UNIVERSITE DE BOURGOGNE

UFR SVTE, Laboratoire Biogéosciences, UMR-CNRS 6282

THESE
Pour obtenir le grade de
Docteur de l'Université de Bourgogne
Discipline : Sciences de la vie
Spécialité : Ecologie évolutive

Par

**Coraline Bichet**

Soutenance le 18 décembre 2012

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# Ecologie évolutive de la malaria aviaire :

# Effets des caractéristiques de l'hôte et de l'environnement

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***« Celui qui trouve sans chercher est celui qui a longtemps cherché sans trouver.»***
***Gaston Bachelard.***

# Résumé

L'étude des interactions hôtes-parasites est devenue un thème de recherche incontournable pour les sciences de l'évolution. Cette coévolution complexe dépend de nombreux compromis évolutifs et peut être grandement influencée par les facteurs environnementaux. Nous nous proposons ici d'étudier les interactions hôtes-parasites à plusieurs échelles, à travers des approches expérimentales et des études en populations naturelles, en étudiant les parasites de la malaria aviaire. Dans un premier temps, nous nous sommes intéressés à l'influence des caractéristiques de l'hôte et notamment au système immunitaire. Le système immunitaire est bénéfique pour l'hôte dans sa lutte contre le parasite, mais peut également engendrer des coûts immunopathologiques. Des traits d'histoire de vie, comme l'âge ou le statut social peuvent modifier la parasitémie au sein des hôtes, sans toutefois avoir d'effet sur la prévalence. Dans un second temps, l'effet de certains facteurs environnementaux a été évalué au sein des interactions hôtes-parasites. La température et la contamination en métaux lourds ont un effet sur la prévalence dans les populations, mais n'affectent pas la parasitémie. Au cours de cette thèse, nous avons également montré l'influence directe des parasites sanguins sur la structure génétique des populations hôtes, notamment au niveau des gènes du CMH.

**Mots-clés :** malaria aviaire, canari domestique, moineau domestique, complexe majeur d'histocompatibilité, système immunitaire, traits d'histoire de vie, environnement, choix de partenaire.

# Abstract

Host-parasite interactions are one of the main topics in evolutionary sciences. This complex coevolution depends on several trade-offs and can be influenced by environmental factors. Here, we propose to study host-parasite interactions with a multi-level approach, using experimental and natural population studies, focusing on avian malaria parasites. First, we studied the effect of host characteristics, and more precisely the immune system. The immune system confers benefits in terms of protection against the parasite, but can also generated immunopathological costs. Life history traits, like age or social status, appear to modify parasitemia but not prevalence. In a second part, we evaluated the effect of environmental factors on host-parasite interactions. We found that temperature and heavy metal contamination had an effect on population prevalence, but not on host parasitemia. We also showed the direct parasite influence on host population genetic structure, and more precisely on MHC genes.

**Keywords :** avian malaria, domestic canaries, house sparrow, major histocompatibility complex, immune system, life history traits, environment, mate choice.

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# Introduction générale

*Les parasites sont des composantes ubiquistes, quoiqu'habituellement invisibles, des communautés naturelles. L'écologiste ne l'a que trop ignoré.*

Robert Barbault, 1994.

## 1. Les interactions hôtes-parasites

Le parasitisme est le mode de vie le plus répandu sur Terre, avec plus de 50% des espèces considérées comme parasites (de Meeus, Renaud, 2002). Au cours de l'évolution est apparue, plusieurs fois et de façon indépendante, une remarquable diversité de formes de parasites, de cycles et de milieux de vie (Figure 1).

Le parasitisme a été longtemps étudié d'un point de vue purement médical et donc associé aux maladies humaines et aux épidémies. La recherche scientifique se cantonnait alors à étudier les parasites dans le but de les éliminer. Les agronomes et les vétérinaires se sont également penchés sur l'étude des parasites chez les animaux domestiques, de part leurs impacts économiques et alimentaires certains.

Depuis le début des années 80, la parasitologie est devenu un thème incontournable en écologie évolutive (Anderson, May, 1982; Combes, 2001b; Poulin *et al.*, 2000). De part la position centrale des parasites au sein des écosystèmes, les scientifiques s'intéressent aujourd'hui à comprendre leurs rôles et leurs impacts sur les communautés naturelles, par exemple, leur effet sur la reproduction sexuée ou sur la structure génétique des hôtes (Dobson *et al.*, 2008; Hudson *et al.*, 2006). Aujourd'hui, le parasitisme est étudié sous bien des aspects (médecine, écologie évolutive, écologie comportementale, immunologie). Cette multi-disciplinarité permet d'obtenir une vision et compréhension globale du parasitisme, avec notamment pour but de prédire l'émergence et ré-émergence de maladies (Grenfell, Dobson, 1995; Harvell, 2004).

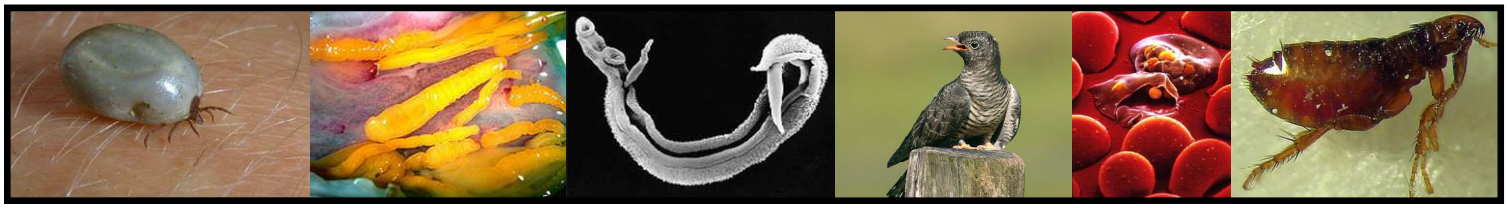


*Figure 1 : Ilustration de l'extraordinaire diversité des formes parasitaires. De gauche à droite : tique, acanthocéphales, schistosomes, coucou gris, Plasmodium et puce.*

Les interactions hôtes-parasites sont qualifiées de durables. Les hôtes et parasites sont associés de manière pérenne et confrontés régulièrement l'un à l'autre (Combes, 1995). D'un point de vue évolutif, l'intérêt du parasite est d'infecter l'hôte, de s'y multiplier et de se transmettre à d'autres hôtes. Inversement, l'intérêt de l'hôte est de réussir à éviter le parasite ou bien de l'éliminer si la rencontre a lieu. Hôtes et parasites sont donc soumis à des pressions de sélection antagonistes et réciproques. C'est ce que l'on appelle la course aux armements (Figure 2).

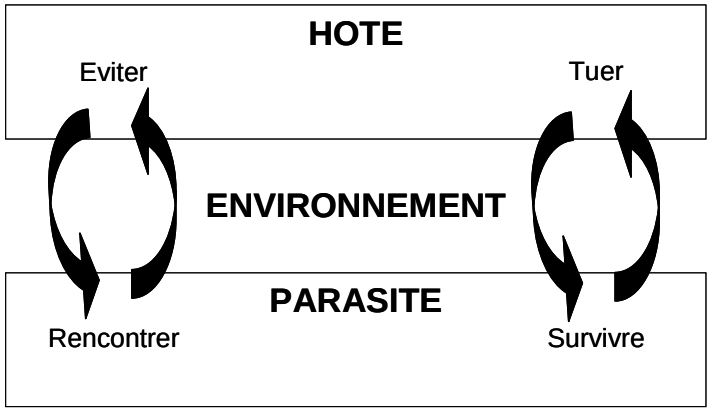



*Figure 2 : Illustration de la course aux armements dans un système hôte-parasite. L'environnement constitue un filtre pouvant modifier la dynamique de cette co-évolution. (D'après Combes, 2001a).*

Ainsi, le système immunitaire apparaît comme un des mécanismes les plus complexes et performants qui existent pour lutter contre les pathogènes. L'étude de l'évolution du système immunitaire a conduit à l'émergence d'une nouvelle discipline, l'immuno-écologie. Cette discipline s'attache à expliquer l'importante variabilité inter-spécifique et inter-populationnelle qui existe autour de la capacité de résistance aux pathogènes ; alors que la sélection naturelle tendrait à maximiser cette résistance. La variabilité génétique liée au système immunitaire est très étudiée, et, avec les techniques actuelles de biologie moléculaire, il est aujourd'hui facile d'accéder à cette variabilité dans les populations naturelles.

Les interactions hôtes-parasites sont également soumises à de nombeux compromis, ou trade-offs. Au niveau du parasite, tuer son hôte trop tôt pourrait empêcher la transmission à d'autres hôtes. Le parasite est donc soumis à un compromis entre transmission et virulence. Au niveau de l'hôte, de nombreux trade-off existent également. L'énergie allouée à se défendre contre le parasite n'est alors plus disponible pour d'autres fonctions, telles que la

reproduction, la croissance, la défence du territoire ou encore la prospection alimentaire. Identifier ces compromis est indispensable pour comprendre les mécanismes mis en place lors des interactions hôtes-parasites. Un certain nombre de ces compromis ont été identifiés au cours de cette thèse.

L'environnement a également un rôle clé dans les interactions hôtes-parasites et explique lui aussi une part de la variabilité observée. Le challenge de l'immuno-écologie est donc aujourd'hui d'appréhender les interactions hôtes-parasites dans leur environnement (Figure 3). Cet objectif est également celui de ma thèse, avec l'étude d'un cas particulier de pathogènes : les parasites de la malaria aviaire.

## 2. La malaria aviaire

La malaria est une maladie infectieuse parasitaire provoquée par des parasites haemosporidiens, transmis à des hôtes vertébrés via des insectes piqueurs servant de vecteurs (plus d'information dans la partie modèles biologiques et méthodologies).

La malaria humaine est considérée comme une pandémie, qui a causé, en 2010, environ 700 000 morts (World Health Organization, 2011). De part son très fort impact sur les populations humaines, la malaria est considérablement étudiée (Doolan *et al.*, 2009; Langhorne *et al.*, 2008). L'expérimentation chez les humains restant très limitée, les chercheurs ont très tôt utilisé une alternative en étudiant cette maladie chez d'autres modèles animaux (Artavanis-Tsakonas *et al.*, 2003). Les oiseaux ont été parmi les premiers à être utilisés, conduisant à la découverte des premiers traitements (Ball, Chao, 1961; Coatney *et al.*, 1953; Davey, 1951; McGhee *et al.*, 1977). Toutefois, après la découverte de parasites de malaria chez les rongeurs (Killickk.R, 1974) et du transfert de la malaria humaine chez le singe (Young *et al.*, 1966), le modèle aviaire a rencontré nettement moins de succès.

Depuis ces deux dernières décennies, la malaria aviaire rencontre un regain d'intérêt, principalement en écologie évolutive. Ces parasites sont ubiquistes et présents dans toutes les régions tropicales et tempérées du globe (Fallon *et al.*, 2005; Valkiūnas, 2005), ce qui en fait d'excellents candidats pour l'étude de l'évolution des interactions hôtes-parasites. De plus, certains parasites de la malaria aviaire sont particulièrement intéressants à étudier car ils sont généralistes et donc capables d'infecter plusieurs espèces d'oiseaux. Par exemple, lors de mon étude, certaines souches identifiées dans les populations de moineaux domestiques avaient préalablement été décrites chez des limicoles et des turdidés. La prévalence de ces parasites

varie beaucoup selon les régions du globe, mais également à des échelles géographiques beaucoup plus fines (Hellgren *et al.*, 2007; Marzal *et al.*, 2008). Ainsi, à l'échelle de la France, pour les populations de moineaux domestiques étudiées durant ma thèse, la prévalence de l'espèce *Plasmodium relictum* varie de 0 à 79% (**Manuscrit 4**), et de 0 à 24% en Bretagne (**Manuscrit 7**).

En constatant que ces parasites étaient présents dans la plupart des populations et des espèces d'oiseaux, les chercheurs se sont interrogés sur leur réelle pathogénicité. Les nombreux suivis épidémiologiques réalisés n'ont pas toujours pu mettre en évidence un effet létal fort, certainement à cause du long passé coévolutif des parasites avec leurs hôtes (Fallis, 1977). Toutefois, des expériences d'infections expérimentales ont montré que ces parasites pouvaient être couteux, diminuer la fitness de leurs hôtes (Cellier-Holzem *et al.*, 2010; Knowles *et al.*, 2010; Palinauskas *et al.*, 2008; Zehtindjiev *et al.*, 2008) et augmenter leur mortalité (Williams, 2005). De plus, ces parasites se sont révélés particulièrement virulents pour des populations hôtes naïves. L'exemple classique de l'impact des parasites de la malaria aviaire en population naturelle est celui de l'introduction de *Plasmodium relictum* et de son vecteur *Culex quiquefasciatus* dans l'archipel d'Hawaii. Ce parasite est à l'origine d'un déclin dramatique, voire de l'extinction locale de nombreuses populations qui entraient pour la première fois en contact avec ce parasite (Atkinson *et al.*, 1995; Van Riper, 1986). Le fort pouvoir sélectif des parasites de la malaria est illustré par le fait que quelques années seulement après l'introduction, les populations aviaires locales semblent déjà capables de mieux tolérer le parasite (Woodworth *et al.*, 2005).

## 3. Objectifs de la thèse

Cette thèse a pour but d'appréhender l'écologie évolutive de la malaria aviaire d'une manière plus globale que celle majoritairement usitée jusqu'ici. Le but est d'englober à la fois les interactions hôtes-parasites, mais également les interactions de ces derniers avec leur environnement (Figure 3). Pour ce faire, nous avons choisi d'utiliser à la fois une approche expérimentale (hôte canari) et une approche en populations naturelles (hôte moineau).

Dans un premier temps nous nous sommes demandé comment certaines caractéristiques de l'hôte, son système immunitaire, ses traits d'histoire de vie (âge, sexe, statut social, statut nutritif), pouvaient influencer l'évolution de la virulence des parasites. ➔ **CHAPITRE 1**

Ensuite, nous nous sommes intéressés aux caractéristiques de l'environnement, telles que le climat, les facteurs anthropiques, les caractéristiques géographiques, et de quelle manière ces facteurs pouvaient intervenir dans l'interaction moineaux-malaria. ➔ **CHAPITRE 2**

Dans un dernier chapitre, nous nous sommes intéressés au choix du partenaire dans un contexte géographique particulier, l'insularité. Nous nous sommes demandé si le choix de partenaire pouvait illustrer des stratégies de résistance aux parasites présents dans la population. ➔ **CHAPITRE 3**

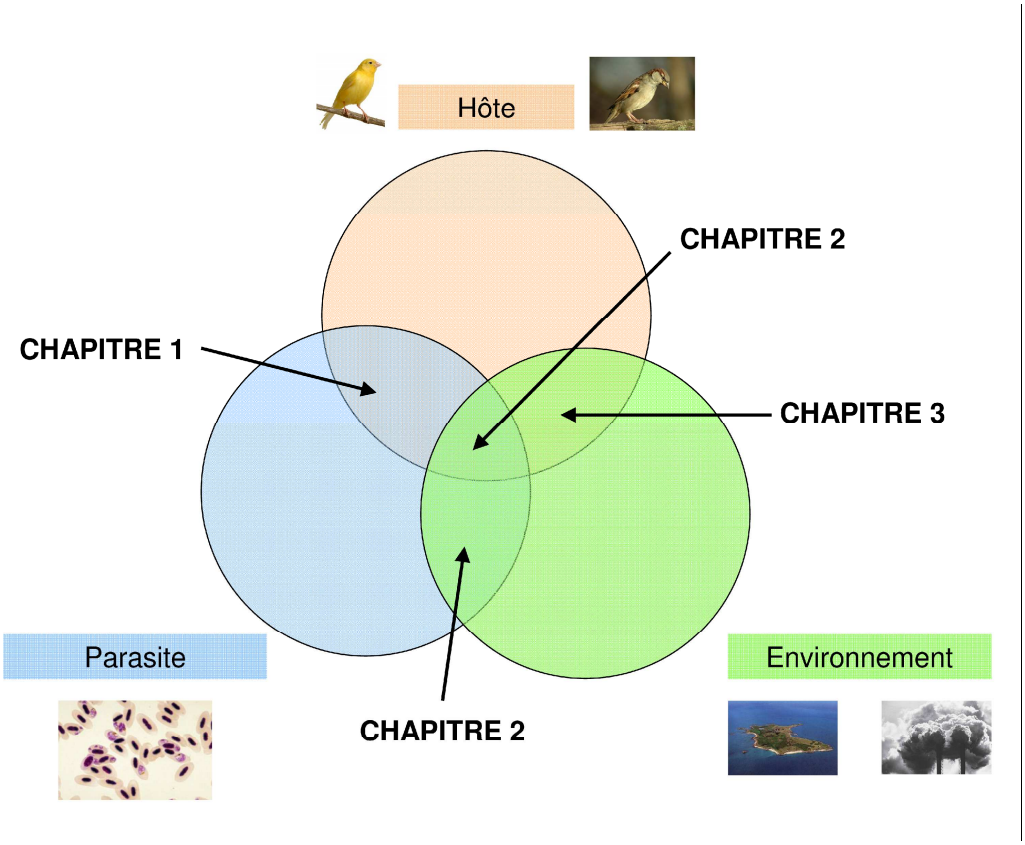



*Figure 3 : Les interactions hôtes-parasites-environnement. C'est dans ce réseau d'interactions que se placent les différents chapitres de cette thèse.*

# Modèles biologiques et méthodologies

# 1. Modèles biologiques

## 1.1 Le modèle aviaire

### 1.1.1 Le canari domestique (*Serinus canaria*)

Le canari domestique a été utilisé lors de nos études en laboratoire consistant en des infections expérimentales de *Plasmodium*. Le canari domestique a déjà été utilisé à plusieurs reprises par d'autres membres de mon équipe, comme modèle biologique, ce qui en fait un organisme de référence pour nos travaux (Cellier-Holzem *et al.*, 2010, **Manuscrits 2 et 3**). C'est un passereau originaire des îles Canaries (de son vrai nom serin des canaries), cependant, les individus utilisés dans nos expériences étaient tous issus d'élevage. Ces oiseaux ont l'avantage de pouvoir être facilement élevés en volière ou en cage individuelle (Figure 4). De plus, le canari est actuellement, avec le poulet, l'hôte expérimental le plus utilisé pour les études de laboratoire sur la malaria. Ils sont également capables, naturellement ou artificiellement, d'être infectés par *Plasmodium* et autres parasites haemosporidiens (Corradeti *et al.*, 1970; Koch, 1899; Sergent, Sergent, 1952; Spencer *et al.*, 2005).

