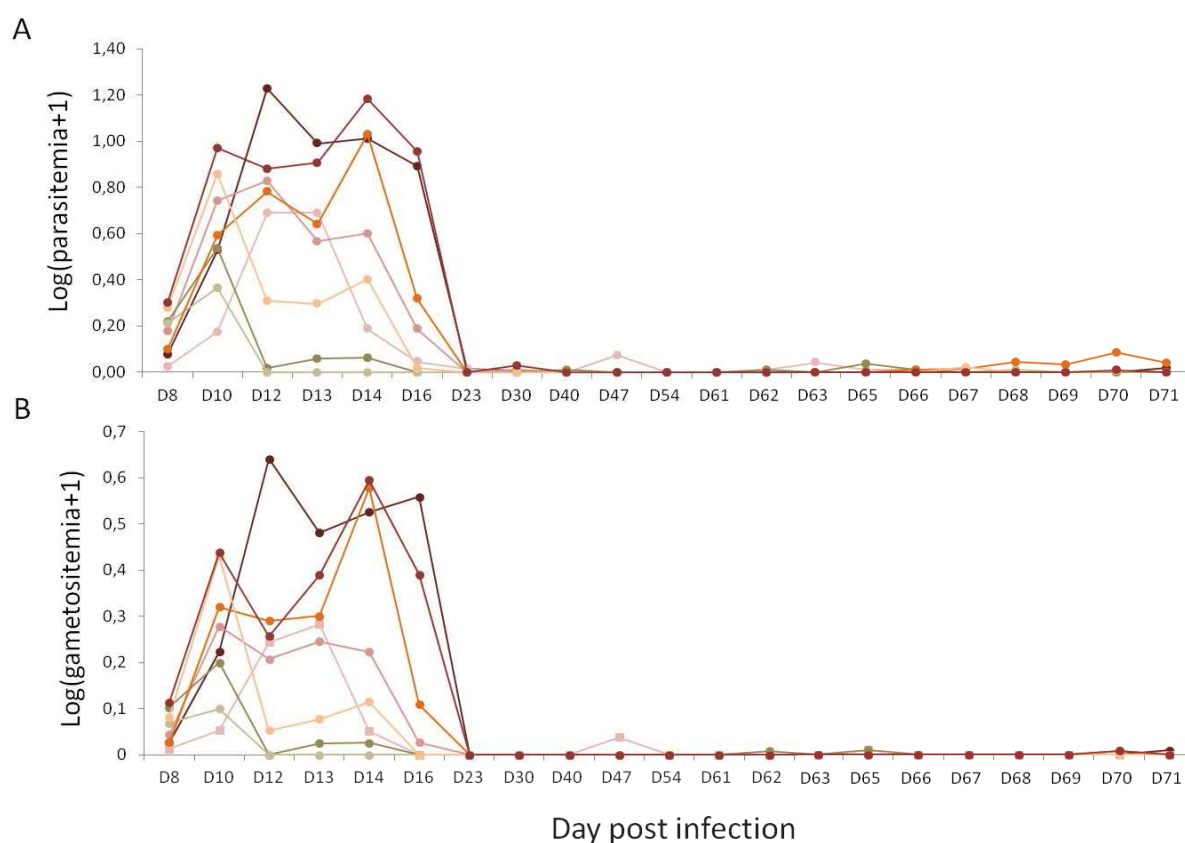


Figure 1: Evolution of the parasitaemia and gametocytaemia in the 8 infected birds between the day 8 and 71 post blood meal. Each bird is represented by one colour.

Circadian rhythm (analysis conducted only on day 13)

There is a very significant effect of time of day on the total number of parasites in the blood of infected birds (model 1: $\chi^2_1 = 11.58$, $p = 0.009$, **Figure 2A**). The parasitaemia was highest in the evenings (18h and 00h) and lowest early in the morning (6h, **Figure 2A**). There was also a highly significant effect of the time of day on the number of parasites ingested by mosquitoes (model 2: $\chi^2_1 = 40.79$, $p < 0.0001$). The lowest ingestion of parasites occurs early in the morning (6h) and the highest late at night (00h, **Figure 2B**). As expected, there was a strong positive correlation between parasitaemia in the birds' blood and the number of parasites ingested by the mosquitoes ($F_{1,10}=184.4$, $p < 0.0001$, R-Squared = 0.949). All fed females were infected at each time of day. However, blood feeding time had a very significant effect on the mosquito oocyst burden (model 3: $\chi^2_1 = 42.69$, $p < 0.0001$, **Figure 2C**). Females that feed at 6h in the morning have significantly fewer oocysts (mean $\pm$ s.e: 62 ± 14) than those that feed at noon (108 ± 25). Females that feed in the evening (18h and 00h) have between twice and 3 times as many oocysts as those feeding at noon (262 ± 51 and 314 ± 52 oocysts respectively). The quantity of excreted haematin also has a strong impact on oocyst burden: females that excrete more haematin produce more oocysts (model 3: $\chi^2_1 = 48.72$, $p < 0.0001$). No interaction between haematin and time of day was, however, observed (model 3: $\chi^2_1 = 3.95$, $p = 0.267$).

A strong impact of the time of day has been observed on the proportion of females taking a blood meal. A significantly higher proportion of females take a blood meal when feeding in the evening (18h, 00h) than in the morning (6h, 12h). By the end of the 135min blood feeding session, more than 90% of females took a blood meal in the evening period against only 68% in morning period (model 4: $\chi^2_1 = 215.24$, $p < 0.0001$, **Figure 3**). This difference holds in mosquitoes captured 45min (model 5: $\chi^2_1 = 298.35$, $p < 0.0001$) and 90min (model 6: $\chi^2_1 = 168.54$, $p < 0.0001$) after the beginning of the feeding bout.

Parasite and Oocyst distribution

During the first part of the day, the distributions of the oocyst number observed in mosquito, seven days after the blood meal, are similar to distribution of the number of parasite ingested (6h: $D = 0.113$ $p = 0.195$, 12h: $D = 0.273$ $p = 0.128$, **Figure 4**). However, in the evening (18h, 00h) the distributions are different (respectively: $D = 0.405$ $p = 0.002$, $D = 0.375$ $p = 0.006$, **Figure 4**). Indeed, oocyst's distribution seems more spread.

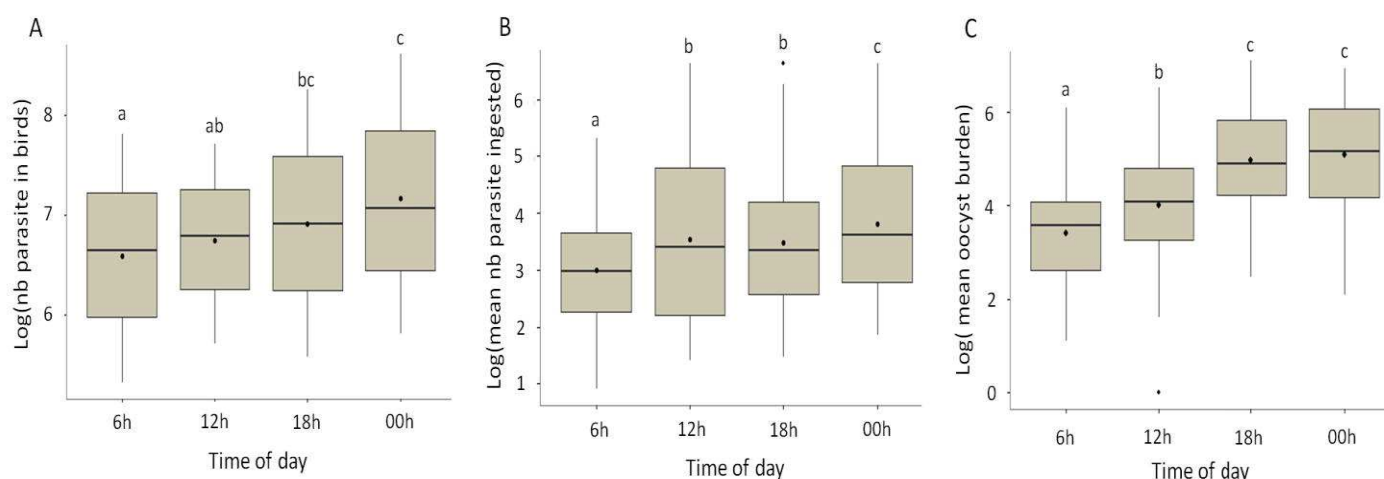


Figure 2: Effect of time of day on the number of bird blood parasite stages (A), amount of parasites ingested by mosquitoes (B) and number of oocysts subsequently produced (C). Boxplots represent the means (points) and medians (horizontal lines). Boxes above and below the medians show the first and third quartiles respectively. Lines above and below the boxes delimit 1.5 times the inter-quartile range. Small letters above the boxes indicate the results of the contrast analyses. Treatments sharing the same letter are not significantly different from each other (cut-off point for significance $p < 0.05$). **Analysis conducted only on day 13.**

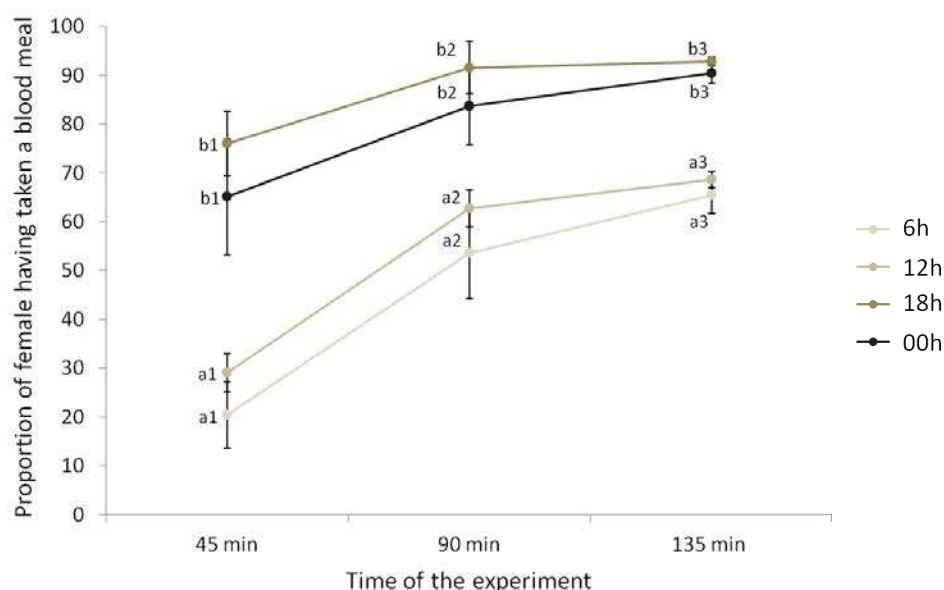


Figure 3: Effect of time of day on the proportion of female having taken a blood meal during the experiment at each captured session. The points represent the means ($\pm$ SE). Two points connected by the same letters is not statically different.

Host heterogeneity

There was a strong heterogeneity between hosts in the efficiency of conversion of parasites ingested in the blood to oocysts in the midgut of mosquitoes. There were highly significant differences in the number of parasites ingested by mosquitoes feeding in the 3 different birds (model 7: $F_{2,170} = 242.22$ $p < 0.0001$, **Figure 5A**). These differences held irrespectively of the time of day. Females feeding on Bird 1 ingested the highest number of

parasites, followed by Bird 2 and Bird 3 (contrast analyses: Bird 1 vs Bird 2: $F_{1,171} = 142.67$, $p < 0.0001$, Bird 2 vs Bird 3: $F_{1,171} = 95.84$, $p < 0.0001$). The number of oocysts produced by these infections were highly dependent on the bird of origin and, as previously described, of blood feeding time (respectively model 8 = $F_{2,138} = 31.38$ $p < 0.0001$, model 6 = $F_{2,138} = 17.22$ $p < 0.0001$ **Figure 5B**), but the pattern does not follow that observed for the blood ingested. Here, the females that fed on Bird 2 are the ones with the highest oocystaemias, followed by Birds 1 and 3 which are not significantly different from each other (contrast analyses: Bird 2 / Bird 1 = $F_{1,139} = 52.02$ $p < 0.0001$, Bird 2 / Bird 3 = $F_{1,139} = 42.67$ $p < 0.0001$, Bird 1 / Bird 3 = $F_{1,139} = 0.66$ $p = 0.416$). As a result, the ratio of conversion from parasite ingestion to oocyst formation is widely different between birds (**Figure 5C**). Firstly, at 6h00 the ratio is higher for all birds. Secondly, the bird with the lowest parasite had the highest ratio.