*Figure 4 : Exemple d'expériences en cages individuelles avec des canaris, dans notre laboratoire de Bretenière (Côte-d'Or, France).*

### 1.1.2 Le moineau domestique (*Passer domesticus*)

Le moineau domestique (Figure 5) a été choisi comme modèle pour les études en populations naturelles. C'est, là encore, un organisme qui a précédemment été étudié par des anciens membres de l'équipe dans laquelle s'est déroulée ma thèse (Bonneaud *et al.*, 2006a; Bonneaud *et al.*, 2004a; Bonneaud *et al.*, 2006b; Loiseau *et al.*, 2009; Loiseau *et al.*, 2008).

Le moineau domestique a une alimentation opportuniste, bien que les jeunes soient presque exclusivement nourris avec des insectes. Il est sédentaire et grégaire, surtout en automne et en hiver. En effet, la distance moyenne de dispersion de cette espèce est de 1.7 km en phase juvénile et de 1.9 km à l'âge adulte (Paradis *et al.*, 1998). Le moineau domestique présente un dimorphisme sexuel (Figure 5) et effectue une à trois pontes par an, pouvant contenir de 1 à 6 œufs. Il peut vivre en moyenne 4 ans, mais sa survie annuelle est relativement faible (80% de mortalité la première année, 43% pour les années suivantes) (Summer-Smith, 1988).

C'est un passereau particulièrement intéressant à étudier car il est mondialement présent et est également commensal de l'Homme. Originaire d'Eurasie et d'Afrique du Nord, il a été introduit en Afrique du Sud, en Amérique, en Australie et en Nouvelle-Zélande. Le moineau domestique est classé LC ("Least Concern") sur la liste rouge de l'IUCN (International Union for Conservation of Nature and Natural Ressources) et est donc considéré comme abondant au niveau international. Cependant, il ne possède pas le même statut dans toutes les régions du globe. Il est considéré comme invasif et nuisible en Amérique, dans le Golfe Persique, ainsi qu'en Australie. A l'inverse, certaines populations européennes semblent être en déclin. Il y a une dizaine d'années, une étude en Angleterre a montré que la population était passée de 12 à 15 millions de couples nicheurs dans les années 70, à 6 millions aujourd'hui (Crick *et al.*, 2002).

*Figure 5 : Moineaux domestiques femelle (à gauche) et mâle (à droite)*

## 1.2 Les parasites sanguins

Les parasites sanguins aviaires (haemosporidiens) se divisent en trois genres : *Plasmodium*, *Haemoproteus* et *Leucocytozoon*. Chaque genre est transmis par des insectes diptères spécifiques (Valkiūnas, 2005). *Haemoproteus* est principalement transmis par des moucherons piqueurs du genre *Culicoides* (Valkiunas, Iezhova, 2004) ; tandis que les mouches du genre *Simuliidae* transmettent plutôt *Leucocytozoon* (Forrester, Greiner, 2009).

Le genre *Plasmodium*, lui, est le seul agent de la malaria aviaire à proprement parler. Il est transmis, entre autres, par les moustiques des genres *Culicidae, Culex* et *Culiseta* (Valkiūnas, 2005). Son cycle de transmission et de reproduction est très proche de celui de la malaria humaine (Figure 6). Dans les régions tempérées, les infections ont surtout lieu au printemps et en été, quand les vecteurs sont présents. Les premières infections sont suivies d'une phase aigüe, où le parasite se multiplie dans les globules rouges de l'oiseau, qui éclatent ensuite pour libérer les parasites, ce qui provoque des anémies. Les parasites peuvent ensuite être éliminés par l'hôte ou perdurer dans l'organisme, sous forme d'infections chroniques (Valkiūnas, 2005).

Pour la majorité des études menées durant ma thèse, nous nous sommes focalisés sur le parasite *Plasmodium relictum* (**Manuscrits 1 à 6**). C'est un des parasites les plus courants dans les populations d'oiseaux et son aire de distribution comprend toutes les régions zoo-géographiques du globe, sauf l'Antarctique (Valkiūnas, 2005). Plus particulièrement, lors de nos études en populations naturelles, nous nous sommes focalisés sur deux souches de *Plasmodium relictum*, SGS1 et GRW11 (**Manuscrits 4 à 6**). Ce sont les deux souches identifiées comme étant les plus abondantes dans les populations françaises de moineaux domestiques (Loiseau *et al.*, 2011). Lors des infections expérimentales nous avons sélectionné une souche unique, SGS1, afin d'éviter la compétition intra-hôte (**Manuscrits 1 à 3**). Cette souche à été décrite chez de nombreuses espèces européennes et africaines d'oiseaux (Beadell *et al.*, 2006; Hellgren *et al.*, 2007; Palinauskas *et al.*, 2007; Waldenstrom *et al.*, 2002) et est, en particulier, très présente chez le moineau domestique.

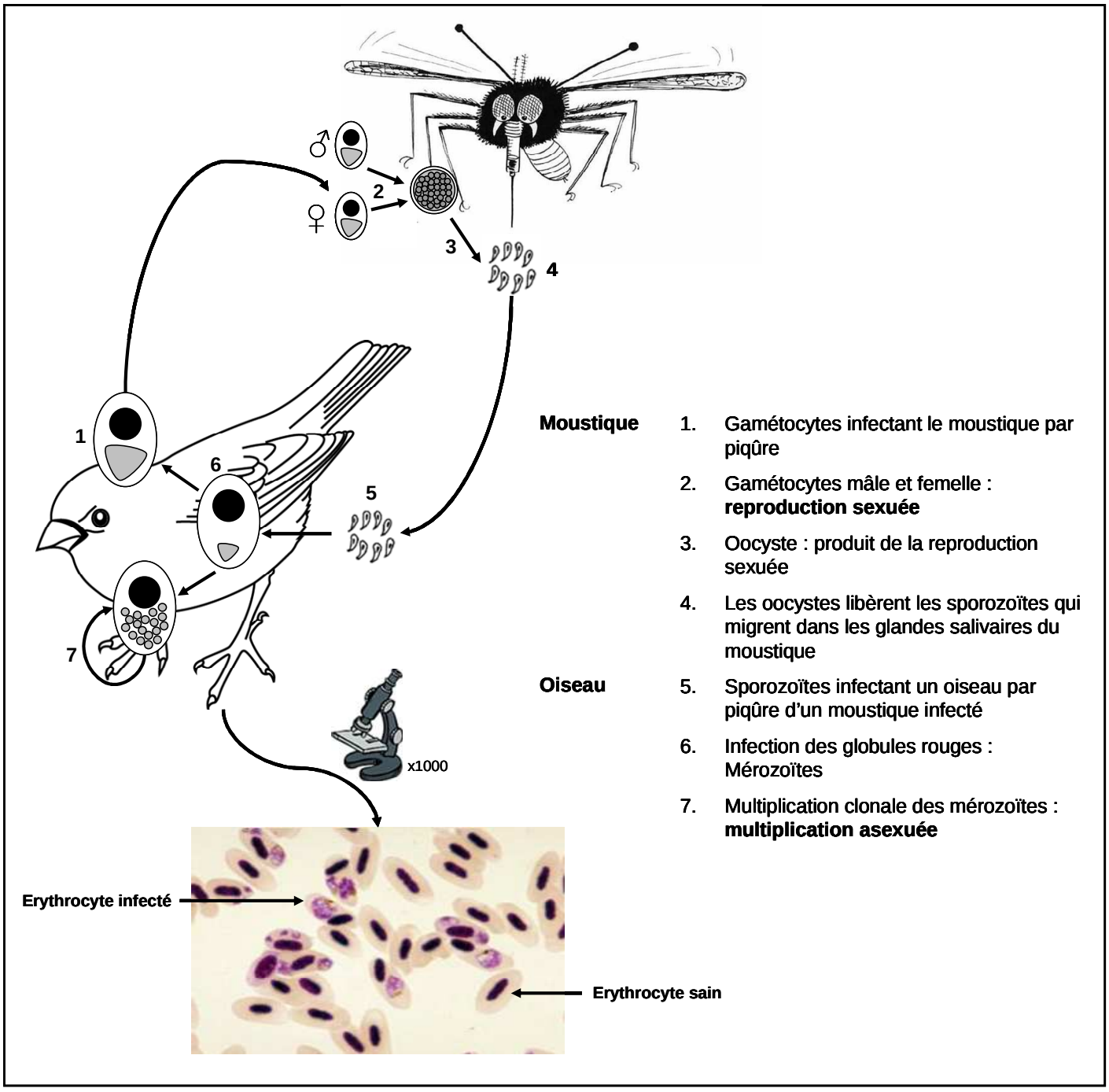



*Figure 6 : Cycle simplifié de la malaria aviaire. L'accent a été mis sur la phase sanguine de l'infection chez l'oiseau. L'infection est alors visible par microscopie au niveau des globules rouges de l'oiseau, après un frottis sanguin et une coloration méthanol-Giemsa. Il est à noter que le parasite peut également infecter le foie, la rate ou même le cerveau.*

# 2. Méthodologies

## 2.1 Détermination du statut parasitaire

### 2.1.1 Mesure qualitative (présence/absence)

Le statut parasitaire des individus a été déterminé par méthode moléculaire à partir des échantillons de sang. La méthode utilisée lors de mes études, une Polymerase Chain Reaction (PCR) nichée, a été décrite par (Waldenstrom *et al.*, 2004) et consiste en deux PCR successives, utilisant deux jeux d'amorces différents (Figure 7). Cette PCR amplifie un fragment du gène du cytochrome b des parasites des genres *Plasmodium* et *Haemoproteus*. Une PCR similaire avec un autre jeu d'amorces permet également de détecter les infections pour les parasites du genre *Leucocytozoon* (Hellgren *et al.*, 2004).

La PCR nichée permet de détecter la présence d'un érythrocyte infecté sur 100 000 ; ce qui correspond à une parasitémie de $10^{-5}$ (Waldenstrom *et al.*, 2004). Toutefois, cette méthode sous-estime toujours la prévalence dans les populations naturelles (Jarvi *et al.*, 2002). En effet, les méthodes moléculaires ne permettent de détecter que les parasites aux stades sanguins gamétocytes et mérozoïtes. Les infections en dormance peuvent alors ne pas être détectées.

Nous avons utilisé cette méthode de PCR nichée pour détecter le statut infectieux des oiseaux en populations naturelles, et aussi lors de nos infections expérimentales. Les produits des PCR sont ensuite directement utilisés pour déterminer la souche du parasite par séquençage du fragment amplifié.

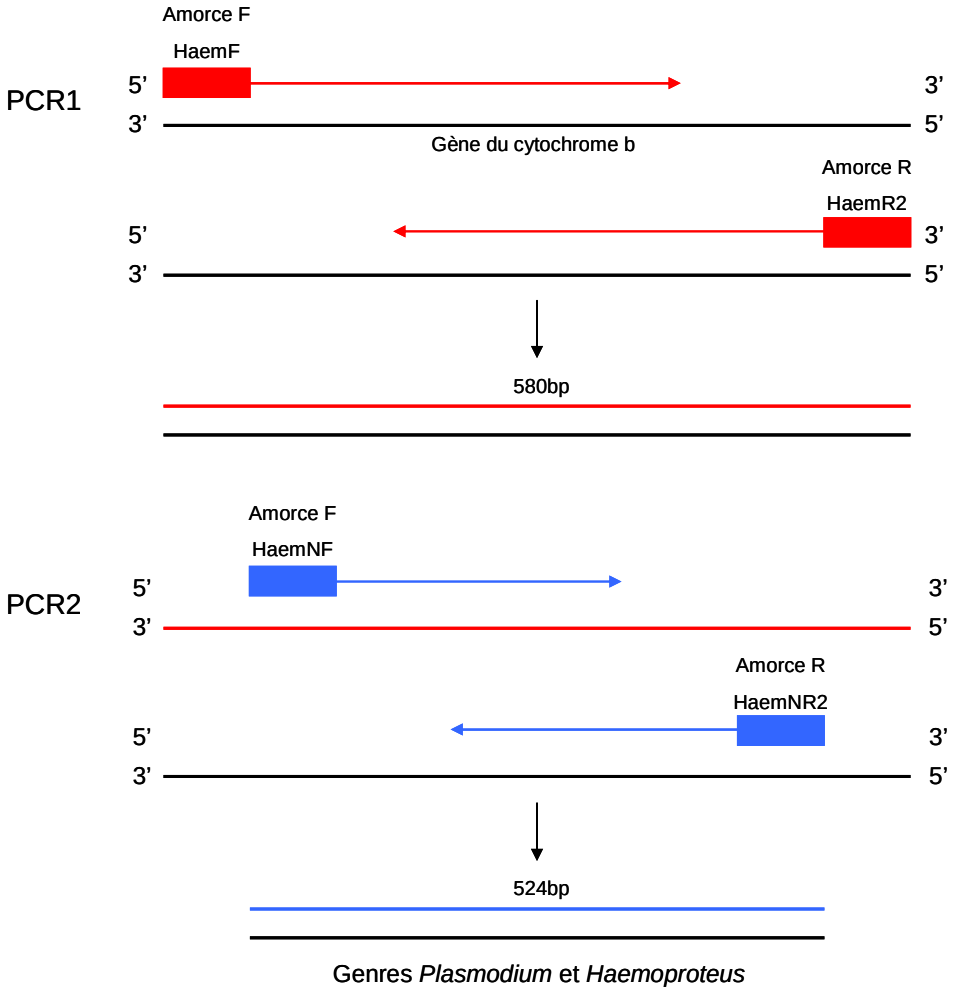



*Figure 7 : Principe de la PCR nichée. Deux PCR successives amplifient le gène du cytochrome b des parasites des genres Plasmodium et Haemoproteus. La deuxième PCR se réalise sur les produits d'amplification de la première.*

### 2.1.2 Mesure quantitative

La PCR quantitative (qPCR) permet de mesurer la quantité de parasites présents dans le sang des oiseaux parasités, appelée intensité parasitaire ou parasitémie. Nous avons utilisé le protocole décrit dans Cellier-Holzem *et al.* (2010). La qPCR est basée sur la détection en temps réel d'une fluorescence. Cette fluorescence augmente avec le nombre de cycles d'amplification de l'ADN cible. Cette quantification de l'ADN du parasite doit être mise en relation avec la quantification en parallèle de l'ADN de l'hôte. En effet, lors des extractions, une quantité variable d'ADN peut être extraite. Pour chaque individu, deux PCRs ont donc été réalisées lors d'un même run, une pour l'oiseau et une pour le parasite. Le principe de la

qPCR est résumé dans la Figure 8. Une sonde simple brin spécifique de l'ADN cible est hybridée. Aux extrémités de cette sonde un reporter (un fluorochrome) est fixé, ainsi qu'un quencher (ou extincteur). La présence du quencher à proximité du reporter inhibe sa fluorescence. Lors de l'amplification, la sonde ADN est détruite par la Taq polymerase. Quencher et reporter se retrouvent éloignés et la fluorescence peut alors être émise.

Plus la quantité de parasites dans l'hôte est élevée, plus la fluorescence sera détectée tôt dans les cycles de PCR. Ce nombre de cycle minimal de détection est appelé Ct. Pour chaque individu, il y a donc un Ct oiseau et un Ct parasite. La parasitémie est ensuite mesurée par l'indice RQ qui se calcule comme suit :

$$\mathbf{RQ = 2^{-(Ct\ parasite - Ct\ oiseau)}}$$

Plus le RQ est élevé, plus la quantité d'ADN du parasite est importante, par rapport à la quantité d'ADN de l'oiseau.

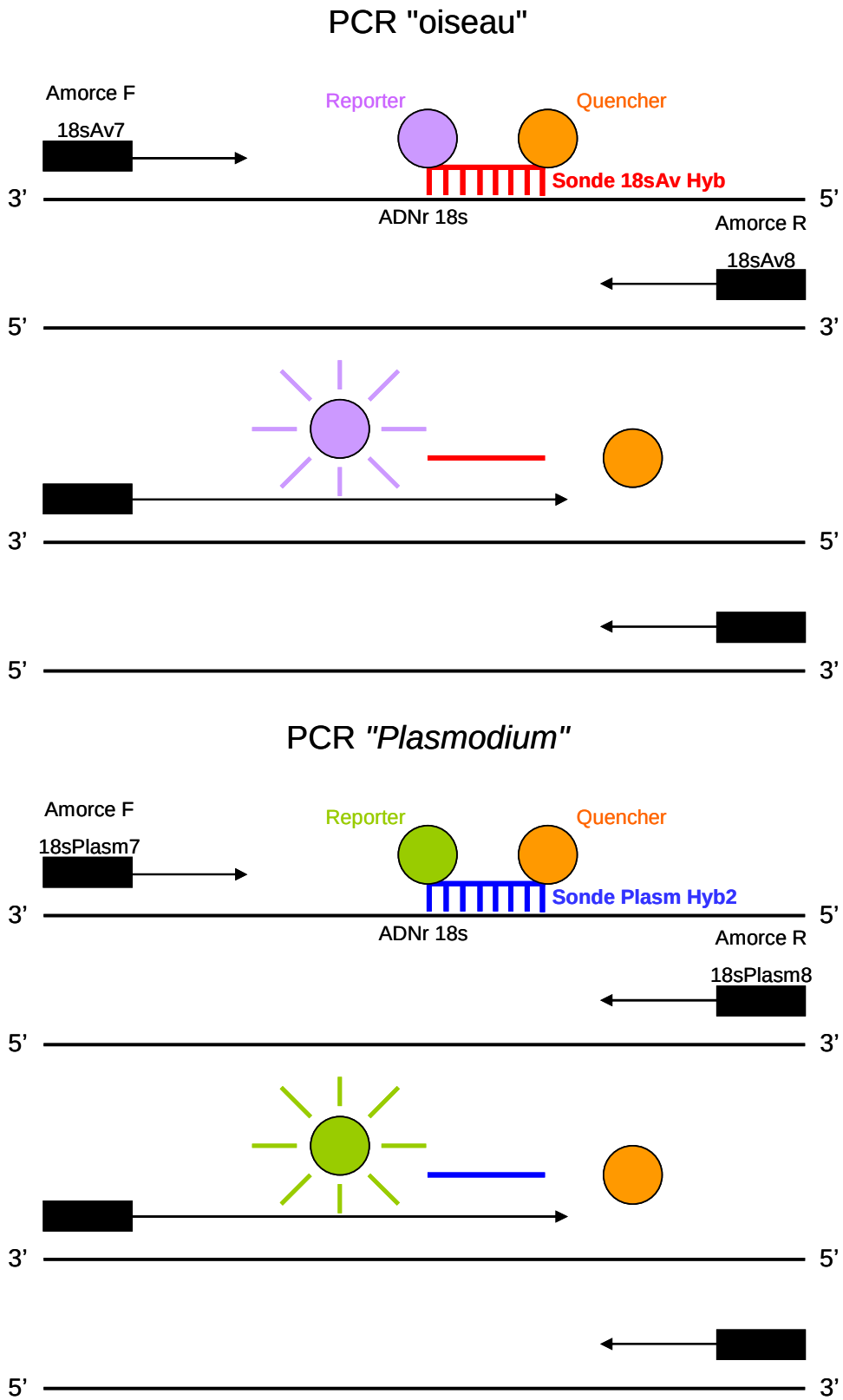



*Figure 8 : Principe de la PCR quantitative. Une sonde spécifique, marquée de deux fluorochromes, se fixe sur l'ADN cible. Lors de l'amplification, cette sonde est dégradée, ce qui permet au reporter et au quencher d'être séparés. De la fluorescence, proportionnelle à la quantité d'ADN présente, est alors émise.*

## 2.2 Les infections expérimentales

Durant ma thèse, les infections expérimentales ont été effectuées avec le modèle canari. Les parasites utilisés ont été extraits d'une population naturelle de moineaux domestiques de Dijon. Ces moineaux, capturés à l'aide de filets japonais, ont été maintenus en volières, le temps de déterminer leur statut parasitaire. Une prise de sang à donc été réalisée au niveau de la veine brachiale, et préservée dans un tampon de Lyse (Seutin *et al.*, 1991). A partir de ce sang, l'ADN a été extrait par la méthode Phénol-Chloroforme (Hillis *et al.*, 1996). La présence du parasite a été détectée par une PCR nichée (Waldenstrom *et al.*, 2004). Si l'individu était parasité, la souche a été déterminée en séquençant le fragment du cytochrome b amplifié. Les séquences ont ensuite été alignées avec le logiciel MEGA4 (Tamura *et al.*, 2007), et comparées aux séquences disponibles dans GenBank et dans la base de données publique MalAvi (Bensch *et al.*, 2009). Sur les moineaux parasités par la souche focale, SGS1, 200µl de sang ont été prélevés. Les autres individus ont été relâchés dans leur population d'origine. Le nombre de parasites par hôte est souvent trop faible pour pouvoir réaliser des infections directement à partir de sang de moineau. Ce sang est donc préalablement transféré en intrapéritonéal chez des canaris, afin que le parasite se multiplie.

A 10 jours post-infection, le sang récolté chez les canaris infectés a été cryopréservé à -80°C, selon le protocole décrit par Diggs *et al.* (1975), qui consiste à ajouter une solution de cryopréservation contenant du glycérol, du sodium lactate, du chlorure de potassium et du PBS. Avant le transfert, les parasites ont été décongelés et réactivés en réchauffant le sang à 37°C et en ajoutant des solutions de chlorure de sodium à 12% et à 1.6%.

Pour les infections expérimentales, nous standardisons la dose de parasites injectée à chaque hôte. Pour cela, il est nécessaire d'évaluer la parasitémie des oiseaux donneurs. Des frottis sanguins des donneurs ont été réalisés et colorés avec des solutions de méthanol et de Giemsa. Les frottis ont ensuite été observés en microscopie à immersion. Comme la détermination de la parasitémie par qPCR demande un certain temps, nous l'avons quantifiée ici en dénombrant les érythrocytes infectés (Figure 6), sur un total de 10.000 érythrocytes, et en tenant compte de l'hématocrite de chaque individu. Le nombre de parasites, ainsi que le volume injecté ont été homogénéisés pour chaque canari receveur.

## 2.3 Mesurer la virulence

La définition de la virulence ne rencontre pas de consensus au sein de la communauté scientifique. Elle était traditionnellement définie comme l'augmentation de mortalité de l'hôte suite à l'infection (Anderson, May, 1982). Du point de vue de l'hôte, la virulence peut-être définie comme les dommages que le parasite peut causer à l'hôte, résultant en une diminution de sa *fitness*. Du point de vue du parasite, la virulence est plutôt représentée par sa capacité à se transmettre, à se multiplier, et à exploiter son hôte (Bull, 1994; Poulin, 1999).
Nous avons choisi de mesurer la virulence du point de vue de l'hôte en suivant la mortalité, le nombre de parasites présents dans l'hôte, la parasitémie, ainsi que deux mesures considérées comme proxy du coût de l'infection et de la virulence : les variations de poids et les variations d'hématocrite (Mackinnon, Read, 2003). Lors des infections expérimentales, nous avons mesuré la masse de chaque oiseau et l'hématocrite à intervalles réguliers durant l'infection, afin de suivre son évolution.

### 2.3.1 Les variations de poids

De nombreuses études ont montré que les pathogènes diminuent la masse de leurs hôtes durant l'infection. Toutefois, concernant la malaria aviaire, ce patron n'a pas toujours été observé. En effet, dans des conditions expérimentales souvent favorables à l'hôte (nourriture et eau *ad libitum*, température constante), les diminutions de poids sont souvent absentes (Palinauskas *et al.*, 2008; Zehtindjiev *et al.*, 2008). Plus généralement, il semble que la perte de poids ne soit pas incluse dans les coûts physiologiques de la malaria pour des oiseaux ayant une histoire de coévolution avec le parasite assez longue. A l'inverse, pour des oiseaux naïfs, une perte de poids consécutive aux infections a été observée (Atkinson *et al.*, 2001; Atkinson *et al.*, 2000).

### 2.3.2 Les variations d'hématocrite

L'hématocrite d'un individu correspond à la proportion de cellules sanguines (les érythrocytes) par rapport au volume total (cellules sanguines + plasma). Plus un individu possède de cellules sanguines, plus son hématocrite est élevé. Les valeurs d'hématocrite ont été mesurées par centrifugation d'un capillaire hépariné contenant du sang. Nous avons

ensuite mesuré la hauteur de cellules sanguines par rapport à la hauteur totale du sang dans le capillaire.

Dans notre système, une réduction d'hématocrite est, en partie, la conséquence directe de la multiplication asexuée des parasites dans les érythrocytes de l'hôte. Cette multiplication est suivie de la lyse de ces globules rouges et du relargage des mérozoïtes dans le sang. Ainsi, plus la multiplication parasitaire sera importante, plus l'anémie sera élevée (et donc plus l'hématocrite sera faible). Mais, en plus de l'action directe du parasite, une réduction de l'hématocrite peut également être une conséquence de l'action du système immunitaire, qui détruit les globules rouges infectés. Une étude menée sur la malaria chez les rongeurs à montré que 10% de l'anémie provoquée par le parasite pouvait être imputée à la réponse immunitaire (Graham *et al.*, 2005b).

Une diminution de l'hématocrite peut avoir de lourdes conséquences chez les oiseaux. Cela diminue le transport de l'oxygène, ce qui peut, par exemple, impacter les performances de vols des individus.

## 2.4 Etudes en populations naturelles

### 2.4.1 Capture et baguage

Pour nos études en populations naturelles de moineau domestique, nous avons eu besoin de capturer un certain nombre d'individus adultes (Figure 9). Ces individus ont été capturés avec des filets japonais. Pour chaque nouvel individu capturé, une bague métal, fournie par le CRBPO (Centre de Recherches par le Baguage des Populations d'Oiseaux), comportant un numéro d'individu unique a été posée. Une prise de sang (20 µl) a également été réalisée au niveau de la veine brachiale. Ce sang a été conservé dans un tampon de lyse (QLB, Seutin *et al.*, 1991). Des mesures morphologiques ont été effectuées pour chaque oiseau : poids, longueur de l'aile pliée, longueur du tarse, et longueur entre l'extrémité du bec et l'arrière de la tête (Figure 9).

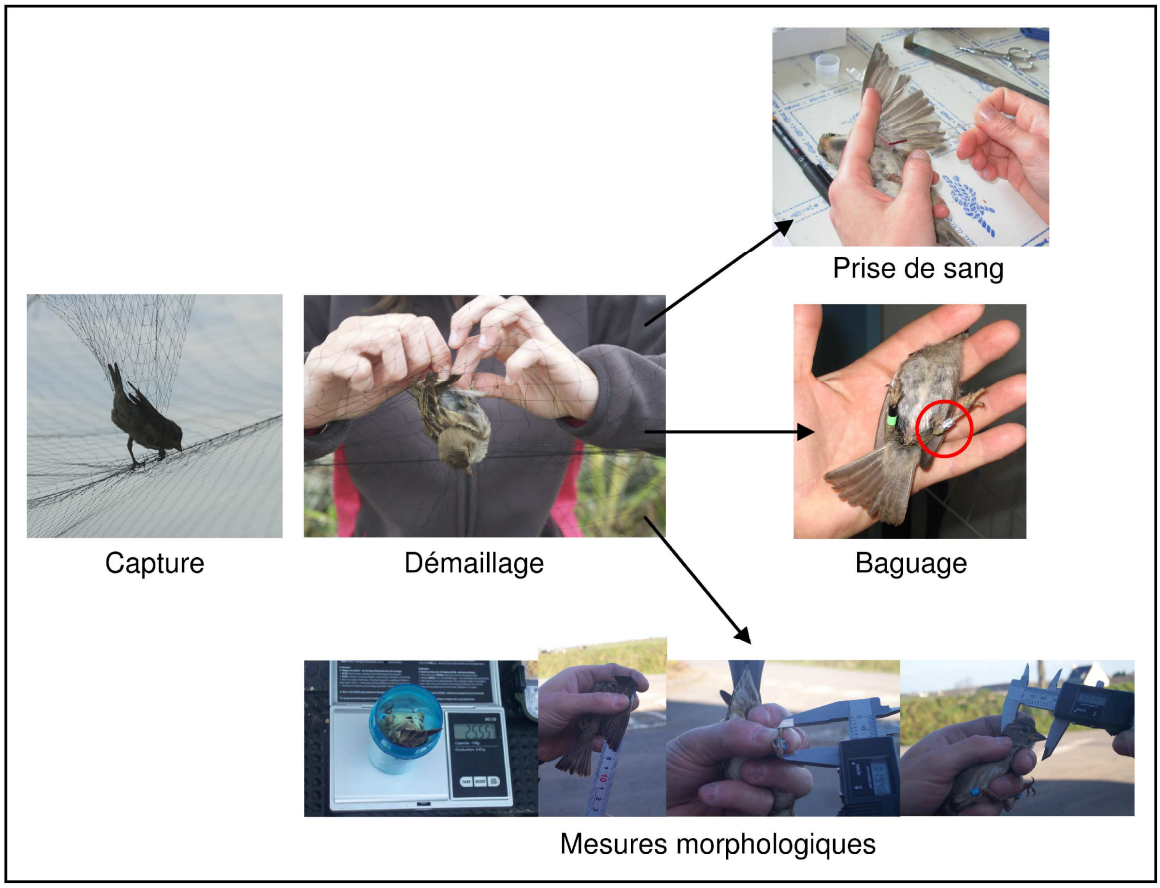



*Figure 9 : Capture, baguage, prise de sang et mesures morphologiques dans des populations naturelles de moineau domestique.*

### 2.4.2 Suivi de reproduction

Au cours de ma thèse, nous avons également suivi la reproduction du moineau domestique dans une population insulaire située sur l'île d'Hoëdic. Pour cela, une soixantaine de nichoirs artificiels a été placée dans le village dans le but de suivre l'avancement de la reproduction. Le nombre d'œufs, le nombre de poussins éclos et le nombre de poussins à l'envol a été déterminé pour chaque nichée, nous permettant de calculer des mesures phénologiques telles que le taux d'éclosion et le taux d'envol. Les dates de ponte et d'éclosion ont également été relevées. Une fois les poussins suffisamment grands et emplumés, soit à 10 jours post-éclosion environ, nous les avons bagués avec une bague métal unique. Nous avons également réalisé une prise de sang pour chaque poussin. Pour chaque nichée, le but était également d'identifier le mâle et la femelle. Cette détermination passait par une phase d'observation aux jumelles. Une femelle était considérée comme mère de la nichée quand elle était observée lors de la couvaison et/ou si elle apportait de la nourriture au nid pour les

poussins ; et ce à plusieurs reprises. Un mâle était considéré comme le père social de la nichée s'il était observé à plusieurs reprises nourrissant les poussins. L'identification du couple était rendue possible car chaque individu adulte capturé au filet dans cette population a été, en plus de sa bague métal, bagué avec une combinaison unique de bague(s) couleur(s). A cette combinaison est associée une bague métal et son numéro unique, ainsi qu'une prise de sang réalisée lors de la première capture. Par contre, les individus non bagués du couple ont dû être capturés directement au nichoir. Toutes les étapes décrites précédemment doivent être réalisées pour chaque nichée de chaque nichoir, car les couples peuvent changer entre deux événements de reproduction. La figure 10 résume de façon chronologique les différentes étapes de notre suivi.

Le but de ce suivi était donc de connaître parents, poussins et succès de chaque nichée. Les prises de sang ont été réalisées en vue de futures études génétiques sur ces individus.