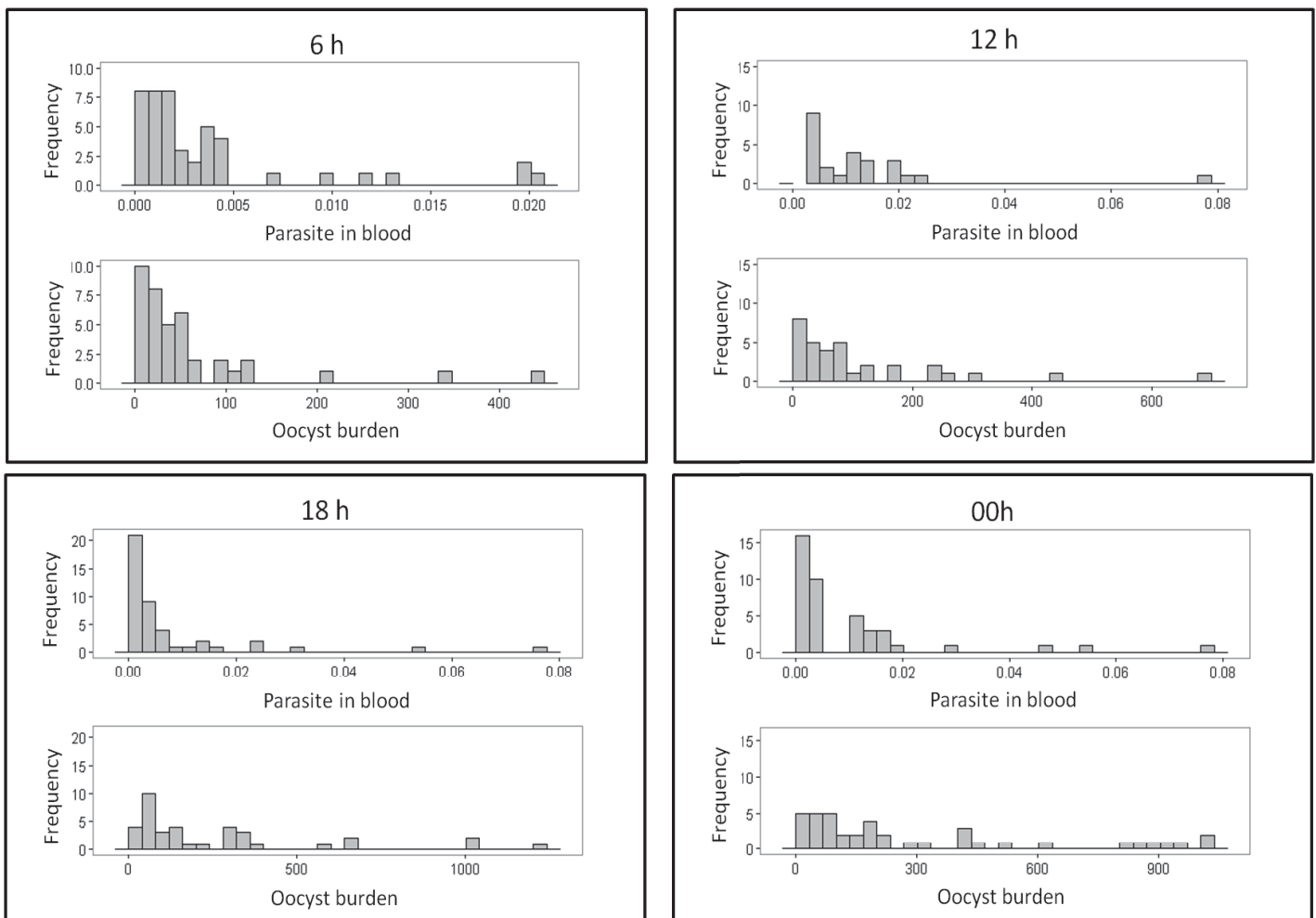


Figure 4: Distribution, at each time of day, of parasite in blood ingested by mosquito and oocyst burden seven days after the blood meal. **Analysis conducted only on day 13.**

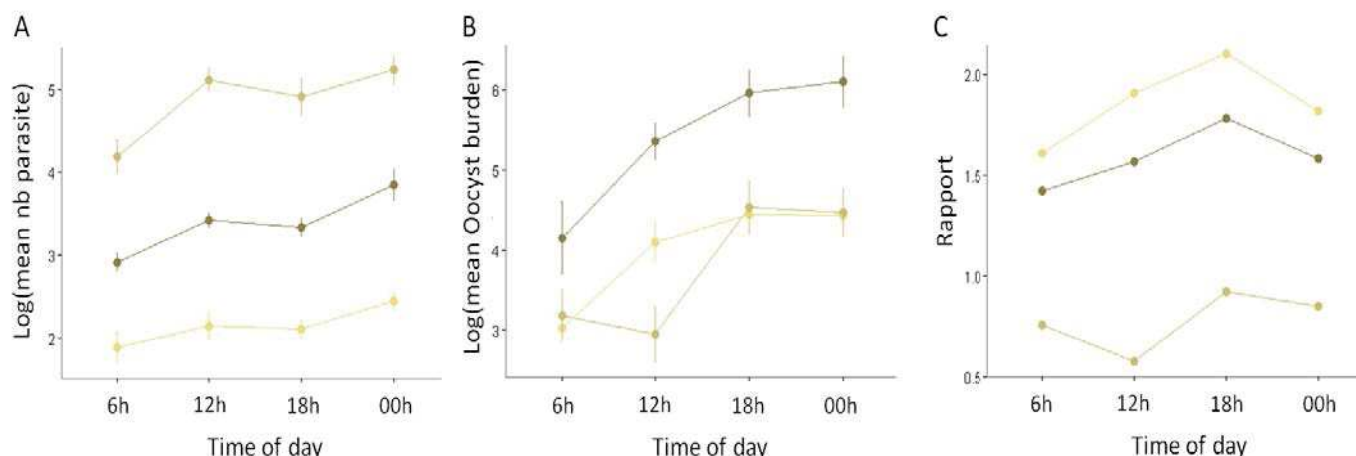


Figure 5: Effect of bird and time of day on the amount of parasites ingested by mosquitoes (A), number of oocysts subsequently produced (B) and the rapport measured between the amount of parasites ingested and the oocysts burden (C). Boxplots represent the means (points) and medians (horizontal lines). Boxes above and below the medians show the first and third quartiles respectively. Lines above and below the boxes delimit 1.5 times the inter-quartile range. **Analysis conducted only on day 13.**

Discussion

A first finding of our study is that, despite a relatively similar pattern observed during the course of the infection, the intensity of the parasitemia and gametocytemia vary drastically between birds. These variations had significant effects on parasite transmission to the vector. However, surprisingly, this was not the bird with the highest parasitemia and gametocytemia (measured only at noon) which was the most infectious. Regarding the relationship between the quantity of parasite ingested by mosquito and the number of oocyst observed seven days later, the highest ratio was measured in the lowest infected bird. This result may be explained in several ways. In the one hand, it is possible that compounds present in the blood of this bird (nutrient, micro-element) promote mosquitoes' infection by *Plasmodium* (Noden *et al.*, 2011; Gendrin *et al.*, 2015). On the other hand, it is also possible that our measured of gametocyte by microscopy was not perfect. Many studies have shown that sub-microscopic gametocytemia values can have significant effects on the mosquito infection rate (Schneider *et al.*, 2007; Churcher *et al.*, 2013).

A strong variation of host's infectivity has also been observed within the same individual during the course of a day. We have shown that birds were more infectious between the nightfall and midnight. This result is consistent with the "Cinderella hypothesis": *P. relictum* appears to be more infective to mosquitoes during the period

corresponding to the peak of vector's activity. Indeed, regarding the activity rhythm of *Cx pipiens*, we have observed that the probability of females to take a blood meal was distinctly higher during the evening; several studies confirmed this observation (Chiba *et al.*, 1993; Barrozo *et al.*, 2004; Fritz *et al.*, 2014). The increase of host infectivity is, in part, explained by the increase in bird parasitemia throughout the day and so on the amount of parasites ingested by females. However, other parameters, independent of the total parasite burden, must be implicated. Indeed, at 18h, females had not ingested more parasite than those at noon, yet, their oocyst burden was clearly higher. Variations in the gametocyte density may be involved (Hogg & Hurd, 1995; Collins & Jeffery, 2003). Indeed, irrespective of the total parasitaemia (asexual and sexual stages), the quantity and / or the maturity of gametocyte could also vary during the time of the day (Gautret & Motard, 1999, we are currently trying to investigate this possibility in our biological system).

Finally, strong infection heterogeneity between mosquitoes fed on the same bird was also observed at each time of day. The analysis of the variance observed on the mosquitoes' infection intensity reveals that 24% of the total variance is due to the "bird effect" and 20% is due to the "biting time". The remaining 56% of the total variance is associated with variation between mosquitoes feeding on the same bird at the same time. Blood meal size explains 20% of total variation observed in the infection intensity. The remaining 36% cannot be explained by genetic variations in mosquitoes' susceptibility because we use isogonic lines. Although one might think that this is due to the distribution of the parasite in the peripheral blood of the host (Smalley *et al.*, 1981), Pichon and collaborators (2000) have shown that the distribution of parasites in the vertebrate blood was random. Currently, this strong variation remains unexplained.

The Cinderella hypothesis, proposed more than 50 years, fails to win unanimous support in the scientific community (Bray *et al.*, 1976; Gautret & Motard, 1999; Magesa *et al.*, 2000; Mideo *et al.*, 2013). A major difficulty in the test of this hypothesis lies on the existence of alternative explanations for the existence of periodic variations in parasitemia and infectivity. For instance circadian variations in immunity could also lead to this periodic fluctuations (Mideo *et al.*, 2013; Martinez-Bakker & Helm, 2015). The present study is an attempt to explore the validity of this hypothesis with a combination of approaches. First, we develop a mathematical model to identify the selective forces driving the evolution of rhythmicity in malaria transmission. Second, we monitor the short-term variations of

parasitemia across different vertebrate hosts. Third, we relate these variations to the activity of its main mosquito vector. Beyond the exploration of this hypothesis, the present study is also an attempt to disentangle the different factors that yield variations in infections intensities among infected mosquitoes.

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Supplementary Materials

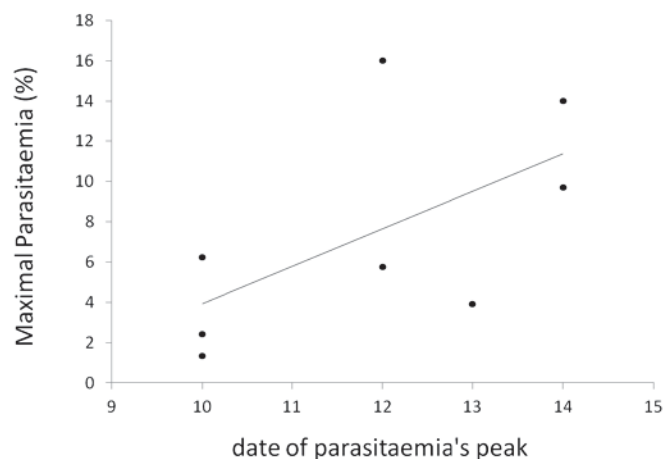


Figure S1: Correlation between maximal parasitaemia and the date of the infection peak in the vertebrate host

Table S1: Description of statistical models used to analyze the data. The response variable was not transformed unless otherwise stated. N gives the number of mosquitoes or bird included in each analysis. "Maximal model" gives the complete set of explanatory variables (and their interactions) included in the model. "Minimal model" gives the model containing only the significant variables and their interactions. Round brackets indicate variables fitted as random factors (1/bird denotes that the effect of bird is fitted as a random variable). Square brackets indicate the error structure used (n: normal, b: binomial).