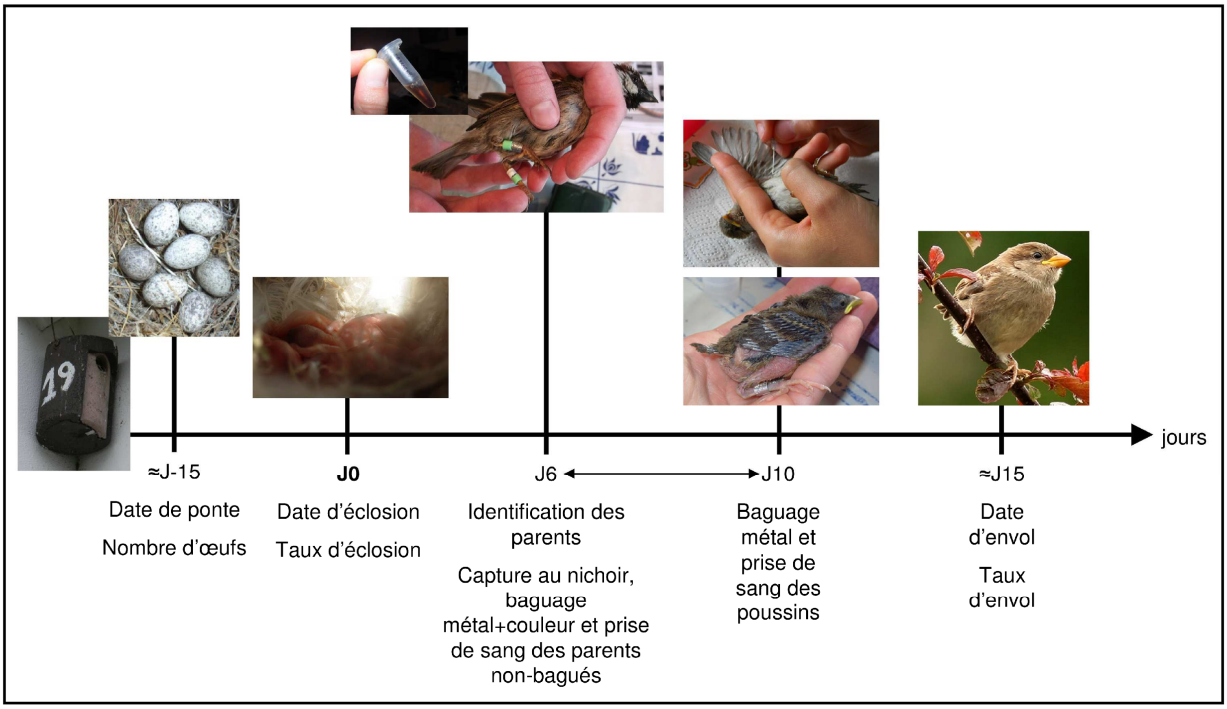



*Figure 10 : Protocole de suivi de reproduction du moineau domestique sur l'île d'Hoëdic.*

## 2.5 Génotypage

### 2.5.1 Marqueurs neutres

Estimer la variabilité génétique dans les populations naturelles est un moyen d'estimer leur viabilité (Allendorf, Luikart, 2007; Altizer *et al.*, 2003; Frankham, 1996). La mise au point de marqueurs moléculaires a permis l'essor de ce type d'études. La variabilité génétique des populations peut être influencée par deux grands types de facteurs : les facteurs démographiques et les facteurs sélectifs. Les facteurs démographiques comprennent la dérive génétique (fixation ou disparition aléatoire de certains allèles qui dépend fortement de la taille de la population) et les flux de gènes. La variabilité génétique est d'autant plus grande que la taille de la population est importante et les flux de gènes élevés (Frankham, 1996; Frankham, 1998; Keller, Waller, 2002; Luikart, Cornuet, 1998). Cette variabilité génétique, dite neutre, peut-être mesurée à partir de marqueurs génétiques non soumis à l'action de la sélection naturelle.

Les marqueurs neutres les plus couramment utilisés, et utilisés dans mon étude, sont les microsatellites. Les microsatellites sont des séquences d'ADN formée par des répétitions continues de motifs composés de 2 à 10 nucléotides. Ce sont des marqueurs en général très polymorphes : le nombre de répétitions du motif varie grandement d'un individu à l'autre. La transmission de ce nombre de répétitions suit les lois Mendéliennes de l'hérédité.

Nous avons utilisé un total de 16 loci différents lors de ma thèse (**Manuscrits 7 et 8**). Ces loci ont été mis au point chez le moineau domestique ou chez d'autres espèces de passereau (Dawson *et al.*, 2010; Garnier *et al.*, 2009; Griffith *et al.*, 1999; Li *et al.*, 1997; Neumann, Wetton, 1996; Richardson *et al.*, 2000). Ces loci ont été génotypés grâce à un séquenceur automatique à capillaire (ABI PRISM 3130 xl automated DNA Sequencer, Applied Biosystems). Les amorces spécifiques de chaque locus ont été couplées (en 5') à des fluorochromes de couleurs différentes. Le séquenceur est capable de détecter la fluorescence de ces amorces plus ou moins tôt dans la séquence, ce qui nous informe sur la taille de chaque allèle. Ainsi, les individus homozygotes seront visualisés (via le logiciel Genemapper) par la présence d'un pic à une taille donnée, tandis que les hétérozygotes possèderont deux pics à deux tailles différentes (Figure 11).

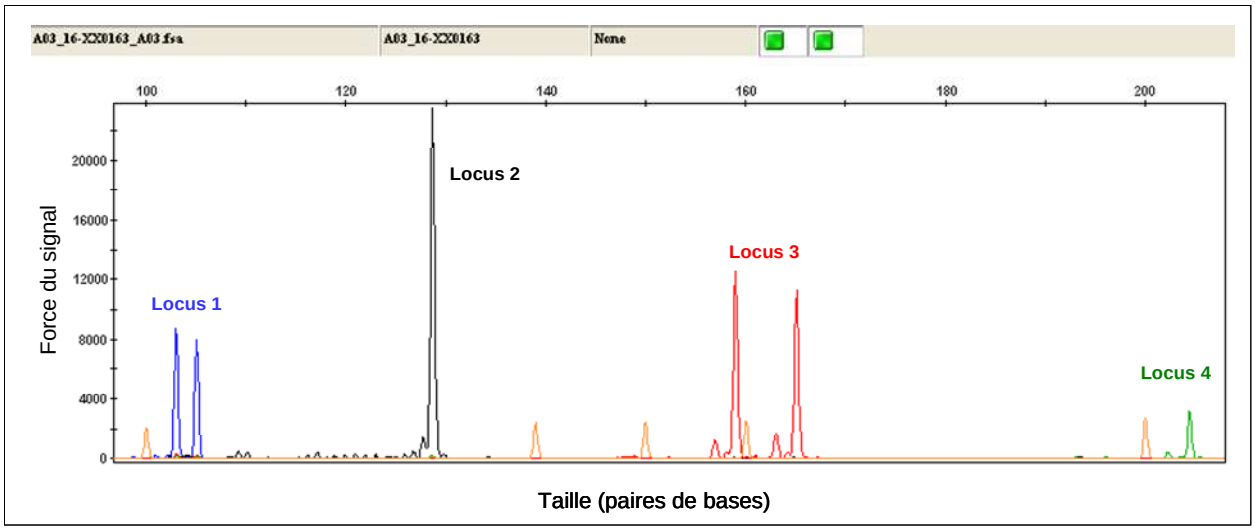



*Figure 11 : Exemple de sortie donnée par GeneMapper V4.0 après un génotypage microsatellites. 4 loci ont été génotypés simultanément et sont distinguables par 4 fluorochromes de couleurs différentes. L'individu représenté est hétérozygote pour les loci 1 et 3, et homozygote pour les loci 2 et 4. Les pics orange correspondent au marqueur de taille.*

### 2.5.2 Marqueurs sélectionnés

Si la variabilité génétique neutre reflète l'action des processus démographiques, elle n'apporte en revanche aucune information sur la variabilité génétique adaptative (Sommer, 2005). Cette variabilité est plus difficile à évaluer, notamment chez les organismes non modèles. Elle consiste à étudier le polymorphisme de gènes soumis à la sélection. Dans ce cadre, l'utilisation de gènes du Complexe Majeur d'Histocompatibilité (CMH) connaît un essor important. C'est une famille de gènes codant pour des protéines de surface cellulaire responsable de la reconnaissance et de la présentation des antigènes aux cellules du système immunitaire des vertébrés, ce qui déclenche la réponse immunitaire (Hedrick, 1994) (Figure 12). On distingue les molécules CMH de classe I, présentes à la surface de toutes les cellules nucléés et impliquées dans la défense contre les pathogènes intracellulaires, et les molécules CMH de classe II, présentes uniquement sur les cellules présentatrices d'antigènes (macrophages, lymphocytes, cellules dendritiques) et impliquées dans la défense contre les pathogènes et parasites extracellulaires (Piertney, Oliver, 2006) (Figure 12 et Chapitre 2).

Les gènes du CMH sont très polymorphes et ce polymorphisme peut être déterminé par plusieurs méthodes moléculaires. Les amorces et le protocole de PCR que nous avons utilisé a été décrit chez le moineau domestique par Bonneaud *et al.* (2004b). Nous avons amplifié

l'exon 3 du CMH de classe I, ce qui correspond à la région de reconnaissance des antigènes (PBR pour peptide-binding region). Comme pour les microsatellites, l'amorce en 5' a été couplée avec un fluorochrome, ce qui a permis le génotypage sur séquenceur automatique (ABI PRISM 3130 xl automated DNA Sequencer, Applied Biosystems). Cette étape a été réalisée lors d'une collaboration d'un mois au laboratoire KLIVV à Vienne, en Autriche. Il existe différentes méthodes, plus ou moins récentes, de génotypage du CMH (Babik, 2010). Nous avons choisi la méthode du CE-SSCP (Capillary Electrophoresis Single-Strand Conformation Polymorphism). Cette méthode est basée sur le fait que la mobilité électrophorétique de l'ADN simple brin dépend de sa conformation, et que cette conformation dépend de sa séquence. Cette méthode permet de distinguer des allèles ne différant que d'une seule paire de base. Cette méthode a déjà été utilisée chez le moineau domestique (Griggio *et al.*, 2011). Le nombre de pics obtenus pour un individu détermine alors le nombre d'allèles CMH de cet individu. Toutefois, avec cette méthode, nous ne pouvons déterminer le nombre de locus amplifié, et donc savoir si un individu est homozygote ou hétérozygote pour tel ou tel locus. Les fréquences alléliques ont alors été estimées par le nombre d'individus portant un certain allèle, divisé par le nombre total d'allèles observés dans la population concernée (Loiseau *et al.*, 2009). Cette procédure conduit à une surestimation de la fréquence des allèles rares et une sous-estimation de la fréquence des allèles communs (Ekblom *et al.*, 2007). Une méthode plus récente de séquençage haut débit (le pyroséquençage, 4-5-4) permet de contourner ce problème. Cette méthode très couteuse et qui demande une longue phase de mise au point n'a pu être utilisée durant ma thèse.

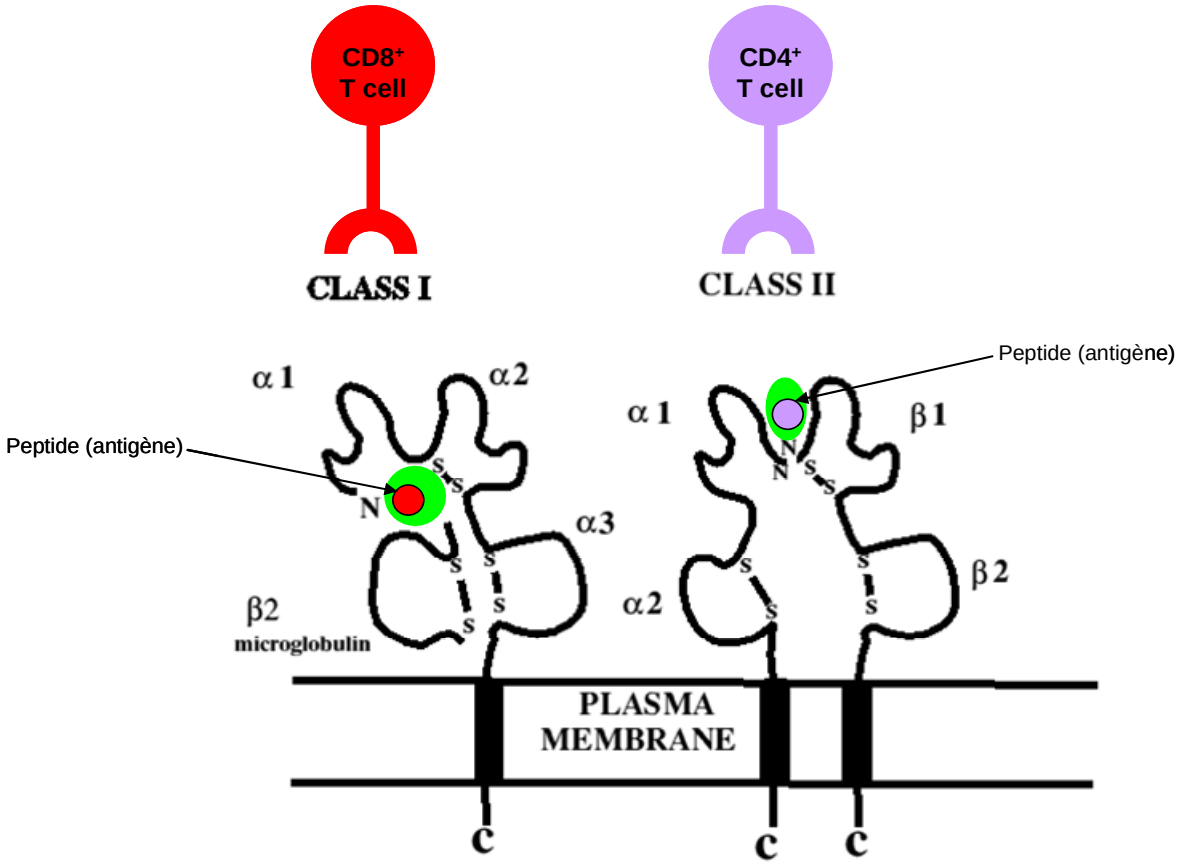



*Figure 12 : Représentation schématique des molécules CMH de classe I et II, présentes à la surface des cellules. La région colorée en vert est la région de fixation des peptides antigéniques (peptide-binding region, PBR). Pour le CMH de classe I, cette région est codée par l'exon 3, très polymorphe. Le CMH de classe I présente les antigènes aux lymphocytes T cytotoxiques (CD8), qui détruisent les cellules infectées. Le CMH de classe II présente les antigènes aux lymphocytes T helpers (CD8) qui vont participer au recrutement d'autres cellules du système immunitaire.*

## 2.6 Méthodes statistiques

### 2.6.1 Analyses de choix de partenaire et bootstrap

Nos analyses de choix de partenaire menées sur une population naturelle de moineau domestique (**Manuscrit 8**) ont principalement consistées à savoir si l'appariement observé dans la population était compatible ou non avec un appariement aléatoire (plus d'informations chapitre 3). Nous voulions notamment savoir si la variabilité génétique observée chez les poussins pouvait être produite suite à un choix aléatoire du partenaire de reproduction. De la

même manière, nous voulions savoir si l'apparentement moyen observé chez nos couples correspondait ou non à un choix aléatoire. La diversité génétique des poussins et des couples a été estimée avec des marqueurs microsatellites. L'apparentement a été mesuré avec ces mêmes marqueurs, mais aussi avec les gènes du CMH de classe I. Ce dernier correspond au nombre d'allèles CMH communs entre deux individus, divisé par la somme du nombre d'allèles de chaque individu (allele-sharing).

Voici comment ces différents indices ont été calculés :

*Diversité génétique*

- **IR, Internal Relatedness** : Il correspond au nombre de loci homozygotes pour un individu, pondéré par la fréquence des allèles dans la population (Amos *et al.*, 2001).

$$\frac{2H - \sum f_i}{2N - \sum f_i}$$

H : nombre de loci pour lesquels l'individu focal est homozygote
N : nombre de loci génotypés pour cet individu
$f_i$ : fréquence de l'allèle *i*

- **He, hétérozygotie multilocus** : Cet indice correspond simplement au nombre de loci hétérozygotes divisé par le nombre de loci génotypés pour un individu (Chapman *et al.*, 2009).

*Apparentement, proximité génétique*

- ***r*, coefficient d'apparentement** : Ce coefficient a été calculé pour chaque couple. Son calcul est basé sur la méthode décrite par Li *et al.* (1993). Un poids a aussi été attribué à chaque locus, selon Lynch, Ritland (1999) et Van de Casteele *et al.* (2001). Cette méthode est assez souvent utilisée car elle est dite non-biaisée par rapport à d'autres indices d'apparentement (Frasier, 2008; Krutzen *et al.*, 2003; Van de Casteele *et al.*, 2001). Pour un locus (*l*), le coefficient d'apparentement a pour équation :

$$r_{xy}(l) = \frac{S_{xy} - S_0}{1 - S_0}$$

*x* : génotype femelle

*y* : génotype mâle

$S_{xy}$ = 1 quand *x* = *y* = *ii* ou *ij* ; $S_{xy}$ = 0.75 quand *x* = *ii* et *y* = *ij* ; $S_{xy}$ = 0.5 quand *x* = *ij* et *y* = *ik* ; et $S_{xy}$ = 0 quand *x* = *ij* et *y* = *kl*

*i, j, k, l* : allèles à différentes positions allélique

$S_0$ correspond à la somme des fréquences alléliques pour un locus et se calcule comme suit :

$$S_0 = \sum_{i=1}^{n} p_i^2 (2\text{-}p_i)$$

$p_i$ : fréquence de l'allèle i dans la population

Le coefficient d'apparentement multi-locus est ensuite obtenu en multipliant le coefficient d'apparentement d'un locus par son poids, et en faisant la somme de ces valeurs obtenues pour tous les loci. Il faut ensuite diviser par la somme des poids de tous les loci.

$$r_{xy} = \frac{1}{W} \sum w(l) r_{xy}(l)$$

W : sommes des poids de tous les loci utilisés

w(*l*) est le poids de chaque locus et se calcule :

$$w(l) = \frac{n_j - 1}{\sum n_j - 1}$$

$n_j$ : nombre d'allèles au locus *j*

- **D, allele-sharing** : contrairement aux autres indices, il est calculé en utilisant les gènes du CMH de classe I comme marqueur moléculaire. Il nous informe sur le nombre d'allèles CMH que chaque couple a en commun. Il correspond au double

du nombre d'allèles partagés, divisé par la somme des allèles de chaque individu (Bonneaud *et al.*, 2006b; Wetton *et al.*, 1987).

$$D = \frac{2F_{ab}}{F_a + F_b}$$

$F_{ab}$ : nombre d'allèles communs entre l'individu *a* et l'individu *b*

$F_a$ : nombre d'allèles possédés par *a*

$F_b$ : nombre d'allèles possédés par *b*

L'appariement aléatoire a été simulé par la distribution de la diversité génétique des poussins, ou de l'apparentement des couples, attendue sous hypothèse d'appariement aléatoire. Pour les poussins, la méthode consiste à simuler, par un ré-échantillonnage, des reproductions aléatoires de mâles et de femelles de génotypes connus. Cela nous permet d'obtenir de manière aléatoire le même nombre de génotypes de poussins que sur le terrain. La diversité génétique des poussins ainsi simulée est ensuite calculée. Cette simulation est répétée par une méthode de bootstrap (1000 itérations) (Nakagawa, Cuthill, 2007). La distribution de la diversité génétique attendue des poussins est alors générée. Pour les mesures d'apparentement, le principe est le même : mâles et femelles sont associés aléatoirement afin de créer un nombre de couples identique à celui observé sur le terrain. La distribution attendue de l'apparentement entre mâles et femelles est alors obtenue. Nous pouvons alors savoir si les valeurs observées (sur le terrain) sont significativement plus faibles ou plus élevées que les valeurs attendues (simulées) sous l'hypothèse d'un appariement aléatoire (au seuil de significativité de 5%) (Figure 13).

Ces méthodes de bootstrap ont été réalisées avec le logiciel STORM (Frasier, 2008) ou avec le logiciel R 2.15.0 (R Developement Core Team, 2011).

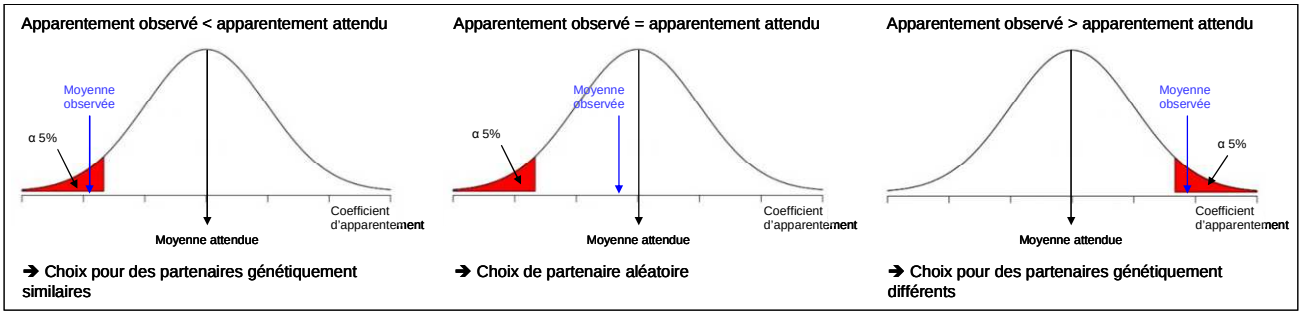



*Figure 13 : Principe de la méthode de bootstrap pour les analyses de choix de partenaires. Les trois cas de figures possibles sont représentés : un appariement préférentiel entre individus génétiquement similaires (à gauche), génétiquement différents (à droite), ou un appariement aléatoire (au centre).*

### 2.6.2 Modèles à effets mixtes

Les différentes analyses statistiques réalisées pour mes études sont détaillées dans chaque manuscrit. Toutefois, nous avons sensiblement utilisé le même type de modèle statistique dans un grand nombre de cas et la justification de son utilisation sera donnée dans ce paragraphe.

Pour les études menées en laboratoire (**Manuscrits 1 à 3**), le but était de suivre l'évolution de la virulence de *Plasmodium relictum* chez des groupes d'hôtes ayant subits différents traitements. Les oiseaux étaient suivis durant un cycle parasitaire, c'est-à-dire jusqu'à 20 jours post-infection. Plusieurs mesures (poids, hématocrite et parasitémie) ont été réalisées plusieurs fois pour un même individu, à des intervalles de temps réguliers. Nous nous trouvions donc dans un cas de pseudo-réplication temporelle. Cette non-indépendance statistique des données ne nous permet pas d'utiliser des modèles à effets fixes classiques. Nous avons alors utilisé des modèles mixtes (LMMs pour Linear Mixed Models).

Ces modèles sont ainsi nommés car les variables explicatives peuvent être à la fois à effets fixes et à effets aléatoires. Ces modèles nous ont permis de tenir compte du fait que plusieurs mesures avaient été réalisées sur le même individu en ajoutant l'identité de l'oiseau en variable à effet aléatoire.

De la même manière, nos études en populations naturelles nous ont conduits à utiliser les LMMs. Dans l'étude concernant le choix de partenaire chez le moineau domestique (**Manuscrit 8**), le suivi de reproduction s'est déroulé pendant trois années. Les femelles pouvaient donc s'être reproduites plusieurs fois au cours de ces trois années, mais également

plusieurs fois au sein d'une année. Nous avions ici affaire à plusieurs facteurs à effets aléatoires nichés les uns dans les autres (l'évènement de reproduction pour une même année, nichée dans l'identité de la femelle, nichée dans l'année de reproduction).

En réalité, nous avons bien souvent utilisé des GLMMs (General Linear Mixed Models) pour ces mêmes analyses. En effet, nous avions la plupart du temps affaire à une distribution non-normale. Les GLMMs permettent de spécifier la nature de la distribution de la variable à expliquer (binomiale, poisson…).

Les analyses ont été réalisées avec les logiciels SAS v.9.2 (SAS, 2002) et R 2.15.0 (R Development Core Team, 2011). Pour le logiciel R, nous avons utilisé le package lme4 (Bates *et al.*, 2011).

### 2.6.3 Approche IT et AIC

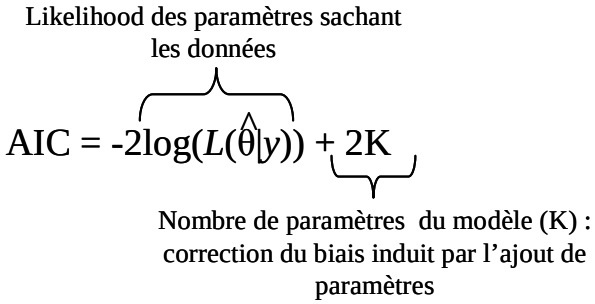
Dans ce paragraphe, je vais tenter de résumer et de simplifier le principe de l'approche IT ("Information-Theoretic Approach"). Je vais également expliquer l'utilité de cette approche statistique et pourquoi nous l'avons choisie dans plusieurs études de ma thèse. Je ne détaillerai pas toutes les formules mathématiques correspondantes (pour plus de détails, voir Burnham, Anderson, 2002). Pour ce type d'approche, le logiciel R 2.15.0 (R Development Core Team 2011) a été utilisé, et plus précisément le package MuMin (Barton, 2012).

*Cadre conceptuel*

Le but des statistiques est d'exprimer et de résumer l'information contenue dans les données par un modèle. Les modèles sont des approximations, ils ne correspondent pas parfaitement à la réalité. Par contre, il est possible de trouver le modèle qui permet de perdre le moins d'informations par rapport à la réalité. Cette perte d'information est formalisée par l'information de Kullback-Leibler (K-L information) qui correspond à la quantité d'information perdue quand un modèle *i* est utilisé pour approximer la réalité *f*. Mais cette réalité *f* est en général inconnue. Voilà pourquoi Akaike, en 1973, a développé un estimateur de cette information K-L, basé sur une fonction de log-likelihood : le Critère d'Information d'Akaike (AIC).

*Akaike's Information Criterion*

La formule de l'AIC est la suivante :




En pratique, l'AIC est calculé pour chaque modèle généré, et le modèle avec la plus faible valeur d'AIC est considéré comme le plus proche de la réalité qui a généré les données de départ, parmi les modèles candidats. L'AIC n'est pas une valeur absolue, mais une valeur relative à celle des autres modèles considérés. En fait, nous utilisons le plus souvent en écologie les valeurs d'$AIC_c$ (Second-Order Information Criterion), qui correspondent à des AIC corrigés pour les biais engendrés lorsque la taille d'échantillon est faible par rapport au nombre de paramètres des modèles (Hurvich, Tsai, 1989).

*Les différences d'AIC, ΔAIC*

Cette valeur est calculée en faisant la différence des valeurs d'AIC entre le modèle considéré et le modèle ayant l'AIC le plus faible. Plus le ΔAIC est élevé, moins le modèle considéré est probable pour expliquer les données. Les modèles ayant un ΔAIC compris entre 1 et 2 par rapport au meilleur modèle, peuvent être considérés comme apportant une information qui devra être prise en compte lors de l'interprétation. Des modèles ayant des ΔAIC entre 4 et 7 en apportent beaucoup moins, tandis qu'un ΔAIC supérieur à 10 correspond à des modèles qui n'expliquent par de variation substantielle dans les données et peuvent donc être écartés.

*Akaike Weights, $\omega_i$*

Afin de mieux interpréter le likelihood relatif d'un modèle, il est possible de calculer son poids, $\omega_i$. $\omega_i$ est considéré comme le poids de l'évidence, c'est-à-dire une estimation de la probabilité que le modèle considéré soit le meilleur modèle, parmi les modèles considérés. Plus ΔAIC est élevé, plus $\omega_i$ est faible, moins le modèle a de chance d'être le meilleur.

*Précautions*

L'approche IT est basée sur le principe de parcimonie. L'efficacité de cette approche dépend très fortement au départ de la construction des modèles possibles. Il nous appartient de bien étudier la structure des données et de ne placer dans les modèles que les paramètres jugés importants dans notre étude et pour répondre à la problématique de départ. Les AIC sont utiles pour sélectionner le meilleur modèle, mais, si tous les modèles sont faibles, l'approche IT fera juste ressortir le modèle le moins faible.

L'approche IT et l'utilisation de l'AIC ne constituent pas un test statistique. Dans des situations complexes, ces deux types d'approches peuvent donner des résultats très contrastés. L'approche IT a au moins l'avantage d'être exhaustive et de présenter tous les modèles possibles. Les valeurs d'AIC, de $\Delta$AIC et de $\omega_i$ permettent ensuite de discuter et d'interpréter les résultats en s'affranchissant du dogme des p-values. Cette approche est maintenant devenue obligatoire pour prétendre publier dans certaines revues médicales. Elle est fortement recommandée dans notre domaine, notamment dans les études en populations naturelles (Burnham, Anderson, 2002). Elle permet de visualiser le fait que les paramètres mesurés lors de l'étude ne sont peut-être pas les plus importants en réalité, que d'autres interviennent, ce qui est invisible avec le calcul des p-values.

# CHAPITRE 1

# Effets des caractéristiques intrinsèques de l'hôte sur les interactions hôtes-parasites

*Les parasites sont présents dans l'environnement de la très grande majorité des espèces animales et végétales, et les pressions qu'ils exercent sur elles sont conséquentes. Les espèces hôtes ont donc développé des mécanismes de défense afin de limiter la rencontre avec les parasites, de les éliminer ou de limiter leur prolifération. Dans ce chapitre, nous tenterons de détailler ces différents mécanismes. Nous nous intéresserons aux facteurs intrinsèques de l'hôte qui peuvent avoir une influence sur ces mécanismes, et donc modifier l'interaction avec le parasite.*

# 1. Les mécanismes de défense de l'hôte

## 1.1 Les mécanismes comportementaux

Le comportement joue un rôle non négligeable dans les interactions hôtes-parasites. L'évitement de la rencontre avec le parasite peut être dû à une série de choix comportementaux, tels que la décision de s'installer dans un habitat, de s'accoupler avec tel ou tel partenaire, ou encore de se nourrir de telle ou telle proie (Moore, 2002). Certains comportements, comme le comportement alimentaire, peuvent moduler la prolifération des pathogènes, et donc, essentiellement, ajuster la réponse immunitaire (paragraphe 2.4). Certaines espèces d'oiseaux ont également adopté des comportements "d'automédication" en introduisant dans leurs nids des espèces végétales aux capacités antimicrobiennes et antiparasitaires (Clark, 1990; Clark, Mason, 1985; Lafuma *et al.*, 2001; Petit *et al.*, 2002). Pour les espèces animales sociales, il apparaît aussi que le statut social de chaque individu joue un rôle important dans les interactions avec le parasite, en modulant, entre autre, l'accès à la nourriture, au territoire ou aux partenaires de reproduction. Nous y reviendrons également dans ce chapitre (paragraphe 2.3).

## 1.2 Les mécanismes immunitaires

Chez les vertébrés, nous distinguons deux voies majeures de l'immunité : l'immunité innée et l'immunité spécifique (Delves *et al.*, 2006).

L'immunité innée représente la voie ancestrale et est sensiblement similaire entre les invertébrés, les oiseaux, les mammifères ou même les plantes. L'immunité innée est la première ligne de défense lors d'une infection et se caractérise par une absence de spécificité,

une certaine rapidité et l'absence de mémoire. L'immunité innée regroupe des barrières physiques telles que la peau, des protéines antibiotiques circulant dans le sang ou la salive, et des cellules du système immunitaire (macrophages, neutrophiles, granulocytes). Ces cellules sont activées lors de la reconnaissance de peptides microbiens par les récepteurs PAMPs (pathogen-associated molecular patterns). Les cellules peuvent alors secréter des cytokines pro-inflammatoires, ainsi que des composés réactifs de l'oxygène et de l'azote, par exemple, l'oxyde nitrique (NO) (Janeway, Medzhitov, 2002; Sorci, Faivre, 2009). L'oxyde nitrique est un radical libre, produit de l'oxydation de la L-arginine en citrulline par l'enzyme NO synthase (NOS) (Vincendeau *et al.*, 2003). Cette substance est rapidement produite dans de nombreux tissus et cellules, suite à la production des cytokines pro-inflammatoires (Rivero, 2006). Le NO est toxique pour de très nombreux pathogènes : virus, champignons, bactéries, parasites intra et extracellulaires (Colasanti *et al.*, 2002).

L'immunité spécifique ou immunité acquise se caractérise par une grande spécificité, ainsi qu'une capacité d'induction et de mémorisation. Cette voie est principalement basée sur des médiateurs cellulaires, tels que les lymphocytes T et B, ainsi que sur la production d'anticorps. L'immunité à médiation cellulaire joue un rôle contre les pathogènes intracellulaires en détruisant les cellules infectées via la production de cytokines qui activent les lymphocytes T cytotoxiques et les cellules NK (natural killers). Cette réponse immunitaire à un antigène spécifique est mémorisée par des lymphocytes T-mémoires qui pourront être mobilisés lors d'éventuelles infections par un antigène similaire. L'immunité à médiation humorale est, quant à elle, impliquée dans la destruction des pathogènes extracellulaires. La reconnaissance des antigènes est assurée par les lymphocytes B. Les lymphocytes T-helpers vont activer la production des anticorps spécifiques par les lymphocytes B. Les anticorps vont se fixer sur le pathogène qui pourra ensuite être phagocyté. Lors des primo-infections, certains lymphocytes B vont se différencier en cellules mémoires. Si le système immunitaire rencontre à nouveau l'antigène, la production d'anticorps sera alors plus rapide et plus intense.

## 2. Les caractéristiques de l'hôte pouvant influencer ses mécanismes de défense

Au vu de l'immense bénéfice que confère une défense efficace contre les pathogènes, la sélection naturelle devrait favoriser les individus présentant une immunité de plus en plus

performante. Pourtant, il existe une très grande variabilité (entre individus et entre espèces) dans la force, l'efficacité et la rapidité de la réponse immunitaire (Schmid-Hempel, 2003). Cette variabilité est expliquée par l'existence de coûts à l'immunité. Cette notion de coûts découle de la théorie des traits d'histoire de vie (Roff, 1992; Stearns, 1992). Ces coûts peuvent être de plusieurs natures. Le plus intuitif est le coût en termes d'allocation des ressources ou *coût physiologique*. En effet, la théorie de l'allocation de ressources entre les composantes rattachées à l'aptitude des individus prévoit que l'énergie dépensée dans le maintien et le déploiement de la réponse immunitaire n'est ensuite plus disponible pour d'autres fonctions (Figure 14). D'autres types de coûts peuvent également être mis en évidence, comme les *coûts immunopathologiques*. Nous détaillerons ces deux types de coûts au cours de ce chapitre (Figure 15).