Variable of interest	Response variable	Model Nb.	N	Maximal model	Minimal model	Rsubroutine [err struct.]
Parasite in bird	log(Par)	1	12	time	time	glm[n]
Parasite in mosquito	log(Par)	2	175	time+(1/Bird)	time+(1/Bird)	lme[n]
Oocyst	log(Oo)	3	144	time+(1/Bird)	time+(1/Bird)	lme[n]
Blood meal rate (135min)	BMR	4	38	time+(1/Bird)	time+(1/Bird)	lmer[b]
Blood meal rate (45min)	BMR	5	38	time+(1/Bird)	time+(1/Bird)	lmer[b]
Blood meal rate (90min)	BMR	6	38	time+(1/Bird)	time+(1/Bird)	lmer[b]
Parasite in mosquito	log(Par)	7	175	Bird*time	Bird+time	glm[n]
Oocyst	log(Oo)	8	144	Bird*time	Bird+time	glm[n]

Discussion générale

Discussion générale

Les moustiques, à la fois vecteurs et lieux de reproduction sexuée de *Plasmodium*, jouent un rôle essentiel dans la dynamique de transmission du paludisme. En effet, leur nombre de piqûres par jour, leur longévité, ou encore leur taux d'infection sont des variables qui vont influencer de manière importante la transmission du parasite. Cependant, ces différents paramètres ne sont pas fixes et vont être soumis à de nombreux facteurs génétiques et environnementaux (abiotiques, biotiques) générant une réelle hétérogénéité de la capacité vectorielle individuelle des moustiques. Identifier les sources de variations responsables de cette hétérogénéité est essentiel pour comprendre la dynamique de transmission de *Plasmodium*. Au cours de ma thèse, j'ai axé mes travaux sur la variabilité observée au niveau de l'infection des moustiques. Alors que certains facteurs ont clairement été décrits comme influençant la prévalence et l'intensité d'infection, d'autres tels que les effets maternels, les effets de la sénescence ou de l'hôte vertébré ont été beaucoup moins étudiés. Au cours de cette discussion générale, j'ai essayé de recenser les différents paramètres décrits comme impactant le taux d'infection des moustiques par *Plasmodium* en y incorporant les différents résultats obtenus durant ma thèse.

1- Hétérogénéité d'infection : sources de variations liées aux vecteurs

1-1 Déterminants génétiques

Des campagnes de capture de moustiques sur le terrain ont mis en évidence qu'il existait, au sein des populations naturelles, une réelle hétérogénéité d'infection à *Plasmodium* (Kilama, 1973; Niaré *et al.*, 2002; Pruck-Ngern *et al.*, 2014, **Chapitre 5**). Au laboratoire, cela fait de nombreuses années que les chercheurs s'intéressent aux bases génétiques responsables de ces variations d'infections. En effet, dès les années 1930, des expériences de sélection expérimentales ont permis de créer des lignées de moustiques soit complètement susceptibles ou à l'inverse résistantes à *Plasmodium* (Huff, 1929, 1931; Ward, 1963; Collins *et al.*, 1986; Yan *et al.*, 1997; Lambrechts *et al.*, 2006). Le développement des nouvelles méthodes de séquençage, ainsi que l'utilisation de l'ARN interférence, ont permis

de localiser les gènes et d'identifier les mutations responsables de ces variations de résistance. De nombreux gènes intervenant dans la réponse immunitaire ont alors été identifiés comme jouant un rôle clé dans la susceptibilité des moustiques (Riehle *et al.*, 2006, 2008; Mitri *et al.*, 2009; Harris *et al.*, 2010). Cependant la résistance à la *Plasmodium* est coûteuse. Au laboratoire comme sur le terrain, il a été mis en évidence que les phénotypes de résistance (total ou partiel) sont associés à des coûts sur la fécondité et la longévité des moustiques (Yan *et al.*, 1997; Hurd *et al.*, 2005; Pompon & Levashina, 2015). Cette réduction de fitness, mise en regard avec le coût induit par le parasite (Hogg & Hurd, 1995; Vézilier *et al.*, 2012) peut expliquer, en partie, le maintien de l'hétérogénéité d'infection observé au sein des populations naturelles (Yan *et al.*, 1997, Discussion **Chapitre 5**).

Alors que l'immunité est un facteur qui impacte la probabilité et l'intensité d'infection en agissant directement sur le parasite, d'autres traits d'histoire de vie, tels que le comportement de piqûre, vont pouvoir avoir un effet indirect. Effectivement, on peut s'attendre à ce que la probabilité d'infection d'une femelle soit positivement corrélée au nombre de repas de sang pris au cours de sa vie. Néanmoins, la part génétique responsable de la variabilité observée au niveau du nombre de piqûres n'a jamais été étudiée. Au sein du **Chapitre 5** nous avons mis en évidence, chez *Cx pipiens*, que la variabilité en termes de proportion de femelles qui vont prendre un repas de sang sur un hôte vertébré est expliquée par une composante génétique non négligeable. Il est possible de penser que cette « motivation » à piquer impacte positivement le nombre de repas de sang pris par une femelle au cours de sa vie et donc sa probabilité d'infection.

Le niveau d'attraction des moustiques envers un hôte infecté est également un facteur qu'il serait pertinent d'étudier (Lacroix *et al.*, 2005; Cornet *et al.*, 2013b). En effet, les chercheurs ont mis en évidence que l'attraction des vecteurs pour un hôte donné était en partie sous contrôle génétique (Kilpatrick *et al.*, 2007; McBride *et al.*, 2014; Fernández-Grandon *et al.*, 2015). On pourrait alors s'attendre à ce que des variations du niveau d'attirance des moustiques pour un hôte infecté apparaissent suite à des modifications d'expression de gènes codant pour des récepteurs olfactifs (Mcbride et al 2014). Cependant aucune étude n'a encore été réalisée sur le sujet.

La sénescence va également pouvoir impacter la susceptibilité des moustiques à *Plasmodium*. Le vieillissement est caractérisé par une dégradation des capacités motrices, reproductives mais également immunitaires des organismes (Doums et al 2002, Augustin &

Partidge 2009). Chez les invertébrés, cette immunosénescence est observée chez la très grande majorité des espèces (**Chapitre 2**). De par le rôle essentiel que joue l'immunité dans la défense des organismes contre une infection, on peut s'attendre à ce que les individus les plus âgés soient plus sensibles aux agents pathogènes que leurs homologues plus jeunes. Bien que cette prédiction soit confirmée dans certains cas (Hillyer et al 2005, Roberts & Hughes 2014), de nombreuses études rapportent un effet positif du vieillissement sur la résistance des individus (**Chapitre 2**). Mes résultats vont également dans ce sens. En effet, j'ai pu mettre en évidence que, bien qu'une immunosénescence soit observée, la susceptibilité des moustiques au parasite de la malaria diminue drastiquement avec leur âge. Des résultats similaires ont été observés chez *Ae. Aegypti* infecté par *P. galinaceum* (Terzian et al 1956). Il semblerait alors que des modifications physiques et/ou physiologiques, associées au vieillissement des moustiques, soient davantage responsables de l'augmentation de résistance à *Plasmodium* que leur système immunitaire (**Chapitre 2**). Cependant, davantage d'études sont nécessaires pour confirmer ces hypothèses.

1-2 Déterminants non génétiques

Le fond génétique et la sénescence des moustiques ne sont pas les seuls déterminants responsables de la variabilité d'infection par *Plasmodium*. Dans leur habitat naturel ces insectes sont également constamment impactés par diverses pressions abiotiques et biotiques qui vont pouvoir affecter leur reproduction, leur survie mais également leur taux d'infection par le parasite de la malaria (Lefèvre *et al.*, 2013). Alors que les effets de la température ou des ressources alimentaires sont relativement bien documentés, d'autres paramètres comme le rôle des symbiotes ou des effets maternels le sont beaucoup moins.

Les moustiques étant des insectes ectothermes, il est bien évident que leurs traits d'histoire de vie, incluant la réponse immunitaire et donc la susceptibilité à une infection, vont être fortement impactés par la température ambiante mais également par ses fluctuations journalière (voir **Introduction partie 3-2-2**, Rueda *et al.*, 1990; Suwanchaichinda & Paskewitz, 1998; Murdock *et al.*, 2012; Murdock *et al.*, 2013; Ciota *et al.*, 2014). De plus, le développement des agents pathogènes au sein de ces diptères est aussi influencé par la température (Okech *et al.*, 2004; Murdock *et al.*, 2012; Mordecai *et al.*, 2013). Concernant le parasite de la malaria, l'augmentation de la température ambiante accélère la vitesse de développement de *Plasmodium* au sein des vecteurs, mais cela jusqu'à atteindre un certain

seuil au-delà duquel sa survie décroît nettement (Mitzmain, 1917; Noden *et al.*, 1995; Okech *et al.*, 2004; Mordecai *et al.*, 2013).

Pour ce qui est de l'effet de l'alimentation, il a clairement été mis en évidence que l'accessibilité et la qualité des ressources nutritives acquises au stade adulte mais également larvaire ont un impact important sur l'immunité (voir **Introduction partie 3-2-2**, Suwanchaichinda & Paskewitz, 1998; Koella & Sørensen, 2002, Telang *et al.*, 2012; Breaux *et al.*, 2014) et donc sur l'infection des vecteurs (Ferguson & Read, 2002a; Lambrechts *et al.*, 2006). La qualité de la nourriture acquise par les larves va notamment influencer l'expression de gènes immunitaires au stade adulte (Fellous & Lazzaro, 2010). Cependant, les relations entre nutrition, immunité et infection sont relativement complexes (Ponton *et al.*, 2011). Elles vont notamment pouvoir être influencées par d'autres paramètres environnementaux comme la température (Murdock *et al.*, 2014).

Alors que l'alimentation des moustiques adultes peut avoir un effet direct sur le développement de *Plasmodium* (Scott & Takken, 2012, **Chapitre 2**), elle peut également avoir un effet indirect en agissant sur le microbiote (Wang *et al.*, 2011; Ricci *et al.*, 2012). Il a notamment été mis en évidence qu'à la suite d'un repas de sang le nombre de bactéries du tube digestif pouvait être multiplié par quarante (Pumpuni *et al.*, 1996). Depuis quelques années les chercheurs s'intéressent à la composition de cette flore intestinale et à l'impact qu'elle pourrait avoir sur la susceptibilité à l'infection par *Plasmodium* (Dong *et al.*, 2009; Meister *et al.*, 2009; Cirimotich *et al.*, 2011; Boissière *et al.*, 2012, Minard *et al.*, 2013). Il a été montré que l'abondance et la diversité de la flore bactérienne pouvaient jouer un rôle important sur le développement précoce du parasite de la malaria (Dong *et al.*, 2009; Meister *et al.*, 2009; Cirimotich *et al.*, 2011). Alors que certaines bactéries vont avoir un effet direct, (Cirimotich *et al.*, 2011) en produisant notamment des composés cytotoxiques, d'autres vont pouvoir agir de manière indirecte en induisant un amorçage du système immunitaire de leur hôte (Dong *et al.*, 2009; Meister *et al.*, 2009).