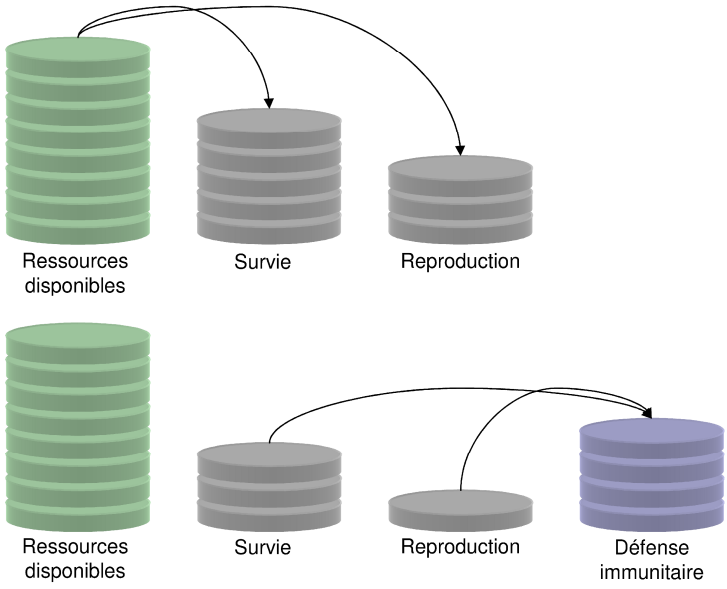



*Figure 14 : Illustration du principe d'allocation des ressources et du coût physiologique des défenses immunitaires. D'après Combes (2001b).*

## 2.1 Les caractéristiques génétiques

Les études cherchant à mettre en évidence le déterminisme génétique de la réponse immunitaire sont en essor depuis plusieurs dizaines d'années. Ce déterminisme commence à être bien connu chez les organismes modèles, mais il est beaucoup plus difficile de l'appréhender sur des organismes tels que les oiseaux. L'effet de la génétique sur la réponse immunitaire peut-être mis en évidence de manière indirecte, en étudiant la réponse de lignées

hôtes (Lambrechts *et al.*, 2006; Tinsley *et al.*, 2006) ou de clones (Carius *et al.*, 2001; Mucklow *et al.*, 2004), différant génétiquement entre eux. Certains gènes sont connus pour être directement impliqués dans la réponse immunitaire. Par exemple, chez la drosophile, les capacités antimicrobiennes sont associées à seize gènes de l'immunité innée (Lazzaro *et al.*, 2004). Chez le saumon, le gène *TAP* (Transporteur associated with Antigen Processing) est impliqué dans la reconnaissance des peptides antigéniques (Jensen *et al.*, 2008).

Chez les oiseaux, les gènes du Complexe Majeur d'Histocompatibilité (CMH) sont des gènes codants pour des glycoprotéines de surface cellulaire, responsables de la reconnaissance et de la présentation des antigènes, issus de pathogènes ou de parasites, aux cellules du système immunitaire, telles que les lymphocytes T, déclenchant ainsi une réponse immunitaire appropriée (Hedrick, 1994) (voir la partie modèles biologiques et méthodologies et le chapitre 2 pour plus d'informations). La variabilité des molécules du CMH est corrélée avec la diversité des récepteurs des cellules, ce qui détermine en retour la résistance aux pathogènes d'un individu (Sommer, 2005).

Le polymorphisme des gènes impliqués dans l'immunité est donc une notion primordiale. Les gènes du CMH constituent l'une des familles de gènes les plus polymorphes dans le génome des vertébrés (Bernatchez, Landry, 2003). Par exemple, les gènes du CMH humain, tels que HLA-A, HLA-B et HLA-DRB1 possèdent respectivement 243, 499 et 321 allèles (Piertney, Oliver, 2006). L'étude des mécanismes impliqués dans le maintien de ce polymorphisme est une question importante en écologie évolutive. Nous y reviendrons plus précisément dans les chapitres 2 et 3.

Par exemple, chez les épinoches, les individus exprimant une plus faible diversité allélique CMH souffrent d'une plus forte infection par un schistosome (Kurtz *et al.*, 2004). Cependant, un polymorphisme maximal, n'est pas toujours optimal (Woelfing *et al.*, 2009). Toujours chez l'épinoche, les individus les moins parasités sont ceux possédant un nombre intermédiaire d'allèles CMH (Wegner *et al.*, 2003a). En effet, un nombre trop important d'allèles CMH peut augmenter la reconnaissance des peptides du soi et entraîner l'élimination des lymphocytes T correspondants, lors de la sélection thymique, ce qui aurait en retour un impact négatif sur le système immunitaire (Nowak *et al.*, 1992). La présence d'allèles CMH particuliers semble également être liée à une résistance/susceptibilité à certains parasites et pathogènes. Cela a notamment été mis en évidence chez le moineau domestique (Bonneaud *et al.*, 2006b; Bonneaud *et al.*, 2005; Loiseau *et al.*, 2008). De manière plus indirecte, des lignées congéniques de poulets apparaissent plus résistantes à certains pathogènes plutôt qu'à d'autres. Par exemple, il a été montré que la lignée B21 était résistante à une mite

(*Ornithonyssus sylviarum*) (Owen *et al.*, 2008), au Rous sarcomavirus (Aeed *et al.*, 1993), à la leucose aviaire (Mays *et al.*, 2005), ainsi qu'au virus H5N1 (Boonyanuwat *et al.*, 2006). Contre le virus de Marek, la lignée B21 apparaît également résistante, tandis que la lignée B19 est sensible (Briles *et al.*, 1977; Plachy *et al.*, 1992).

Les gènes du CMH apparaissent donc comme de très bons candidats pour étudier l'influence des caractéristiques génétiques sur les interactions hôtes-parasites.

### 2.2 Système immunitaire et coûts immunopathologiques

Les voies immunitaires impliquées lors des infections par la malaria sont bien connues chez l'homme et chez les rongeurs, mais beaucoup moins en ce qui concerne le modèle aviaire.

Depuis quelques années, l'équipe de recherche dans laquelle s'est déroulée ma thèse s'attache à étudier les mécanismes immunitaires mis en œuvre lors de cette infection. Nous savons, par exemple, que des infections répétées chez un même individu par la malaria aviaire conduisent à une sorte de prémunition contre ce parasite (Cellier-Holzem *et al.*, 2010). Nous savons également que des individus immunodéprimés seront moins capables de résister à la malaria aviaire, ce qui résulte en une parasitémie plus élevée chez ces oiseaux (Cellier-Holzem *et al.*, en préparation.). Ce résultat a été obtenu en comparant la virulence de parasites infectant des hôtes totalement libres de monter leur réponse immunitaire, avec la virulence de parasites infectant des hôtes immunodéprimés. Cette immunodépression a été obtenue en utilisant la cyclophosphamide, molécule capable de détruire tous les types de cellules immunitaires sans distinction. Les hôtes ayant reçu ce traitement sont donc globalement immunodéprimés. Cependant, cela ne nous permet pas de comprendre finement les voies immunologiques clés mobilisées dans ce type d'infection. Pour cela, nous avons réalisé le même genre d'expérience en utilisant un autre traitement, l'aminoguanidine. Cette molécule est capable d'inhiber spécifiquement une enzyme, la iNOS (pour inductible nitric oxide synthase), responsable de la synthèse d'oxyde nitrique (NO) (Allen, 1997; Vincendeau *et al.*, 2003; Wideman *et al.*, 2006). Le NO est un composé actif de l'oxygène ayant un effet cytotoxique sur de très nombreux pathogènes (Colasanti *et al.*, 2002; Rivero, 2006). La voie du NO est donc particulièrement intéressante à étudier ici, car elle représente une échappatoire potentielle pour le parasite, comme cela a déjà été montré pour des protozoaires comme *Trypanosoma cruzi* et *Leishmania major* (Vincendeau *et al.*, 2003).

Dans le **Manuscrit 1**, nous avons mis en évidence que le NO contribuait aux défenses immunologiques lors d'une infection par la malaria aviaire (*Plasmodium relictum*), puisque les hôtes (*Serinus canaria*) ayant une synthèse de NO limitée par l'amminoguanidine présentaient une parasitémie plus élevée. Cette implication de la voie du NO dans les défenses contre *Plasmodium* avait déjà été montrée chez d'autres organismes (Anstey *et al.*, 1996; Hobbs *et al.*, 2002; Luckhart *et al.*, 1998; Macchi *et al.*, 2010; Peterson *et al.*, 2007; Taylor-Robinson, 2010; Wang *et al.*, 2009).

Le point intéressant de notre expérience est que, même si les individus NO-inhibés ont montré une plus forte parasitémie, ils n'ont pas pour autant payé un coût supplémentaire à l'infection par rapport aux individus contrôles. Les niveaux d'hématocrite, le poids et la mortalité ne diffèrent pas entre ces deux groupes d'oiseaux. Ce résultat pourrait illustrer les *coûts immunopathologiques* associés à la voie immunitaire du NO contre *Plasmodium*. En effet, une diminution de l'hématocrite est la conséquence directe de la lyse des érythrocytes provoquée par le relargage des mérozoïtes des parasites, mais également la conséquence du système immunitaire lui même qui s'attaque aux globules rouges infectés. Dans le système rongeur-malaria, il a été estimé que 10% de l'anémie était due à une sur-activation de la réponse immunitaire (Graham *et al.*, 2005b). Les pathologies liées à l'immunité sont en fait assez courantes, aussi bien chez les vertébrés (Cooke *et al.*, 2004; Graham *et al.*, 2005a; Kobasa *et al.*, 2007; Sorci, Faivre, 2009), que chez les invertébrés (Brandt *et al.*, 2004; Sadd, Siva-Jothy, 2006; Sugumaran *et al.*, 2000).

Dans notre système hôte-parasite, inhiber la production de NO a augmenté les coûts de l'infection, mais réduit ceux induits par les défenses immunitaires. Les couts immunopathologiques apparaissent comme un déterminant majeur de l'évolution de la virulence, capable d'affecter le trade-off existant entre multiplication et virulence (Alizon *et al.*, 2009; Day *et al.*, 2007; Graham *et al.*, 2005a; Long, Graham, 2011). En effet, si le but d'un parasite est de se multiplier, c'est aussi de se transmettre à d'autres hôtes. Dans le cas de cycles parasitaires complexes, comme celui de la malaria aviaire, tuer son hôte trop tôt peut empêcher la transmission au vecteur. Les parasites doivent donc adapter rapidement leur virulence en fonction des capacités immunitaires de leurs hôtes (Day *et al.*, 2007).

### 2.3 Le statut social de l'hôte

Chez les espèces sociales vertébrées, les relations dominants-subordonnés et le statut de chaque individu peuvent être associés à une virulence des parasites plus ou moins forte. Chez

les oiseaux, la dominance comporte un certain nombre de bénéfices, tels qu'un meilleur accès à la nourriture (Parisot *et al.*, 2004), le choix de territoires sans prédateurs, ou un meilleur succès reproducteur (Post, 1992). La dominance est aussi couteuse, engendre un stress et la production des hormones associées (Creel *et al.*, 1996; Goymann, Wingfield, 2004), ainsi qu'une augmentation des coûts énergétiques liés à la protection de la ressource alimentaire (Hogstad, Stenberg, 1997). Les ressources dépensées pour maintenir un statut de dominance seront alors moins disponibles pour les fonctions immunitaires (Li *et al.*, 2007). Cependant, comme la dominance assure un meilleur accès aux ressources alimentaires, les individus dominants pourront être en meilleure condition, ce qui peut avoir un effet bénéfique sur le système immunitaire.

Une étude récente, menée par notre équipe a, dans ce contexte, permis de montrer que les parasites (*Plasmodium relictum*) infectant des canaris dominants étaient plus virulents que les parasites infectant des individus subordonnés (Larcombe *et al.*, en préparation), illustrant ainsi le coût de la dominance. Cette étude a montré que la virulence, si elle dépend du statut social de l'hôte, dépend également du statut infectieux des autres individus du groupe. Il faut noter que cette expérience avait été réalisée dans des conditions d'accès à la nourriture limitée.

Dans le **Manuscrit 2,** nous présentons la suite logique de ces résultats, où nous avons regardé si la virulence du parasite différait entre dominants et subordonnés, selon que la quantité de nourriture était illimitée ou restreinte. Les résultats montrent encore une fois que les individus dominants paient un coût plus élevé que les subordonnés. La parasitémie est plus élevée et l'hématocrite plus faible chez ces individus. De plus, il n'y a pas de différence d'hématocrite entre subordonnés parasités et subordonnés non parasités. Les relations de compétition entre individus semblent peu affecter la virulence dans notre modèle d'étude.

### 2.4 Le statut nutritionnel de l'hôte

La théorie d'allocation des ressources prédit un trade-off entre les fonctions immunitaires et les autres fonctions de l'organisme. Si ces ressources sont essentiellement récupérées dans l'alimentation, le statut nutritionnel de l'hôte pourra avoir de grandes conséquences sur la virulence du parasite (Bedhomme *et al.*, 2004; Brown *et al.*, 2000; Krist *et al.*, 2004; Restif, Kaltz, 2006; Seppala *et al.*, 2008). Il est à noter que, si la condition nutritionnelle peut être considérée comme un caractère intrinsèque à l'hôte, elle est aussi très dépendante de la qualité de l'habitat dans lequel l'hôte évolue. L'effet de l'environnement sur les interactions hôtes-parasites sera développé dans le chapitre 2.

Si les parasites peuvent être plus virulents chez les hôtes ayant une faible condition corporelle, probablement à cause d'un système immunitaire moins performant (Krasnov *et al.*, 2005), ils peuvent être limités par le fait que ces hôtes en moins bonne condition leur fournissent moins de ressources à exploiter (Bedhomme *et al.*, 2004; Tschirren *et al.*, 2007; Tseng, 2006). Dans ce cas, la virulence du parasite sera soumise à un trade-off entre la quantité de ressources à exploiter et les défenses immunitaires de l'hôte.

La quantité et la qualité de la nourriture sont connues pour affecter l'immunocompétence des oiseaux (Gonzalez *et al.*, 1999; Smith *et al.*, 2007). Les fonctions immunitaires sont également capables d'affecter les traits parasitaires (Buckling, Read, 2001; Mackinnon, Read, 2003), mais la relation entre statut nutritionnel et stratégies d'exploitation du parasite n'a jamais été étudiée pour le cas de la malaria aviaire (pour les modèles rongeur et moustique, voir Bell *et al.*, 2006 et Tseng, 2006). Dans le **Manuscrit 3,** nous avons comparé la virulence de *Plasmodium relictum* entre deux groupes de canaris : un groupe recevant une nourriture à base de graines *ad libitum*, et un groupe recevant un apport supplémentaire de protéines et de vitamines. De plus, l'effet de la provenance des parasites (parasites ayant infectés des hôtes supplémentés ou non) sur la virulence a été testé en infectant ces deux types de parasites à deux autres groupes d'hôtes, supplémentés ou non. En accord avec notre prédiction, la supplémentation a conduit à une masse des canaris plus élevée et à une plus faible parasitémie, illustrant le fait que des oiseaux mieux nourris sont capables de monter une réponse immunitaire plus efficace. L'origine des parasites semble également jouer un rôle dans la virulence puisque des parasites originaires d'hôtes non supplémentés sont responsables d'une augmentation de la parasitémie et de l'anémie. Cela est contradictoire avec d'autres études similaires sur d'autres modèles, où les parasites ayant précédemment infectés des hôtes en bonne condition étaient plus virulents (Little *et al.*, 2007; Tseng, 2006). Dans notre système canaris-*Plasmodium*, il semble que les capacités immunitaires de l'hôte, plus que les ressources disponibles pour le parasite, affectent la virulence. De plus, si les hôtes supplémentés ont une parasitémie plus faible, ils présentent en revanche une anémie plus forte. Ce résultat rejoint celui du **Manuscrit 1** et illustre encore une fois l'importance de l'immunopathologie dans l'évolution de la virulence. Les individus non supplémentés résistent moins à la multiplication du parasite, mais les *coûts immunopathologiques* sont limités.

Dans le **Manuscrit 2**, nous avions également testé l'effet de l'alimentation sur l'interaction entre statut social des hôtes et virulence du parasite. Cet effet n'a pas été mis en évidence, les canaris dominants paient un coût supplémentaire à l'infection, quel que soit

l'apport alimentaire (limité ou *ad libitum*). Cette expérience montre, qu'en rajoutant un seul degré de complexité (ici le statut social), l'interaction hôte-parasite peut s'en trouver totalement modifiée. Est-il besoin de rappeler que les expériences de laboratoires en conditions contrôlées ne permettent bien souvent d'étudier qu'une caractéristique de l'hôte à la fois et que ce qui se passe en populations naturelles est beaucoup plus complexe. Ainsi, nous avons également voulu savoir s'il était possible de mettre en évidence l'effet de caractéristiques hôtes sur la virulence des parasites de la malaria aviaire dans des populations naturelles. Ces études seront détaillées dans les paragraphes suivants (2.4 et 2.5) et concernent des populations naturelles de moineaux domestiques (*Passer domesticus*).

### 2.5 L'âge de l'hôte

Parmi les caractéristiques de l'hôte, l'âge est un facteur intrinsèque à l'hôte souvent évoqué pour ses effets sur les dynamiques d'infection (McCurdy *et al.*, 1998; Weatherhead, Bennett, 1992). Le patron généralement observé, en particulier pour la malaria, est que la parasitémie est plus élevée chez les jeunes que chez les adultes (Hudson, Dobson, 1997). Au contraire, la prévalence est plus forte chez les adultes que chez les juvéniles. Cela reflète une plus grande durée d'exposition pour les adultes et le maintien d'infections chroniques (Allander, Bennett, 1994; Stjernman *et al.*, 2004; Wood *et al.*, 2007). Ce patron a été trouvé dans de nombreuses études sur les parasites sanguins aussi bien chez l'homme (Graves *et al.*, 1988; Syafruddin *et al.*, 2009), chez d'autres mammifères (Gregory *et al.*, 1992), chez les reptiles (Amo *et al.*, 2005) et chez les oiseaux (Allander, Bennett, 1994; Dawson, Bortolotti, 2000; Hasselquist *et al.*, 2007; Merilä *et al.*, 1995; Sol *et al.*, 2000; Sol *et al.*, 2003).