Les co-infections, associant *Plasmodium* à d'autres micro-organismes, vont également pouvoir influencer la susceptibilité des moustiques face au parasite de la malaria. La présence au sein des vecteurs de microsporidies, de filaires, de bactérie endosymbiotique exogène, mais également d'autres souches de *Plasmodium*, est connue pour impacter négativement le développement de *Plasmodium* lors d'une réinfection (Bargielowski & Koella, 2009; Moreira *et al.*, 2009; Rodrigues *et al.*, 2010; Kambris *et al.*, 2010; Hughes *et al.*, 2011; Aliota *et al.*, 2011; Zélé, 2012). Dans la majorité des cas ces micro-organismes

semblent ne pas agir directement contre le parasite (Bargielowski & Koella 2009, voir Zélé, 2012). Leur présence au sein des moustiques induit une réponse immunitaire qui, suite à une nouvelle exposition à un agent pathogène, va permettre aux insectes d'être déjà armés et prêts à lutter plus efficacement contre ce nouvel envahisseur (amorçage immunitaire). Cependant des cas de compétitions par interférences ou bien par exploitations peuvent également être envisagés (Mideo, 2009).

La protection face à *Plasmodium*, liée à un amorçage immunitaire, va, en plus de protéger les individus, pouvoir être transférée à la génération suivante (Lorenz & Koella, 2011). Ce mécanisme de protection confère aux descendants un réel avantage adaptatif. Il est cependant important de noter que cet effet transgénérationnelle d'une infection n'est pas observé chez l'ensemble des espèces d'invertébrés (**Chapitre 4**). Nous avons pu mettre en évidence qu'il était plus susceptible d'évoluer chez les espèces phylopatriques et présentant une longue durée de vie (**Chapitre 4**). De plus, l'infection des parents par un agent pathogène peut également avoir des effets néfastes pour les descendants (Vantaux *et al.*, 2014, **Chapitre 3**). C'est notamment ce qui est observé dans le cas des infections par *Plasmodium* (*P. falciparum* et *P. relictum*). En effet, il a été montré que les femelles infectées par le parasite de la malaria vont produire des descendants avec une fécondité nettement plus basse et une susceptibilité plus haute à ce même parasite que des descendants issus de femelles non infectées. (Vantaux *et al.*, 2014, **Chapitre 3**).

Ainsi, de nombreux paramètres génétiques et environnementaux (biotiques et abiotiques), agissant directement sur les vecteurs, ont donc été identifiés comme pouvant impacter de manière importante la prévalence et l'intensité d'infection des moustiques. Cependant, au cours de mes différentes expériences, j'ai pu observer une autre source de variation non négligeable: l'hôte vertébré.

2- Hétérogénéité d'infection : sources de variations liées à l'hôte vertébré

L'intensité d'infection des hôtes vertébrés va, tout comme celle des moustiques, être impactée par différents paramètres d'ordres génétiques et environnementaux. Du fait de la corrélation positive entre la charge en parasite au sein de l'hôte (notamment en stade sexué) et l'intensité d'infection des vecteurs (Huff, 1954; Tchuinkam *et al.*, 1993; Hogg & Hurd, 1995; Schall, 2000; Collins & Jeffery, 2003 mais voir Ross *et al.*, 2006; Schneider *et al.*,

2007; Churcher *et al.*, 2013; Da *et al.*, 2015), il semble évident que l'identification des paramètres responsables des variations de parasitémie entre hôtes, mais aussi au sein d'un même individu, permettrait d'expliquer, en partie, l'hétérogénéité d'infection observée au sein d'une population de moustiques.

2-1 Déterminants génétiques

Actuellement, nous savons qu'un grand nombre de mutations génétiques jouent un rôle essentiel dans la résistance des hôtes vertébrés à *Plasmodium* (Longley *et al.*, 2011). Ces mutations ciblent principalement des gènes codant pour des protéines associées aux globules rouges, intervenant dans la réponse inflammatoire précoce ainsi que des gènes associés à la réponse immunitaire innée (Aucan *et al.*, 2000; Bongfen *et al.*, 2009; Longley *et al.*, 2011). L'un des cas les plus connus de protection contre le paludisme lié à une mutation génétique est la drépanocytose. Depuis plus d'un demi-siècle, il a été montré que les personnes hétérozygotes, au niveau du gène incriminé, sont en partie protégées contre les effets néfastes de l'infection par *Plasmodium* (Allisons, 1954). Le mécanisme de protection est lié au fait que la mutation induit une surproduction d'une enzyme particulière (hème-oxygénase-1) intervenant dans la dégradation des hèmes (Ferreira *et al.*, 2011). Or la dangerosité de certaines formes de paludisme vient en grande partie d'une sur-libération de ces composés toxiques (Pamplona *et al.*, 2007). On peut également citer les gènes appartenant au complexe majeur d'histocompatibilité (CMH). En effet, à la fin des années 1980, alors qu'une épidémie de paludisme atteignait la république de Gambie, il a été montré que des mutations observées au niveau de cette classe de gènes participaient à la protection d'enfants gambiens face aux infections par *Plasmodium* (Hill *et al.*, 1991). Des résultats similaires ont été observés dans le cas de la malaria aviaire (Bonneaud *et al.*, 2006; Sepil *et al.*, 2013). Au sein de population sauvage d'oiseaux des allèles CMH de classe 1 ont été décrites comme associées à un phénotype de résistance partielle des individus face au paludisme. Cependant, les allèles conférant cette protection étaient différentes d'une population à une autre, ce qui laisse alors suggérer une adaptation locale dans le système hôte parasite (Bonneaud *et al.*, 2006).

Tout comme chez les moustiques, la sénescence va également pouvoir impacter la susceptibilité des hôtes vertébrés à *Plasmodium*. En effet, le vieillissement est associé à une détérioration progressive du système immunitaire affectant à la fois la réponse innée et adaptative (Akbar & Fletcher, 2005; Solana *et al.*, 2006). Ce déficit immunitaire est

généralement caractérisé par une augmentation de la sensibilité des individus aux maladies infectieuses (Gardner, 1980). Dans le cas du paludisme, que ce soit chez l'homme ou au sein de populations d'oiseaux, des prévalences ainsi que des charges en parasite plus importantes sont observées chez les individus âgés (Mühlberger *et al.*, 2003; Wood *et al.*, 2007; Lachish *et al.*, 2011; Podmokła *et al.*, 2014 mais voir Garvin *et al.*, 2006; Bichet *et al.*, 2014). Chez les reptiles, aucun effet du vieillissement n'a été observé sur la prévalence ou l'intensité d'infection par *P. mexicanum* (Bromwich & Schall, 1986; Schall, 2000). Cependant le taux de maturation des gamétocytes a été décrit comme plus rapide au sein des individus âgés (Eisen, 2000; Eisen & Schall, 2000). Il faut savoir que la situation est clairement différente au sein des régions hyperendémiques. En effet, il a été montré que, dans ces zones particulières, les individus les plus jeunes sont les plus susceptibles (Bull *et al.*, 2000; Gavazzi *et al.*, 2004; Mapua *et al.*, 2015; Stanisic *et al.*, 2015). Ceci peut s'expliquer assez facilement grâce à la réponse immunitaire adaptative des vertébrés. Suite à une première exposition à *Plasmodium* les individus vont développer une réponse immunitaire spécifique permettant alors d'être en partie protégés en cas de réinfection.

2-2 Déterminants non génétiques

Différents facteurs environnementaux incluant les variations climatiques, l'altitude ou la distance entre le lieu de vie et un point d'eau permanent ont été décrits comme impactant directement le taux d'infection des hôtes vertébrés par *Plasmodium*. En effet, ces facteurs peuvent à la fois limiter l'abondance et la distribution des vecteurs du paludisme, mais également impacter la vigueur du parasite au sein de ces derniers (Foley *et al.*, 2003; Balls *et al.*, 2004; Freed *et al.*, 2005; Atkinson & LaPointe, 2009; Lachish *et al.*, 2011). D'autres facteurs biotiques, tels que le sexe ou la condition corporelle des individus vont agir directement sur la capacité des hôtes à lutter contre l'infection (Cornet *et al.*, 2014b).

Les effets du sexe sur l'immunité des vertébrés sont étudiés depuis de nombreuses années (Greenberg *et al.*, 1953; Giefing-Kröll *et al.*, 2015). D'une manière générale, les mâles ont été décrits comme plus sensibles aux parasites et comme présentant généralement des charges infectieuses plus importantes que celles observées chez les femelles (Zuk & McKean, 1996; Sheridan *et al.*, 2000; Zuk & Stoehr, 2002; Nunn *et al.*, 2009; Galligan & Fish, 2015). Ce dimorphisme sexuel est généralement attribué à deux causes: d'une part l'existence d'une exposition différentielle des individus aux parasites en lien avec l'existence de comportements sexe-spécifiques (reproduction, comportement d'alimentation ou de

défense du territoire, Arriero & Møller, 2008; Lachish *et al.*, 2011). La seconde, serait l'impact de la testostérone sur l'immunité (Peters, 2000; Casto *et al.*, 2001; Belliure *et al.*, 2004). En effet, cette hormone a été décrite comme ayant un effet immunosuppresseur (Belliure *et al.*, 2004; Krücken *et al.*, 2005). Cependant, dans le cas de l'infection par *Plasmodium*, les effets du sexe ne sont pas aussi clairs et semblent dépendre des couples hôte/parasite. Alors que chez les reptiles, les mâles semblent toujours être plus infectés que les femelles (Schall, 2000; Vardo *et al.*, 2005), chez les oiseaux cela va dépendre des espèces d'hôtes et de parasites étudiés (Garvin *et al.*, 2006; Wood *et al.*, 2007; Arriero & Møller, 2008; Lachish *et al.*, 2011; Shurulinkov *et al.*, 2012; Bichet *et al.*, 2014). A l'inverse, chez les primates humains et non humains, la grande majorité des études n'ont pas mis en évidence de lien particulier entre sexe et taux d'infection (Bernin & Lotter, 2014; Mapua *et al.*, 2015).

D'autres paramètres comme le stress (Cohen & Williamson, 1991) et la condition physique peuvent également influencer la susceptibilité des hôtes à différents parasites (Gershwin *et al.*, 1985; Møller *et al.*, 1998; Navarro *et al.*, 2003, Beldomenico & Begon, 2010). L'une des causes pouvant induire une diminution de la condition physique d'un organisme est son infection par un agent pathogène. Cette réduction de l'état de santé global peut alors faciliter une réinfection par un nouveau parasite (Beldomenico & Begon, 2010). Le résultat des infections multiples, en termes de transmission des différents agents pathogènes, va varier en fonction des différents protagonistes mis en relation (Roode *et al.*, 2005; Choisy & de Roode, 2010; Bashey, 2015). Dans le cas du paludisme, suite à des co-infections inter- mais également intra-spécifiques, trois grandes issues possibles ont été observées : pas d'effet (Rooyen *et al.* 2013), des effets positifs (Spiegel *et al.*, 2003; Sokhna *et al.*, 2004; Graham *et al.*, 2005; Noland *et al.*, 2007; Vardo & Schall, 2007) ou bien des effets négatifs sur la transmission de *Plasmodium* (Lyke *et al.*, 2005; Brutus *et al.*, 2006). Dans le cas des co-infections faisant intervenir plusieurs espèces ou plusieurs clones de *Plasmodium* par exemple, on peut observer que chez les oiseaux, une co-infection par *P. relictum* et *P. ashfordi* aboutit à une augmentation de la parasitémie générale (Palinauskas *et al.*, 2011). A l'inverse chez les reptiles, une multi-infection avec différents clones de *P. mexicanum* aboutit à une diminution de la charge en parasite mais à une augmentation de la proportion de gamétocytes (Vardo-Zalik & Schall, 2008). Des résultats similaires sont observés lors de l'infection en phase aigüe de souris par deux clones de *P. chabaudi* (Taylor *et al.*, 1998; De Roode *et al.*, 2003; Wargo *et al.*, 2007).

2-3 Variations temporelles

Des variations temporelles en termes de prévalence et d'intensité d'infection des hôtes vertébrés vont également pouvoir être observées et impacter le taux d'infection des vecteurs. Concernant la dynamique globale d'infection au sein d'une population d'hôtes, il a été mis en évidence des variations annuelles de prévalences chez de nombreuses espèces (Bromwich & Schall, 1986; Lachish *et al.*, 2011; Podmokła *et al.*, 2014; Reiner *et al.*, 2015). Ces fluctuations sont en partie causées par des variations de densités de vecteurs au cours du temps (Gil *et al.*, 2003; Mbogo *et al.*, 2003), mais pas seulement. Dans le cas de la malaria aviaire, il a été montré que chez les individus âgés, la prévalence d'infection atteignait un pic au moment du printemps, probablement causée par une réactivation des stades dormants du parasite associé au retour des vecteurs (Applegate, 1971; Cosgrove *et al.*, 2008). Un second pic est observé au début de l'automne, en lien avec le moment où les jeunes de l'année quittent le nid, permettant alors à *Plasmodium* de disperser avec ses hôtes (Cosgrove *et al.*, 2008).

A l'échelle d'un individu, une fois infecté par *Plasmodium*, l'intensité d'infection au sein de ce dernier va varier au cours du temps. Ces variations temporelles, qu'elles soient à l'échelle de plusieurs mois ou de la journée, vont pouvoir impacter le taux de transmission du parasite aux moustiques.

Suite à l'injection de *Plasmodium*, deux phases bien définies vont se succéder au sein de l'hôte vertébré. Tout d'abord, durant une vingtaine de jours, une phase aigüe d'infection est observée. Elle est caractérisée par une parasitémie élevée et est associée à un fort potentiel de transmission du parasite (**Chapitre 6**). Après cette première étape, une phase chronique va se mettre en place et durer de plusieurs semaines à plusieurs mois. La parasitémie et la gamétocytémie vont alors diminuer drastiquement en réponse à l'activation du système immunitaire de l'hôte. En conséquence, la probabilité d'infection des vecteurs, suite à la prise d'un repas de sang, sera donc beaucoup plus faible. En effet, au cours du **Chapitre 6**, nous avons pu montrer que la prévalence d'infection était divisée par 10 entre ces deux stades. Si la différence la plus marquée entre phase aigüe et chronique concerne la parasitémie et la gamétocytémie, d'autres différences ont été observées, notamment concernant le sex-ratio des gamétocytes (Reece *et al.*, 2009), ainsi que la condition physiologique (O'Donnell *et al.*, 2009) et immunitaire (Atkinson *et al.*, 2001) des hôtes.

Si l'on s'intéresse plus particulièrement à la phase chronique d'infection, on peut constater que des fluctuations de la parasitémie vont également être observées durant ce stade. Il a en effet été montré qu'en réponse à des piqûres de moustique, le parasite se réactivait rapidement (Cornet *et al.*, 2014a). Dans le cas de la malaria aviaire, cette réactivation induit une augmentation drastique de la densité de parasite présent dans le sang de l'hôte impactant alors positivement la transmission du parasite au vecteur (Cornet *et al.*, 2014a).

Pour terminer, il existe un dernier type de variation temporelle à une échelle plus fine: les variations journalières. En effet, en phase aigüe d'infection, chez la plupart des espèces de *Plasmodium*, une augmentation récurrente de la parasitémie et/ou de la gamétocytémie, suivant un cycle d'une durée associée à un multiple de 24h, a été observée (**Chapitre 7**, Hawking *et al.*, 1968, 1972; Gautret & Motard, 1999). Par exemple, un intervalle de 24h chez *P. relictum* et *P. chabaudi* et de 48h chez *P. falciparum* et *P. vivax* a été mesuré entre deux vagues de production de gamétocytes (Gautret & Motard, 1999). La maturation de ces stades sexués a également été décrite comme périodique et synchrone avec une durée pouvant varier de 12h environ pour *P. falciparum* à plus de 60h pour *P. gallinaceum* (Gautret & Motard, 1999). Chez la majorité des espèces, la production périodique des gamétocytes, la durée de leur cycle de maturation associée à des phénomènes de séquestrations/libérations des stades infectieux (Roller & Desser, 1973; Mons *et al.*, 1985; Taylor & Read, 1997), induisent une rythmicité de l'infectiosité des hôtes vertébrés. En se basant sur les résultats de plusieurs études, Frank Hawking a pu montrer que cette variation périodique de la capacité du parasite à infecter son vecteur était associée au rythme circadien d'activité des moustiques (Hawking, 1975). Cette théorie, appelée « Phénomène de Hawking » (Garnham & Powers, 1974; O'Donnell *et al.*, 2011), a depuis été vérifiée chez certaines espèces (*P. relictum*, *P. knowlesi* **Chapitre 7**) mais semble absente chez d'autres. C'est notamment le cas chez *P. falciparum* (Bray *et al.*, 1976; Gautret & Motard, 1999; Barrozo *et al.*, 2004), *P. chabaudi* et *P. berghei* (Gautret & Motard, 1999) où aucune variation journalière d'infectiosité des hôtes n'a été mesurée.

Concernant la phase chronique, actuellement aucune étude ne s'est intéressée aux caractéristiques des « relapses » engendrés par la mise en contact des hôtes avec des moustiques. Bien que l'on puisse s'attendre à ce que cette réponse rapide du parasite présente les mêmes critères de synchronie et de périodicité que ceux observés lors de la phase aigüe d'infection, les résultats préliminaires de l'étude présentée au **chapitre 7** ne semblent pas aller dans ce sens. Cependant les analyses sont encore en cours.

Afin d'obtenir une image plus complète des processus évolutifs responsables de la rythmicité du cycle parasitaire, davantage d'investigations sont nécessaires. Plus précisément, il serait important de déterminer s'il existe un lien entre le cycle cellulaire du parasite et les rythmes circadiens de l'hôte vertébré. En effet, si le phénomène de Hawking est vérifié, alors, le seul moyen pour le parasite d'identifier les périodes de la journée lors desquelles les vecteurs sont actifs, est d'ajuster le plus finement possible son cycle de développement au rythme circadien de son hôte (O'Donnell *et al.*, 2011).

3- Hétérogénéité d'infection : sources de variations liées au parasite

3-1 Diversité génétique

Comme nous l'avons vu précédemment, des paramètres biotiques et abiotiques agissent directement sur *Plasmodium* et vont induire une variabilité d'infection au sein des populations d'hôtes vertébrés et de vecteurs. Cependant, les interactions génotype / génotype vont également pouvoir contribuer à cette hétérogénéité d'infection (Lambrechts *et al.*, 2005). Au sein d'une espèce de *Plasmodium* il peut exister un polymorphisme plus ou moins important (Prugnolle *et al.*, 2008; Bensch *et al.*, 2009; Neafsey *et al.*, 2012; Schaer *et al.*, 2013) aboutissant à la formation de clones/souches/isolats qui peuvent présenter des traits d'histoire de vie différents (virulence envers l'hôte et/ou le vecteur, taux de transmission, Ferguson & Read, 2002a; Mackinnon *et al.*, 2002; Ferguson *et al.*, 2003). L'exemple actuellement le plus marquant est celui de *P. chabaudi*. Dans cette espèce de malaria des rongeurs il a été montré, à partir de 10 clones différents, que les taux de multiplications des stades asexués et sexués variaient fortement d'un clone à l'autre et impactaient le taux de transmission des parasites aux moustiques (voir Mackinnon & Read 2004).

Il est également important de noter qu'une souche de *Plasmodium* va avoir des effets différents en fonction de l'espèce d'hôte ou de vecteur qu'elle va infecter (Palinauskas *et al.*, 2008). Des variations d'effets entre population d'une même espèce ont également été observées et peuvent être expliquées par des adaptations locales des individus à leur population de parasites et/ou inversement (Bonneaud *et al.*, 2006; Loiseau *et al.*, 2011; Harris *et al.*, 2012).

3-2 Evolution du parasite

Les parasites sont décrits comme pouvant évoluer très rapidement en milieu naturel ce qui leur permet notamment de s'adapter en peu de temps à de nouvelles conditions environnementales (Ebert 1998). Un moyen d'étudier, au laboratoire, l'évolution des agents pathogènes est de réaliser des expériences de passage en série (Ebert 1998). Ce type d'expérience permet notamment de suivre l'évolution de la virulence d'un parasite face à un hôte donné. Depuis 2009, nous maintenons au laboratoire une lignée de *Plasmodium relictum* (SGS1) par passage en série sur canaris. Ce mode de maintien expérimental du parasite a engendré une évolution rapide de ce dernier. En effet, la parasitémie, mesurée au stade aigüe d'infection, a été multipliée par deux en cinq ans. Nos résultats, ainsi que ceux observés dans des études antérieures (Dearsly *et al.*, 1990; Ebert, 1998; Mackinnon & Read, 1999), sont cohérents avec la prédiction selon laquelle en l'absence de moustique, les passages en séries induiraient une sélection des individus avec un taux de reproduction asexué plus important (Mackinnon & Read 2004). Alors que certaines études ont montré que cette augmentation de parasitémie était accompagnée par des variations dans la production de gamétocytes (stade sexué, Mackinnon & Read 1999, Dearsly *et al.* 1990), nos résultats n'ont pas mis en évidence de variation au niveau de ce paramètre. Ceci est plutôt surprenant car, au cours du **Chapitre 6**, nous avons observé une corrélation positive entre parasitémie et gamétocytémie. Nous nous serions alors attendus à ce que, en parallèle de la parasitémie, l'investissement dans la production des stades sexués augmente également. Cependant, il semble que nos mesures de gamétocytémie ne soient pas parfaites. En effet, obtenir une quantification exacte des gamétocytes, par microscopie, est relativement compliqué (Schneider *et al.*, 2007; Ouédraogo *et al.*, 2009; Churcher *et al.*, 2013). Davantage de travaux sur le lien entre production des stades sexués et asexués chez *P. relictum* sont donc nécessaires.

Un moyen de vérifier si l'investissement du parasite dans la production de stades transmissibles (sexués) a été altéré, suite à son maintien sur hôte vertébré, est de regarder l'évolution de la prévalence et de l'intensité d'infection des moustiques au cours des cinq années (**Figure 4**). On peut alors constater que malgré une légère diminution de prévalence après la première année, celle-ci s'est maintenue aux alentours de 85% durant toutes les expériences. Pour ce qui est de l'intensité d'infection chez le moustique, malgré des fluctuations importantes d'une année à l'autre, il ne semble pas y avoir de tendance générale quant à l'évolution de ce paramètre. Ces résultats valident alors en partie notre

observation concernant le maintien d'une valeur de gamétocytémie constante malgré les passages en série sur hôte vertébré.

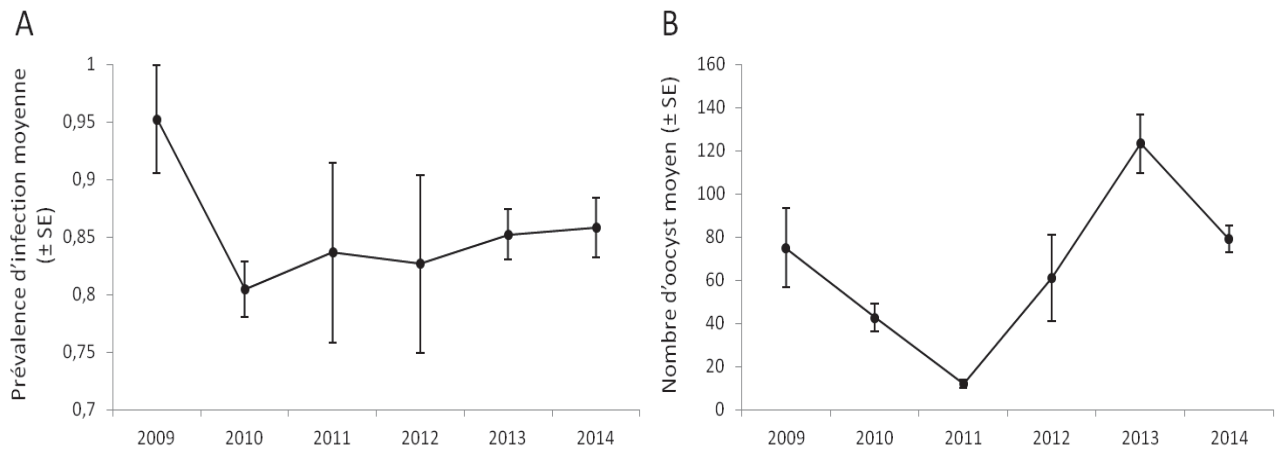


Figure 4: Evolution de (A) la prévalence et de (B) l'intensité d'infection des moustiques par *P. relictum* au cours des cinq années durant lesquelles le parasite a été maintenu au laboratoire. Les points et les barres représentent la moyenne par année ($\pm$ SE).