Trois hypothèses non-exclusives sont évoquées pour expliquer ce patron (Gregory *et al.*, 1992). La première est *l'hypothèse de la sélection* qui suggère une mortalité différentielle entre adultes et juvéniles, où les juvéniles infectés vont mourir avant d'avoir été recrutés dans la population adulte. L'immunité des individus est aussi un paramètre important et constitue *l'hypothèse de l'immunité* (Adamo *et al.*, 2001; Cichon *et al.*, 2003; Lavoie, 2005; Miller, 1996; Palacios *et al.*, 2007; Saino *et al.*, 2003). Durant leur vie, les individus sont soumis à différents pathogènes et renforcent ainsi leur immunité au cours du temps. La dernière hypothèse, *l'hypothèse du vecteur*, suggère que les adultes sont moins exposés aux vecteurs, et donc aux parasites, que les juvéniles.

Si l'effet de l'âge sur les dynamiques d'infection a déjà été étudié chez les oiseaux, il n'est pas facile de déterminer l'influence relative des trois hypothèses. Par exemple, une étude sur

la malaria, menée sur le pigeon bizet (*Columba livia*), a montré que l'exposition au vecteur du parasite *Haemoproteus* était similaire entre adultes et juvéniles (Sol *et al.*, 2000). Les auteurs ont également montré que l'hypothèse de sélection n'était pas assez forte dans ce système pour expliquer les différences de parasitémie observées entre les classes d'âge. Ils ont alors suggéré que l'hypothèse d'une immunité plus efficace chez les adultes était la plus probable. Par contre, l'étude expérimentale menée chez le coq sauvage (*Gallus gallus*) par Williams (2005) trouve que les jeunes sont plus sensibles que les vieux adultes, même après avoir déjà été exposés une première fois à l'infection. De plus, l'influence de l'âge peut être différente selon les classes d'âge qui sont prises en compte. Ainsi, les deux classes d'âge extrêmes (très vieux et très jeunes) sont souvent annoncées comme étant les plus susceptibles aux infections. Dans ce cas là, ce sont les fonctions immunitaires qui sont responsables de ce phénomène, avec les jeunes individus possédant un système immunitaire inexpérimenté, tandis que l'efficacité de celui-ci diminue chez les vieux individus à cause de la sénescence (Hudson, Dobson, 1997).

L'immunosénescence a surtout été étudiée chez les humains et les mammifères (Lavoie, 2005). Chez l'homme, le déclin des fonctions immunitaires avec l'âge est initialement provoqué par une atrophie du thymus. L'immunité acquise est altérée par une réduction de la prolifération des lymphocytes T et de la réponse des lymphocytes B (Malaguarnera *et al.*, 2001). La voie innée de l'immunité est aussi moins efficace avec l'âge, via, par exemple, une diminution de l'activité des cellules NK (Malaguarnera *et al.*, 2001; Plackett *et al.*, 2004). Chez les oiseaux, l'étude des changements immunitaires liés à l'âge a été réalisée chez, notamment, quatre espèces d'oiseaux : l'hirondelle rustique (*Hirundo rustica*, Saino *et al.*, 2002; Saino *et al.*, 2003), le gobe-mouche à collier (*Ficedula albicollis*, Cichon *et al.*, 2003), la sterne pierregarin (*Sterna hirundo*, Apanius, Nisbet, 2003), et le combattant varié (*Philomachus pugnax*, Lozano, Lank, 2003). Les hirondelles "vaccinées" contre le virus de Newcastle (NDV) présentent une réponse secondaire anti-NDV qui diminue avec l'âge (Saino *et al.*, 2003), de même chez les gobe-mouches "vaccinés" contre les globules rouges de moutons (Cichon *et al.*, 2003). Ces études concluent que la survie des individus âgés est conditionnée par la balance entre des fonctions immunitaires déclinantes et la bonne mémoire immunitaire d'un système expérimenté.

Dans le **Manuscrit 4**, nous avons mesuré la prévalence et la parasitémie, dans plusieurs populations naturelles de moineaux domestiques, à l'échelle de la France. Cette dynamique parasitaire a été mise en relation avec plusieurs caractéristiques de l'hôte comme l'âge et le sexe (voir paragraphe 2.6 pour l'effet du sexe). Dans cette étude, seule la parasitémie peut être

expliquée par les caractéristiques de l'hôte, la prévalence semblant plutôt dépendre de facteurs environnementaux (voir chapitre 2). L'hypothèse d'exposition différentielle au vecteur a été écartée dans notre étude car, si les juvéniles étaient plus exposés au vecteur (et donc au parasite), nous aurions observé chez eux des prévalences plus élevées.

Les individus âgés d'un an présentaient donc des parasitémies plus élevées que les adultes. Ce résultat est cohérent avec un grand nombre d'études (Allander, Bennett, 1994; Amo *et al.*, 2005; Dawson, Bortolotti, 2000; Graves *et al.*, 1988; Gregory *et al.*, 1992; Hasselquist *et al.*, 2007; Merilä *et al.*, 1995; Sol *et al.*, 2000; Sol *et al.*, 2003; Syafruddin *et al.*, 2009). Cet effet semble principalement dû à des différences dans les fonctions immunitaires selon l'âge des oiseaux (Hasselquist *et al.*, 2007). En effet, les risques d'expositions et de réexpositions augmentent avec l'âge (Atkinson *et al.*, 2000; Graczyk *et al.*, 1994; Hudson, Dobson, 1997; Sol *et al.*, 2003) et peuvent engendrer des phénomènes d'immunisation et de tolérance envers le parasite au cours du temps. Cela a notamment été démontré dans notre équipe lors d'une expérience d'infection-réinfection chez le canari (Cellier-Holzem *et al.*, 2010).

### 2.6 Le sexe de l'hôte

Comme l'âge, le sexe de l'hôte peut avoir une influence sur les interactions hôtes-parasites. L'effet du sexe sur les interactions hôtes-parasites est essentiellement dû au trade-off entre reproduction et immunité (Rolff, 2002). Il est généralement admis que les capacités immunosuppressives de la testostérone font que les mâles seraient plus sensibles aux infections (McCurdy *et al.*, 1998; Poulin, 1996; Schalk, Forbes, 1997; van Oers *et al.*, 2010).

Pourtant, dans le **Manuscrit 4**, nous avons trouvé que la parasitémie est plus élevée chez les femelles que chez les mâles. Ce résultat a également été trouvé dans d'autres études, en particulier chez les oiseaux et pour les parasites transmis par des vecteurs (Chernin, 1952; McCurdy *et al.*, 1998; Peirce, Marquiss, 1983). Une explication possible est que le coût de la reproduction serait plus fort pour les femelles que pour les mâles, rendant les femelles moins immunocompétentes face à la malaria (Richner *et al.*, 1995; Williams, 2005). Une hypothèse alternative serait que mâles et femelles ne seraient pas exposés de la même manière aux vecteurs de la malaria. Chez le moineau domestique, c'est majoritairement la femelle qui couve les œufs. Cette période d'incubation la rend plus facilement détectable par les vecteurs, et donc plus sujette aux parasites qu'ils transportent (Chernin, 1952; Korpimaki *et al.*, 1993; Norris *et al.*, 1994; Peirce, Marquiss, 1983). Cependant, cette hypothèse tendrait plutôt à être

rejetée dans notre étude car nous n'avons pas détecté de différences de prévalence entre mâles et femelles.

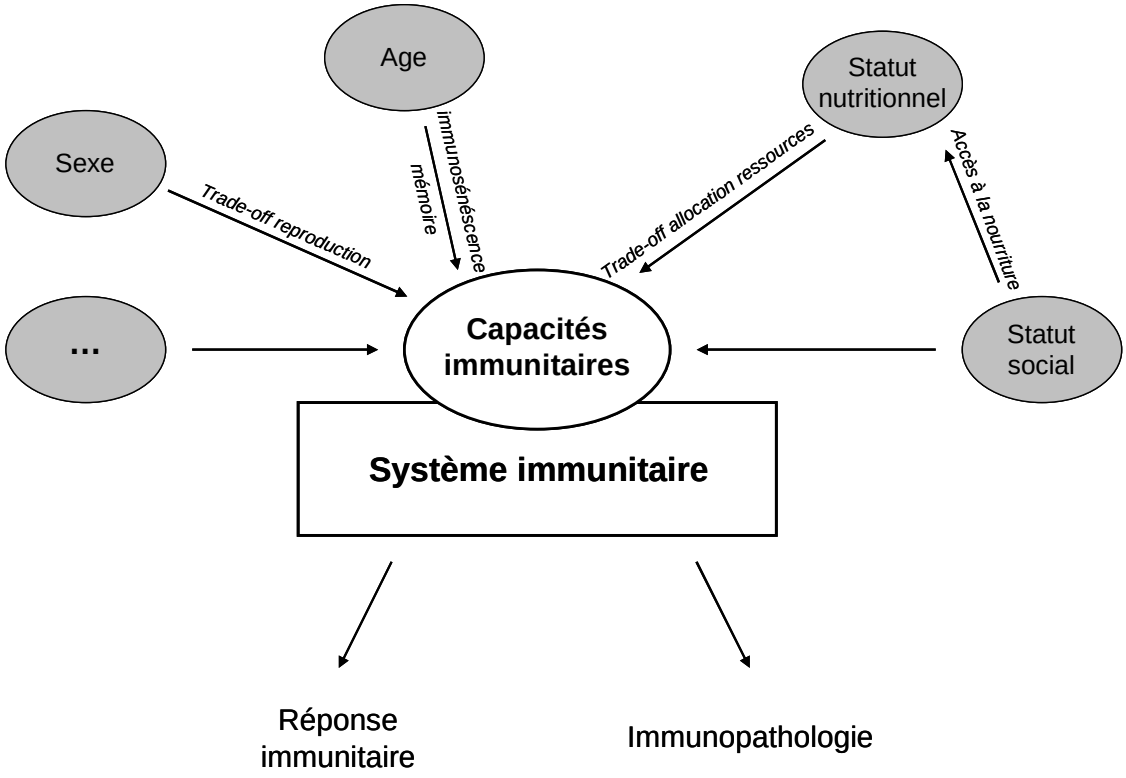



*Figure 15 : Trade-offs entre certaines caractéristiques de l'hôte et les capacités immunitaires, affectant l'interaction avec le parasite. Plus directement, des composantes du système immunitaire, comme l'immunopathologie, sont susceptibles d'affecter l'évolution de la virulence.*

*Pour résumer :*

| Manuscrit | Etude | Hypothèses | Résultats | Conclusion |
|---|---|---|---|---|
| 1 | Rôle de la voie de l'oxyde nitrique lors d'une infection par *P.relictum* chez le canari. | Les individus immunodéprimés pour la voie du NO tolèrent moins l'infection. | Les individus immunodéprimés ont des parasitémies plus élevées.<br><br>Pas de différences d'hématocrite entre individus immunodéprimés et contrôles. | La voie du NO est impliquée dans la résistance à la malaria aviaire chez le canari.<br><br>Cette voie comporte des coûts immunopathologiques. |
| 2 | Rôle du statut social de l'hôte et des ressources alimentaires lors d'une infection par *P.relictum* chez le canari. | La résistance à la malaria dépend de la balance entre les bénéfices d'être dominant (meilleur accès à la nourriture et donc meilleure condition) et les coûts (stress). | Les individus dominants présentent des parasitémies plus élevées que les subordonnés.<br><br>Le régime alimentaire (nourriture limitée ou *ad libitum*) ne change pas la différence entre dominants et subordonnés. | Les individus dominants paient un coût supplémentaire à l'infection de part leurs différences physiologiques avec les subordonnés (hormones liées au stress, dépense d'énergie).<br><br>Le statut nutritionnel ne rentre pas en compte dans l'interaction malaria-statut social. |
| 3 | Rôle du statut nutritionnel des hôtes lors d'une par *P.relictum* chez le canari. | Les individus mieux nourris peuvent allouer plus d'énergie à la lutte contre la malaria. | Les individus supplémentés sont en meilleure condition et présentent des parastemies plus faibles.<br><br>Par contre leur hématocrite est plus faible. | Une meilleure alimentation permet d'allouer plus de ressources à la réponse immunitaire.<br><br>L'augmentation de la réponse immunitaire augmente aussi les coûts immunopathologiques associés. |
| 4 | Rôle de l'âge et du sexe de l'hôte dans l'épidémiologie de la malaria aviaire en populations naturelles. | Différences de prévalences et de parasitémies entre jeunes et adultes et entre mâles et femelles provoquées par :<br>des différences dans la réponse immunitaire,<br>des taux de mortalité différentiels,<br>une exposition différente aux vecteurs. | La prévalence ne dépend ni de l'âge, ni du sexe des moineaux.<br><br>Les jeunes ont des parasitémies plus élevées que les adultes.<br><br>La parasitémie est plus élevée chez les femelles que chez les mâles. | La prévalence dépend plutôt des caractéristiques environnementales.<br><br>Rôle de l'immunité acquise dans la résistance à la malaria.<br><br>Coût de la reproduction plus élevée pour les femelles que pour les mâles (allocation des ressources différente).<br><br>Les hypothèses de taux de mortalité différentiels et d'expositions différentielles au vecteur sont moins probables (pas de différence de prévalence). |

# Manuscrit 1

## Experimental inhibition of nitric oxide increases *Plasmodium relictum* (lineage SGS1) parasitaemia

Coraline Bichet, Stéphane Cornet, Stephen Larcombe and Gabriele Sorci

Sous presse dans *Experimental Parasitology*

# Experimental inhibition of nitric oxide increases *Plasmodium relictum* (lineage SGS1) parasitaemia

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**Abstract:** Malaria is a widespread vector-borne disease infecting a wide range of terrestrial vertebrates including reptiles, birds and mammals. In addition to being one of the most deadly infectious diseases for humans, malaria is a threat to wildlife. The host immune system represents the main defence against malaria parasites. Identifying the immune effectors involved in malaria resistance has therefore become a major focus of research. However, this has mostly involved humans and animal models (rodents) and how the immune system regulates malaria progression in non-model organisms has been largely ignored. The aim of the present study was to investigate the role of nitric oxide (NO) as an immune effector contributing to the control of the acute phase of infection with the avian malaria agent *Plasmodium relictum*. We used experimental infections of domestic canaries in conjunction with the inhibition of the enzyme inducible nitric oxide synthase (iNOS) to assess the protective function of NO during the infection, and the physiological costs paid by the host in the absence of an effective NO response. Our results show that birds treated with the iNOS inhibitor suffered from a higher parasitaemia, but did not pay a higher cost of infection (anaemia). While these findings confirm that NO contributes to the resistance to avian malaria during the acute phase of the infection, they also suggest that parasitaemia and costs of infection can be decoupled.

**Keywords**
Avian malaria, *Plasmodium relictum* lineage SGS1, experimental infection, nitric oxide, immune defence, immunopathology.

## 1. Introduction

Malaria protozoa still severely threaten the health of human populations, causing around 700,000 of deaths worldwide in 2010 (World Health Organization, 2011). The negative health implications of *Plasmodium* infections have stimulated considerable attention on the study of immunity to malaria (see for instance Doolan et al., 2009; Langhorne et al., 2008), in both humans, who are obviously less amenable to experimental approaches, and in animal models (Artavanis-Tsakonas et al., 2003). Thus, the current knowledge of the immunological pathways involved in resistance/tolerance of malaria infection comes mainly from rodent malaria models, *Plasmodium chabaudii*, *P. berghei* and *P. yoelii* (Good and Doolan, 1999; Langhorne et al., 2004; Roetynck et al., 2006).

*Plasmodium* parasites have a complex life cycle involving a mosquito vector, in which sexual reproduction of the pathogen occurs, and a vertebrate host in which the parasite reproduces asexually. Invertebrates and vertebrates differ in many aspects of their immune system which might impose a challenge to the parasite in terms of its ability to adapt to different immunological environments (Hammerschmidt and Kurtz, 2005). However, both hosts share some immunological pathways, in particular the innate arm of the immune system where cytotoxic compounds are released shortly after the infection (i.e. nitric oxide).

Immunity of vertebrate hosts to malaria involves a complex network of immunological effectors. The control of the acute phase of the infection (peak parasitaemia being reached between 8 and 16 days post-infection, depending on the model system considered) depends on the activation of a helper T-cell 1 (Th1) response (Taylor-Robinson et al., 1993). Pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), are produced and released with the further activation of macrophages that release cytotoxic compounds. The acute phase of the infection is then followed by a chronic phase with very low parasitaemia eventually leading to recurrent relapses (Huldén et al., 2008). The shift from acute to chronic infection is paralleled by a shift from a Th1 to a helper T-cell 2 (Th2) response, with the production of immunoglobulins (IgGs) specific to the current parasite strains (Taylor-Robinson and Looker, 1998; Taylor-Robinson et al., 1993). Multiple exposures to malaria parasites therefore lead to a partial immunity that has been called premunition (Soe et al., 2001), whereas total immunity is probably prevented by the antigenic variation of malaria parasites producing variants that escape the pre-existing antibody repertoire (Newbold, 1999).