Pourtant, bien que la capacité de *Plasmodium* à infecter son vecteur n'ait pas évolué, la virulence de ce dernier envers les moustiques a, elle, clairement diminué (**Figure 5A**). Alors qu'en 2009 le parasite induisait une réduction de fécondité de plus de 40%, cinq ans après, ce coût a été divisé par six (**Figure 5A**). N'ayant pas observé de diminution de la charge en parasite, ce résultat laisse alors penser que l'effet de *Plasmodium* sur la fécondité des moustiques n'est pas dépendant de l'intensité d'infection. En regardant ce dernier point de plus près, on peut s'apercevoir qu'il ne semble pas y avoir de lien entre charge en oocystes et nombre d'œufs pondus par les femelles (**Figure 5B**). Le coût de ce parasite sur son vecteur ne semble donc pas être associé à une compétition pour l'utilisation des ressources alimentaires disponibles pour la production des œufs, puisque, dans ce cas, une corrélation positive entre intensité d'infection et coût aurait dû être mesurée. On pourrait alors suspecter un effet direct de *Plasmodium* sur l'ovogenèse des moustiques. Des études antérieures semblent valider cette hypothèse puisqu'elles ont mis en évidence que la présence de ce parasite induisait une perturbation de l'apport en protéine vitelline au niveau des ovaires ainsi qu'une stimulation de l'appose des ovocytes (Hurd, 2003; Hurd *et al.*, 2006).

En regardant tous ces résultats dans leur ensemble, l'évolution de notre parasite, suite au passage en série sur l'hôte vertébré, semble avoir impacté sa capacité à altérer l'ovogenèse du moustique. Est-ce que ceci pourrait avoir des conséquences sur la transmission du parasite ? Il serait désormais essentiel de déterminer si la manipulation de la

fécondité des moustiques par *Plasmodium* joue un rôle dans la dynamique de transmission de cet agent pathogène. Est-ce que, comme proposé par Vézilier et collaborateurs (2012), diminuer la fécondité des moustiques permet, dans un second temps, d'augmenter leur longévité et donc la probabilité de transmission de *Plasmodium* ?

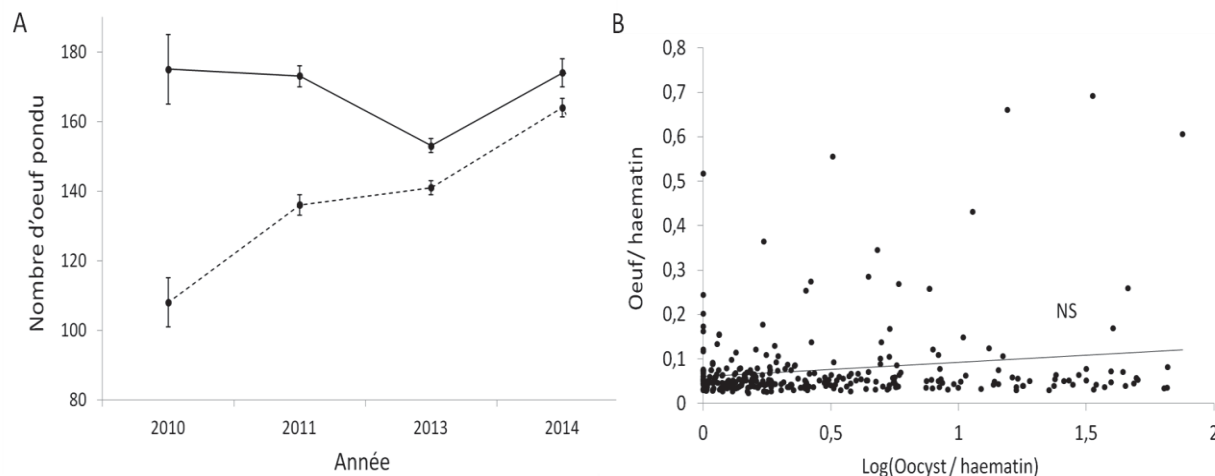


Figure 5 : (A) Evolution du nombre d'œufs pondus par *Cx pipiens* infecté (ligne pointillée) ou non (ligne pleine) par *P. relictum* au cours des cinq années durant lesquelles le parasite a été maintenu au laboratoire. Les points et les barres représentent la moyenne par année ($\pm$ SE). (B) Corrélation entre le nombre d'œufs moyens pondus et la charge en oocystes par femelle. Les deux paramètres ont été corrigés par la taille du repas de sang (haematine ng).

Il est important de noter que toutes ces modifications observées au niveau de la parasitémie et de la fécondité des moustiques semblent bien associées à une évolution du parasite et non des vecteurs ou des hôtes vertébrés. En effet, lorsqu'en Mai 2015 nous avons isolé et installé au laboratoire une nouvelle lignée de *Plasmodium relictum* (SGS1), nous avons pu observer que la parasitémie des oiseaux ainsi que le coût du parasite sur la fécondité des moustiques présentaient de nouveau des taux comparables à ceux observés en 2009 (**Figure 6**).

Ces résultats sur l'évolution rapide de *Plasmodium* peuvent alors laisser songeur quant à l'utilisation de certaines souches de parasite maintenues au laboratoire depuis une cinquantaine d'années (Pigeault *et al.*, 2015). Il serait peut être intéressant de vérifier comment ces souches ont évolué, afin de s'assurer que les résultats issus de ces modèles biologiques peuvent être transposés à ce qui se passe réellement avec des lignées sauvages.

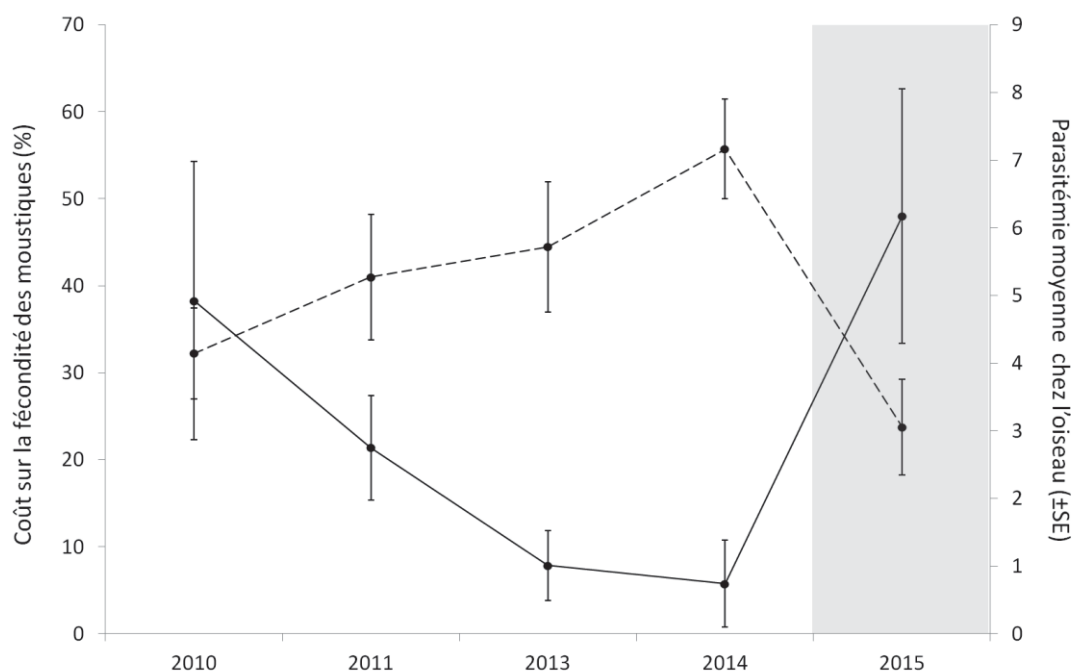


Figure 6 : Progression de la parasitémie dans l'hôte vertébré (ligne pointillée) et des coûts sur la fécondité des moustiques (ligne pleine) induits par *P. relictum* au cours d'une succession d'expériences réalisées entre 2009 et 2015. Les points et les barres représentent la moyenne par année ($\pm$ SE). La zone grise correspond à l'infection réalisée avec le nouveau parasite capturé sur le terrain en mai 2015.

4- Conclusion et perspectives

Les sources d'hétérogénéité de l'infection des moustiques par *Plasmodium* sont multiples. En effet, chaque membre de l'association tripartite intervenant dans le cycle de la malaria va être soumis à un ensemble de variations d'origines génétiques et/ou environnementales, pouvant impacter le taux et l'intensité d'infection des vecteurs. En milieu naturel, des effets synergiques ou au contraire antagonistes pourraient également exister entre ces différents facteurs compliquant alors l'interprétation et l'extrapolation des résultats obtenus expérimentalement. Bien que nos connaissances sur les sources d'hétérogénéité d'infection des moustiques ne cessent d'augmenter, elles ne sont que rarement prises en compte dans les modèles théoriques (Reiner et al 2013). Actuellement, il est difficile de savoir si l'hétérogénéité d'infection observée au sein des populations naturelles de vecteur a un réel impact sur la dynamique de transmission du paludisme.

L'un des facteurs qui semble impacter le plus fortement le taux d'infection des moustiques, mais qui reste aujourd'hui peu compris et peu étudié, est l'effet de l'hôte vertébré. Il est clair que, en plus du lien entre gamétocytemie et taux d'infection des moustiques (mais voir Shute & Maryon, 1951; Graves *et al.*, 1988; Da *et al.*, 2015), d'autres paramètres vont intervenir et influencer la transmission du parasite. A gamétocytemie égale,

tous les hôtes ne semblent pas participer de la même manière à la transmission de *Plasmodium*. Il serait intéressant d'identifier quels profils d'hôtes sont les plus susceptibles d'apporter une contribution importante à la dissémination du parasite. Existe-t-il des facteurs sanguins tels que des nutriments, des micro-éléments ou des facteurs immunitaires produits par l'hôte vertébré qui facilitent ou au contraire impactent négativement le développement du parasite au sein du moustique ? Des arguments semblent en effet aller dans ce sens. Il a récemment été mis en évidence que des anticorps anti-bactériens produits par un hôte (Noden *et al.*, 2011) ainsi que la présence d'antibiotiques dans le sang ingérés par les moustiques (Gendrin *et al.*, 2015) impactent négativement le microbiote intestinal des vecteurs, aboutissant alors à une plus forte infection par *Plasmodium*. N'est il pas possible, à l'inverse, de trouver des molécules, transmises par l'hôte vertébré, qui permettrait de lutter contre le développement du parasite au sein des vecteurs ?

Un autre point important à étudier serait l'impact du stade chronique d'infection sur la dynamique de transmission de *Plasmodium*. En effet, au sein des populations naturelles, la grande majorité des individus présentent une infection chronique et non aiguë ; pourtant, peu d'informations sont disponibles quant à la contribution relative de ce stade à la transmission de la malaria sur le terrain. Vu le faible taux de transmission de *Plasmodium* observé expérimentalement au cours du stade chronique, la transmission de *Plasmodium* ne repose-t-elle pas majoritairement sur les phases aiguës d'infections ? Il serait intéressant de déterminer si la prévalence et l'intensité d'infection, au sein d'une population de vecteurs, sont positivement corrélées à la proportion de stade aiguë observée au sein de la population d'hôtes vertébrés.

Le modèle de la malaria aviaire est un très bon outil pour essayer de répondre à ces questions. De plus, de par la facilité à échantillonner de nouvelles souches de *Plasmodium* sur le terrain, ce modèle biologique pourrait permettre d'étudier les causes responsables de la variabilité génétique observée au niveau de ce parasite et surtout les raisons de son maintien (Bensch *et al.*, 2009). En effet, il serait intéressant de comprendre pourquoi et comment, au sein d'une même population d'hôte, de nombreuses espèces, lignées ou clones de parasites coexistent (Zélé et al 2014b).

L'utilisation d'une association naturelle, l'accumulation des connaissances quant à l'écologie et la distribution de cette maladie sur le terrain, combinées à des recherches expérimentales en laboratoire qui ne cessent d'augmenter font de la malaria aviaire un

modèle biologique de choix pour étudier l'écologie évolutive des interactions hôte-moustique-*Plasmodium*.

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Bibliographie correspondant à l'introduction et à la discussion générale

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Annexe

Annexe 1 :

Coût de l'infection par *Plasmodium* sur la fécondité des moustiques: que ce passe t'il au-delà du premier cycle gonotrophique ?

Plasmodium impact on its vector fecundity: what happens beyond the first gonotrophic cycle

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Introduction

The pattern of transmission of malaria parasites within a population is strongly associated with the fitness of its vector. For this reason, the effect of *Plasmodium* on mosquito fecundity has traditionally received a lot of attention (Hogg and Hurd, 1995, Hurd, 2003, Vézilier et al. 2012). Several species of malaria parasites have been shown to reduce the fecundity and fertility (proportion of hatched larvae) of mosquitoes (Hurd 2003, Gray and Bradley, 2006, Vézilier et al. 2012). However, it is often difficult to determine whether this reduction in fecundity is directly induced by the parasite, or whether it is simply a by-product of the lower quality of the infected blood. Indeed, *Plasmodium* infections induce anaemia in their vertebrate hosts (Bromwich and Schall, 1986, Palinauskas et al. 2008, Pigeault et al. 2015), and red blood cells are a crucial energy resource for egg production in female mosquitoes (Hurd et al. 1995).

Evidence from rodent malaria suggests that *Plasmodium* induces a direct cost on female fecundity. *Plasmodium yoelii* induces apoptosis in mosquito ovaries (Hopwood et al. 2001) and increases eggs resorption (Carwardine and Hurd 1997). In addition, a blood meal containing gametocytes, the only stages that progress to a full mosquito infection, has a greater impact on mosquito fecundity than a meal containing only asexual stages, which do not develop inside the mosquito (Hogg and Hurd 1995). The fecundity reduction therefore requires the successful establishment of *Plasmodium* inside the mosquito, which has led to suggestions that it may be potentially adaptive for the parasite (Hurd 2009).

The *Plasmodium* effect on fecundity has been studied in many mosquito/parasite systems (Hogg and Hurd 1997, Ahmed et al. 1999, Gray and Bradley 2006, Vézilier et al. 2012, Pigeault et al. 2015). Yet, most articles studying the impact of *Plasmodium* on mosquito fecundity have focused on the first *gonotrophic cycle*, which is defined as a blood meal followed by an oviposition event (but see Hogg and Hurd 1995). However, during their lifetime, female mosquitoes go through several gonotrophic cycles, which raises the

question of whether they are able to compensate the fecundity costs induced by an infected blood meal during the following gonotrophic cycles. Investigating the impact of *Plasmodium* on several gonotrophic cycles provides a more complete view of the impact of this parasite on the lifetime fitness of its vector and provides insights into the adaptive nature of the fecundity reduction. Indeed, if the fecundity reduction is due to a low quality bloodmeal (eg *Plasmodium*-triggered anaemia) we may expect it to be more readily compensated for during the following uninfected bloodmeals, than if it is directly triggered or manipulated by the parasite.

Here, we measure the impact of *Plasmodium* on its vector's fecundity during three consecutive gonotrophic cycles (henceforth GC). For this purpose, we have used the avian malaria system consisting of *Plasmodium relictum* and its natural vector in the field *Culex pipiens* (Pigeault et al. 2015).

Materials & Methods

Plasmodium strain, bird infections and mosquito rearing

Plasmodium relictum (lineage SGS1) is the aetiological agent of the most prevalent form of avian malaria in Europe (Valkiunas 2004). The lineage was isolated from infected sparrows in 2009 (Pigeault et al. 2015) and passaged to naïve canaries (*Serinus canaria*). Since then it has been maintained by carrying out regular passages between our stock canaries through intraperitoneal injections. Experimental canaries (n = 2) were infected by injecting them with ca. 80µL of blood from the infected canary stock. Mosquito blood feeding took place 12 days after the injection, to coincide with the acute phase of the *Plasmodium* infection (Vézilier et al. 2010). Mosquitoes were rearing as described by Vézilier et al. 2012. Male and female mosquitoes emerging within a 24- h period were kept together for mating and were provided with 10% sugar solution. Mosquito adults were maintained at 25 ± 1 °C, 80% humidity in a 12: 12 h light/ dark cycle. Five days after emergence, four cages containing 90 females were made. For 12h prior to blood-feeding, access to sugar was denied.

Experiment

For the first GC, six days after emergence, two mosquito cages were provided overnight with an infected bird and two with uninfected one. Four days after the blood meal, cages were provided overnight with a pot containing mineral water. The following morning, egg rafts were collected and photographed using a binocular microscope equipped

with a numeric camera. Egg number was recorded by manually counting the number of eggs on the photographs using the Mesurim Pro freeware (Academie d'Amiens, France). To obtain an estimate of mosquito size (wing length) and of the infection success, on day eight post blood meal 20 females of each cage were haphazardly sampled and dissected.

The mosquitoes were allowed two further GCs, spaced at eight day intervals from each other. The experimental protocol for the second and third GCs was identical to that described above.

Statistical analyses

Analyses were carried out using the R statistical package (v. 3.1.1). Data collected on wing length (proxy of mosquito size) and egg production was tested for normality and homoscedasticity using the Shapiro test and Bartlett test respectively. Differences in mean wing length were tested using a Student's t-test. The impact of *Plasmodium* infection and GC on mosquito fecundity was analysed using a standard general linear model procedure with a normal error structure. *A posteriori* contrasts were carried out by aggregating factor levels together and by testing the fit of the simplified model using a likelihood ratio test.

Results

No differences in size were observed between mosquitoes allocated to the infected and uninfected treatments (t-test = 0.089, $p = 0.9291$). Midgut dissection revealed that almost 95% of the mosquitoes fed on infected bird were infected (mean number of oocysts $\pm$ s.e. = 84 ± 32).

GC and *Plasmodium* infection both had a strong impact on mosquito fecundity (respectively $F_{2,692}=11.855$, $p < 0.0001$, $F_{1,694}=12.062$, $p < 0.001$, **Figure1**). In the first and second GCs, infected females laid 6% fewer eggs than uninfected ones. In the third GC the difference in fecundity between infected and uninfected females increased to 9%. There were no significant differences in the number of eggs laid in the 1st and second GCs by either uninfected (contrast analyses 1st vs 2nd GC: $\chi^2_1 = 0.163$ $p = 0.923$) or infected ($\chi^2_1 = 0.047$ $p = 0.953$) females (**Figure1**). Fecundity, however, decreases drastically between the 2nd and 3rd GCs (contrast analyses: uninfected: $\chi^2_1 = 16.225$ $p = 0.009$, infected: $\chi^2_1 = 19.825$ $p < 0.001$).

Discussion

In accordance with previous studies in several malaria systems (Hogg and Hurd 1997, Ahmed et al. 1999, Gray & Bradley 2006, Vézilier et al. 2012), we observed a negative impact of *Plasmodium* on mosquito fecundity in the first GC following an infected blood meal. Interestingly, despite having taken two subsequent uninfected blood meals, the negative impact of malaria parasite persisted through to the third GC. Similar results have been observed by Hogg and Hurd (1995) albeit using an artificial combination between parasite and vector.

There may be different reasons for the differences in fecundity observed between infected and uninfected females through several consecutive GCs. First, fecundity is strongly correlated with the quantity of the blood meal (Pigeault et al. 2015). Females fed on infected hosts have been shown to excrete less haematin, a product of the degradation of haemoglobin which is correlated with the number of red blood cells ingested (Hogg et al 1995). One potential explanation for our results is that females that become infected during their first blood meal, take smaller blood meals subsequently, even if these are not infected. This could happen if the damage to the midgut epithelium caused by the presence of oocysts (Han and Barillas-Mury 2002) reduces the efficiency of blood digestion. Unfortunately in our experiment haematin was not quantified and thus this hypothesis cannot be tested. However, work carried out in other systems has shown that blood meal size is not affected by the presence of either midgut oocysts (Hogg and Hurd 1995) or sporozoite (Li et al. 1992) in the salivary glands, rendering this explanation unlikely. Second, host blood quality has also been found to be crucial for mosquito fecundity (Hurd et al. 1995). There is abundant evidence that malaria parasites decrease red blood cell density, glucose, lipid, aminoacid and micronutrient composition of host blood (LeRoux et al. 2009), either because these nutrients are scavenged by the parasite or as host's response to the infection. Under this scenario, the negative effects of a low quality (infected) blood meal on mosquito fecundity would not be compensated for by two subsequent healthy blood meals. The limited work available, however, suggests that in *Culex quinquefasciatus* the quality of the first blood meal does not have carry over effects on the second blood meal (Richards et al 2012). Finally, the pervasive effects of a *Plasmodium* infection on mosquito fecundity could be the direct effect of the parasite on mosquito physiology. Hurd and collaborators (Hurd 2003, Hurd et al. 2006) have conclusively shown that the presence of *Plasmodium* within mosquitoes reduces fecundity through a combination of an impaired intake of yolk protein by the ovaries coupled by an

increase in the rate of apoptosis and egg resorption (Hurd et al. 2006). Although no molecule of *Plasmodium* origin has been identified that would justify talking about parasite manipulation, it has been widely assumed that reproductive curtailment is an adaptative strategy of the parasite to increase mosquito survival (Hurd 2009). Recent data has indeed confirmed that a reduction in fecundity in *Cx. pipiens* infected by *P. relictum* during their first GC is accompanied by a significant increase in longevity (Vézilier et al. 2012). While a reduction in fecundity has no consequences for the parasite, mosquito survival is crucial for *Plasmodium* transmission for two reasons. On the one hand because the parasite requires 8-14 days to complete its extrinsic incubation period within the mosquito. On the other hand, because mosquito longevity increases the potential for infective bites to new hosts.

Previous work carried out in our laboratory using the same *P. relictum* and *Cx. pipiens* strains used here (Vézilier et al. 2012), resulted in a much larger reduction in fecundity during the first gonotrophic cycle (infected females laid 40% fewer eggs than their uninfected counterparts) than the 6% reduction we obtain here. This unexpected result, led us to analyse the fecundity costs incurred by *Cx. pipiens* mosquitoes infected by this *P. relictum* SGS1 strain over time, using published and unpublished data collected in our laboratory since 2010 (**Figure 2**). This *a posteriori* analysis shows that, while the fecundity of uninfected mosquitoes has remained unchanged since 2010, the fecundity of *P. relictum*-infected mosquitoes has been reduced 6-fold, strongly suggesting that parasite has evolved towards a lower virulence. During these last 5 years, the parasite has gone through 103 standard (bird to bird) passages and 7 passages through mosquito (see Pigeault et al 2015 for details). The decrease in parasite virulence in the mosquito has been accompanied by a slight but statistically significant increase in parasitaemia in the birds (**Figure 3**, details of the statistical analyses can be found in Pigeault et al 2015). This year we have obtained a new *P. relictum* SGS1 isolate from the field (henceforth *P. relictum* SGS1, line B, to differentiate it from our historic line, renamed line A). Interestingly, fecundity costs to the mosquito and parasitaemias in the bird are similar to those of line A soon after its isolation from the field, further suggesting the ability of the parasite to evolve under laboratory conditions.

In conclusion, here we show that the negative impact of *P. relictum* on *Cx. pipiens* fecundity persists for, at least, three consecutive gonotrophic cycles. Hogg and Hurd (1995) obtained similar results with *An. stephensi* infected with the rodent malaria parasite *P. yoelii* but tempered their conclusions by suggesting that they may have been an artefact of an unnatural parasite-mosquito combination. Our results show that these effects are also

present in parasite-mosquito combinations found in nature, which raises the question of whether the sustained effect of the *Plasmodium* infection across gonotrophic cycles is a mechanistic by-product of taking an infected blood meal or whether it is adaptive for the parasite. Previous work has shown that *Plasmodium*-infected mosquitoes undergo a process of egg apoptosis and resorption during the first gonotrophic cycle (Hurd 2003, Hurd et al. 2006), and that the reduction in fecundity is associated to an increase in mosquito longevity (Vézilier et al 2012). Further work is needed that investigates whether these effects pervade through several gonotrophic cycles.

Author's contributions

MV and RP conceived and designed the experiment. MV performed the experiment. MV and RP analyzed the data. RP and AR wrote the paper.

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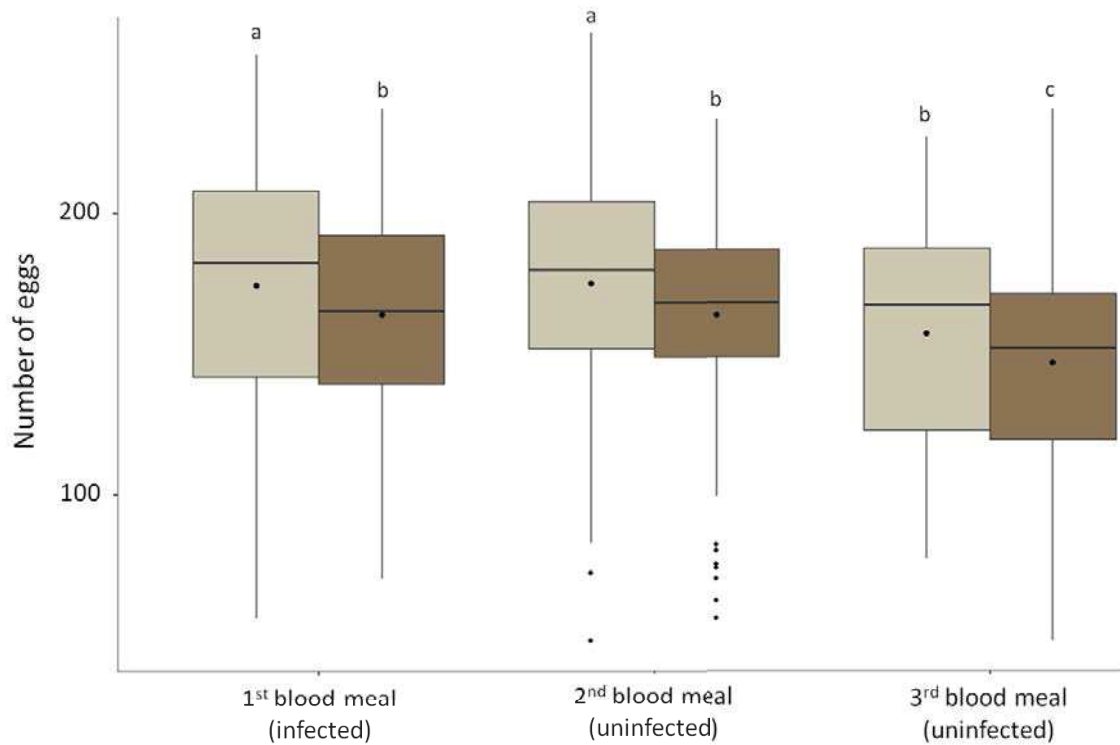


Figure1: Impact of *Plasmodium* infection on the number of eggs laid by mosquitoes during three gonotrophic cycles. Only the 1st blood meal was infected by *P. relictum*. Boxplots represent the means (points) and medians (horizontal lines). Boxes above and below the medians show the first and third quartiles respectively, lines delimit 1.5 times the inter-quartile range, above which individual counts are considered outliers and marked as full circles.

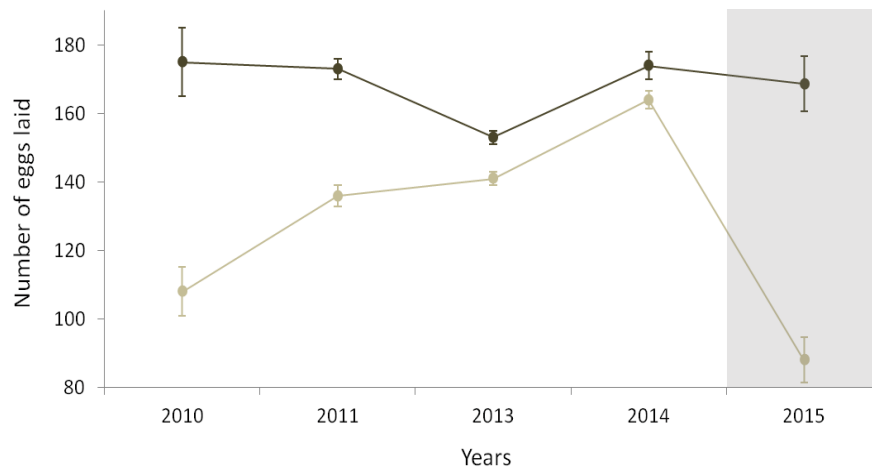


Figure 2: Progression of mosquito fecundity in uninfected (black line) and *Plasmodium*-infected (grey line) mosquitoes in the laboratory from 2010 to 2015. Points represent means by year ($\pm$ SE). The gray area corresponds to a new isolate of *P. relictum* SGS1 obtained from the wild in 2015.

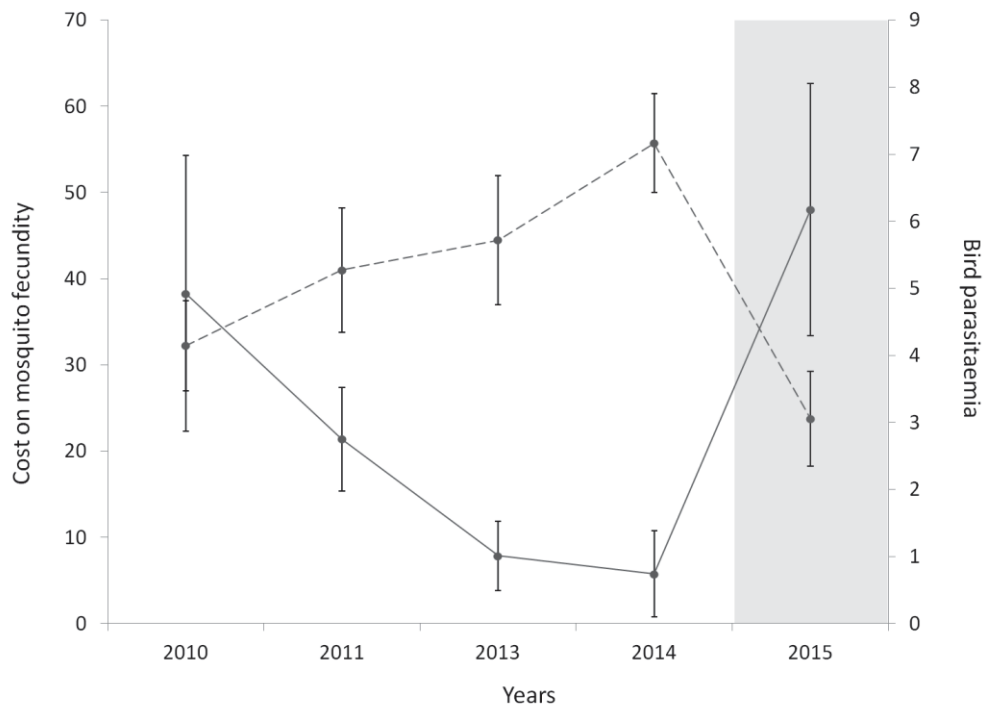


Figure 3: Progression of parasitaemia in the vertebrate host (dashed line) and of fecundity costs in the mosquito (full line) in a succession of experiments carried out between 2010 to 2015. Points and bars represent means by year ($\pm$ SE). The gray area corresponds to a new isolate of *P. relictum* SGS1 obtained from the wild in 2015.

Ecologie évolutive des interactions Hôte/Moustique/*Plasmodium* : Sources d'hétérogénéité de l'infection des vecteurs

Les moustiques jouent un rôle essentiel dans la dynamique de transmission du paludisme. Plusieurs traits d'histoire de vie de ces insectes hématophages vont, en effet, intervenir dans le calcul du taux reproductif de base du parasite. Parmi eux, la probabilité et l'intensité d'infection des vecteurs, suite à la prise d'un repas de sang infectieux, sont des facteurs primordiaux pour la transmission de *Plasmodium*. Pourtant, au sein d'une population de moustiques infectés une grande variabilité a été observée: alors que certains ne sont pas infectés, que d'autres le sont faiblement, une partie des vecteurs vont présenter des intensités d'infection extrêmement fortes. Identifier les sources de variations responsables de cette hétérogénéité est indispensable pour comprendre la dynamique de transmission de *Plasmodium*. Certains paramètres d'origine génétique et environnementale, tels que la température et la nutrition, ont déjà clairement été identifiés comme ayant un impact sur le taux d'infection des moustiques. Néanmoins, de nombreux facteurs restent encore à explorer. En utilisant un système expérimental composé du parasite de la malaria aviaire *Plasmodium relictum*, de son vecteur naturelle le moustique *Culex pipiens* et de l'un de ses hôtes vertébrés *Serinus canaria*, nous nous sommes intéressés aux effets de certains de ces paramètres, peu pris en compte jusqu'à présent, sur l'infection des moustiques. Nous avons notamment pu mettre en évidence des conséquences importantes de l'infection parentale, de l'âge des vecteurs, ainsi que de leur fond génétique. Parallèlement, nous nous sommes intéressés à un facteur souvent laissé de côté, l'effet « hôte vertébré » sur l'infection des moustiques. Dans cette dernière partie, on a pu observer que la dynamique d'infection du parasite au sein de l'hôte influence de manière importante le taux de transmission de *Plasmodium*. Les sources d'hétérogénéité d'infections des moustiques sont donc multiples et l'ensemble des membres de cette association tripartite hôte/parasite/vecteur vont pouvoir influencer de manière importante la dynamique de transmission du parasite.

Mots clés : malaria aviaire, moustique, hôte vertébré, âge, effet maternel, héritabilité, rythmicité, transmission

Evolutionary ecology of the host/mosquito/*Plasmodium* interaction: Sources of heterogeneity of vectors' infection

Mosquitoes play a key role in the dynamics of malaria transmission. Indeed, several life history traits of these bloodsucking insects are closely associated with the basic reproductive rate of the malaria parasite. One of the most important parameters for the transmission of *Plasmodium* is the rate and intensity of the mosquito infection. However, within a single population, a great heterogeneity of mosquito infection levels is often observed: some mosquitoes are not infected, others are only weakly infected, and yet others have extremely high infection intensities. Identifying the sources of variation responsible for this heterogeneity is essential in order to understand the transmission dynamics of *Plasmodium*. Certain genetic and environmental (temperature, nutrition) parameters have already been identified as having an impact on mosquito infection rates. Nevertheless, many factors remain to be explored. Using an experimental system composed of the avian malaria parasite *Plasmodium relictum*, its natural vector *Culex pipiens* and one of its vertebrate host *Serinus canaria*, we have investigated the effect of several seldom investigated parameters on the infection of mosquitoes. We observed surprising effects of the effect of parental infection, mosquito age and genetic background. In parallel, we also studied a rarely investigated parameter: the impact of the vertebrate host on the mosquito infections. We showed that parasite infection dynamics within the vertebrate host, at short but also long time scales, significantly influence the transmission of *Plasmodium* to the vector. The sources of heterogeneity of mosquito infection are therefore multiple and all members of this tripartite partnership (i.e. host / parasite / vector) are able to significantly influence the transmission dynamics of the parasite.

Key words: avian malaria, mosquito, vertebrate host, age, maternal effect, heritability, rhythm, transmission