In addition to humans (and non-human primates) and rodents, malarial parasites are widespread pathogens of birds and reptiles (Valkiūnas, 2005), principally in tropical and temperate areas. Avian malaria is thought to be non-lethal in hosts that have a long co-evolutionary relationship with the parasite (Fallis and Desser, 1977). Nevertheless, recent experimental infections have also shown that avian malaria parasites can be costly and substantially reduce host fitness (Knowles et al., 2010; Palinauskas et al., 2008, 2009; Zehtindjiev et al., 2008). Moreover, immunologically naïve and domestic populations can have large significant negative consequences of infection in terms of mortality, raising both conservation and economic concerns (Atkinson, 1999; Atkinson and Van Riper III, 1991; Cellier-Holzem et al., 2010; Van Riper et al., 1986; Williams, 2005). The introduction of malaria parasites to the Hawaii archipelago is the classical example of the impact of *Plasmodium* on natural populations of birds that have no co-evolutionary history with the parasite. Upon introduction of the mosquito vector, endemic bird species became infected with *Plasmodium relictum* and experienced a dramatic decline in number, due to high infection-induced mortality (Atkinson et al., 1995; Van Riper et al., 1986). Interestingly, a few years after the introduction of the pathogen, local bird populations now seem better able to tolerate the infection while paying much smaller costs (Woodworth et al., 2005). Similarly, infection of domestic birds (i.e., chickens) with *Plasmodium gallinaceum* can lead to very high mortality depending on the age of the host (young chicks may suffer up to 80% mortality) and the inoculum size (Williams, 2005).

In spite of the importance of avian malaria for the functioning of natural populations and domestic animals, immunity to avian malaria has been poorly studied. In the last decade, a few studies have reported a number of associations between major histocompatibility complex (MHC) alleles and *Plasmodium* prevalence and parasitaemia (Bonneaud et al., 2006; Loiseau et al., 2008, 2011; Westerdahl, et al., 2005, 2012). However, experimental approaches are mostly lacking.

Among the possible effectors that might contribute to the control of the acute phase of the infection, nitric oxide is a very good candidate. Nitric oxide (NO) is a highly reactive and unstable free-radical gas that is produced by the oxidation of L-arginine to citrulline by the enzyme inducible NO synthase (iNOS) (Vincendeau et al., 2003). iNOS is rapidly synthesized by a wide array of cells and tissues in response to pro-inflammatory cytokines produced during the infection (Rivero, 2006). NO has both suppressive and stimulatory functions: it inhibits and promotes cell proliferation, it modulates the production of cytokines, chemokines and growth factors, and it directly acts as a non-specific cytotoxic effector molecule (Bogdan et al., 2000). Previous work has shown that NO has a cytostatic (cessation of growth) and cytotoxic effect on different *Plasmodium* species both *in vitro* and *in vivo*) (Taylor-Robinson, 1997; Taylor-Robinson and Looker, 1998; Taylor-Robinson and Smith, 1999). Interestingly, NO is an immune effector shared by both vectors and vertebrate hosts (Rivero, 2006).

Epidemiological studies have also reported negative correlations between severity of malaria infection in children and iNOS expression (Anstey et al., 1996). Similarly, in the mosquito vector, induction of iNOS

expression contributes to control infection with *Plasmodium* parasites (Luckhart et al., 1998; Peterson et al., 2007). Evidence for avian hosts is, however, restricted to a single study where *in vivo* NO production by macrophages isolated from chickens infected with *Plasmodium gallinaceum* was positively correlated with parasitaemia (Macchi et al., 2010).

In this article, we wished to explore experimentally the role plaid by NO in the regulation of the acute phase of the infection with *Plasmodium relictum* in domestic canaries (*Serinus canaria*). This was achieved by using the specific iNOS inhibitor aminoguanidine (AG) (Allen, 1997; Wideman et al., 2006). In addition to assessing parasitaemia in AG-treated and control hosts, we also measured the cost of infection in the absence of a functional NO response.

## 2. **Material and methods**

### *2.1 Bird husbandry*

The experiment was conducted during the autumn of 2009. Birds were kept in individual cages (0.6 x 0.4 x 0.4 m), with food (commercial seed mix, Versele-Laga, Belgium), grit and water provided *ad libitum*. The temperature was kept constant (21 ± 1°C), under a controlled daily light cycles (LD 13:11 h). The birds originated from a bird breeder and were kept under the above conditions three weeks before the start of the experiment.

The experiments were performed under the licence # 21-CAE-085 delivered by the departmental veterinary service.

### *2.2 Experiment 1: Experimental inhibition of the NO response*

To check whether AG has an inhibitory effect on the iNOS as reported for chickens, we performed an experiment where non-infected domestic canaries were either treated with AG [intraperitoneal injection of 1mg AG dissolved in 100 µL of phosphate buffer saline (PBS)] or kept as control and injected with the same volume of PBS. Within each of these treatments, half of the birds had their inflammatory response stimulated by an intraperitoneal injection of *Escherichia coli* lipopolysaccharide (LPS) (0.02mg dissolved in 100 µL of PBS), whereas the other half received a same volume PBS injection. Each experimental group contained 9 birds. We took a blood sample (ca 100 µL) from the brachial vein before the treatment started (h0) and after nine hours (h9) to measure plasmatic concentrations of NO. Nine hours post-challenge corresponds to the peak of LPS-induced NO production (Takahashi et al., 1999).

NO production was indirectly measured using the Griess reaction. Because of the very short half life of nitric oxide (few seconds) in biological tissues, NO was measured by quantifying nitrates ($NO_3^-$) and nitrites ($NO_2^-$) (NOx). NOx were produced during the reaction with different oxygen species (Sild and Horak, 2009). First, $ZnSO_4$ and NaOH solutions were added to deproteinize the plasma. The supernatant produced by this reaction was recovered and a glycine buffer was added. In the second step, nitrate was reduced to nitrite by using cadmium granules, activated with sulfuric acid and $CuSO_4$ solutions. The last step consists in the Griess reaction, in which plasma products were put in a microplate and Griess reagent (sulphanilamide and *N*-naphtylethylene-diamine) was added. The microplate was placed into a spectrophotometer at 25°C, under shaker. Spectrophotometric measurements were done at 540nm and measures were taken every 5 minutes for 30 minutes (SPECTRAMaxPLUS384, Molecular Devices). We used the optical density (OD) values at 30 minutes. NOx concentrations were determined using a standard curve of known nitrate concentration. Standards were obtained by successive dilutions of a $NO3^-$ solution at 100µM. A full description of the method can be found in Sild and Horak (2009).

### 2.3 *Experiment 2: Effects of aminoguanidine on parasitaemia and cost of infection*

Parasites used for the experimental infections were obtained from a natural population of house sparrows (*Passer domesticus*) in Dijon, France and cryopreserved at -80°C. Blood (ca. 200 µl) of SGS1 infected house sparrows [as detected by a nested PCR method (Waldenström et al., 2004) that amplifies a section of the mitochondrial cytochrome b gene and sequencing of the PCR products] was intraperitoneally injected into domestic canaries. At day 10 post-infection, blood of infected canaries was cryopreserved using the protocol described in Diggs et al. (1975). Briefly, fresh infected heparinized blood was centrifuged at 800g for 5 minutes and the supernatant removed. A cryopreserving

solution (6.2M glycerol + 0.14M Na lactate + 0.0005M KCl + PBS to 500ml) was added dropwise with gentle vortexing to packed red blood cells at 4 to 1 volumes. Blood was then stored at -80° C. For the present experiment, cryopreserved blood was thawed at 37° without agitation for 2 minutes. We then added 0.2 volume of 12% NaCl (dropwise with gentle vortexing), allowed to stand for 5 minutes and added 9 volumes of 1.6% NaCl dropwise as above. Blood was then centrifuged for 5 minutes at 650g and the pellet resuspended in PBS.

Thawed blood was directly transferred intraperitoneally into five domestic canaries, using 0.5 ml insulin syringes, in order to increase parasite intensity. Eleven days after infection, we measured the haematocrit of these five birds and prepared blood smears for microscopic examination. Smears were made by spreading a drop of blood from each bird on a glass slide, fixing with absolute methanol and then staining with 10% Giemsa solution (Sigma–Aldrich). We counted the number of asexual infectious stages of the parasite observed in a total of 10,000 erythrocytes. Parasite intensities and haematocrit allowed us to evaluate the number of parasites per µl of blood for each bird (a haematocrit of 50% corresponded approximately to 5,000,000 erythrocytes per microliter of blood). We collected blood from donors, which was subsequently diluted in 0.9% saline solution to obtain the desired number of parasites per inoculum.

In a 2-way factorial design, we investigated the effects of AG, and hence the effect of NO synthesis inhibition, on parasitaemia of *P. relictum*. For this purpose, 60 non-infected canaries were randomly distributed among four experimental groups (n = 15 per group). At day 0, the first group was intraperitoneally inoculated with a dose of 1 x $10^6$ parasites (lineage SGS1) and received a daily injection of 1mg of AG (in 100 µl of PBS) until day 15 post-infection ($AG^+/P^+$). The second group was infected with the same sized parasite inoculum but only received a daily injection of 100 µl of PBS ($AG^-/P^+$). The third group was sham-infected and received the same daily injection of AG as group 1 ($AG^+/P^-$). The final group served as a double negative control since birds were sham infected and received a daily injection of PBS ($AG^-/P^-$). Sham infection was performed by injecting a volume of PBS (50 µl) corresponding to the volume of parasite inoculum. Previous work has shown that injecting PBS represents an appropriate control similar to injecting non-infected blood (Cellier-Holzem et al., 2010).

Birds were monitored at day 5, 8, 10, 14 and 17 post-infection. At each of these time points, we recorded body mass to the nearest 0.1 g and we collected a blood sample from the left brachial vein using heparinized capillaries. Twenty microliters were used to assess haematocrit after centrifugation for 5 min at 10,000 rpm; 20µl were flushed with 500 µl of Queen Lysis Buffer for parasite quantification.

Parasitaemia was assessed using a quantitative PCR, following the protocol described in Cellier-Holzem et al. (2010). For each individual we conducted two qPCR reactions in the same run: one targeting the nuclear 18s rDNA gene of *Plasmodium* (Primers 18sPlasm7 (5'-AGC CTG AGA AAT AGC TAC CAC ATC TA-3'), 18sPlasm8 (5'-TGT TAT TTC TTG TCA CTA CCT CTC TTC TTT-3'), and fluorescent probe Plasm Hyb2 (5'-6FAM-CAG CAG GCG CGT AAA TTA CCC AAT TC-BHQ1-3')); and the other targeting the 18s rDNA gene of birds (Primers 18sAv7 (5'-GAA ACT CGC AAT GGC TCA TTA AAT C-3'), 18sAv8 (5'-TAT TAG CTC TAG AAT TAC CAC AGT TAT CCA-3') and fluorescent probe 18sAv Hyb (5'-VIC-TAT GGT TCC TTT GGT CGC TC-BHQ1-3')). Parasite intensities were calculated as relative quantification values (RQ) as $2^{-(Ct\ 18s\ Plasmodium\ -\ Ct\ 18s\ Bird)}$ using the software SDS 2.2 (Applied Biosystem). Ct represents the number of PCR cycles at which fluorescence is first detected as statistically significant above the baseline and RQ can be interpreted as the fold-amount of target gene (*Plasmodium* 18s rDNA) with respect to the amount of the reference gene (host 18s rDNA). All qPCR reactions were carried out in an ABI Prism 7900 cycler (Applied Biosystem).

### *2.4 Statistical analyses*

#### *2.4.1 Experiment 1: experimental inhibition of the NO response by aminoguanidine*

The effect of treatments on NOx concentration (log-transformed) at h0 and h9 was investigated using a Kruskal-Wallis test.

#### *2.4.2 Experiment 2: Effects of aminoguanidine on parasitaemia and cost of infection*

Changes in log-transformed parasitaemia were modelled using a Generalized Linear Mixed Model (GLMM) with a beta distribution of errors (Duerr et al., 2004). Time post-infection, squared time post-infection, treatment (AG vs control) and the two-way interactions (time * treatment, squared time * treatment) were included as fixed factors. Individual identity was declared as a random factor to take into account the repeated measures of individuals. Degrees of freedom were corrected using the Satterthwaite method. Obviously, this analysis only concerned experimentally infected birds.

The physiological cost of infection was assessed by changes in body mass and haematocrit during the course of the experiment. For our measures of parasitaemia, we used GLMMs with a normal distribution of errors and Satterthwaite correction for degrees of freedom. The models included time post-infection, squared time post-infection, treatment (AG vs. control), infectious status (infected vs non-infected), the two- and three-way interactions as fixed effects. Individual identity was declared as a random effect.

All tests were performed using SAS v.9.2 (SAS 2002)

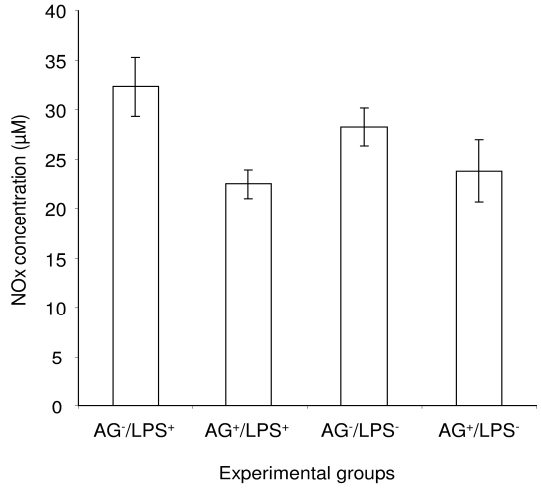


Fig. 1. Mean (± SE) NOx concentration in domestic canaries that were injected with LPS (or PBS as a control) and with aminoguanidine (AG) (or PBS as a control), giving rise to four experimental groups ($AG^-/LPS^+$, $AG^+/LPS^+$, $AG^-/LPS^-$, $AG^+/LPS^-$). NOx was measured 9 hours post-challenge.

## 3. **Results**

### *3.1 Experiment 1: experimental inhibition of the NO response by aminoguanidine*

At time h0, NOx concentration did not differ among the four groups ($X^2_3$=3.21, $P$=0.36). At time h9, the $AG^-/LPS^+$ group had a statistically significant higher NOx concentration than the $AG^+/LPS^+$ group ($X^2_3$=8.57, $P$=0.036) (Fig.1).

### *3.2 Experiment 2: Effects of aminoguanidine on parasitaemia and cost of infection*

Parasitaemia of experimentally infected birds not treated with AG showed the expected bell-shaped variation with time, reaching a peak at day 14 pi. Parasitaemia of AG-treated birds, however showed a steady increase (with the exception of day 11 pi), with peak parasitaemia being reached at day 17 pi. This resulted in a statistically significant interaction between squared time and treatment (Table 1, Fig. 2).

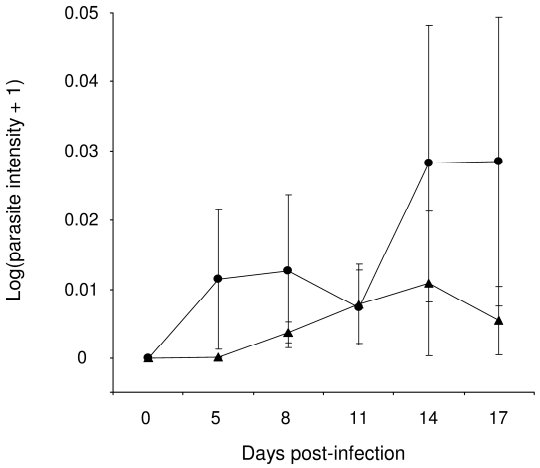


Fig. 2. Changes in parasitaemia (mean ± SE) during the course of the experiment. Triangles represent birds in the $AG^-/P^+$ group, and dots birds in the $AG^+/P^+$ group.

Table 1. Generalized linear mixed model showing the effect of the iNOS inhibitor aminoguanidine on *Plasmodium relictum* parasitaemia. Time refers to days post-infection. Bird identity was declared as a random factor.

| Source of variation | DF | F | P |
|---|---|---|---|
| Time | 1,80.1 | 4.29 | 0.042 |
| Squared time | 1,80.09 | 2.86 | 0.095 |
| Aminoguanidine treatment (AG) | 1,84.67 | 4.32 | 0.041 |
| Time * AG | 1,80.1 | 4.68 | 0.034 |
| Squared time * AG | 1,80.09 | 5.16 | 0.026 |

Infection was costly in terms of haematocrit. Infected birds suffered a clear drop in haematocrit with minimum values reached at day 11 pi, whereas the haematocrit level of non-infected birds remained constant through the experimental period (Fig. 3). This resulted in a statistically significant interaction between squared time and infectious status (Table 2). Interestingly, however, variation in haematocrit did not depend on the

aminoguanidine treatment (Table 2), in spite of infected, AG-treated birds having higher parasitaemia.

Body mass was not affected by either infectious status nor AG treatment (Table 3).

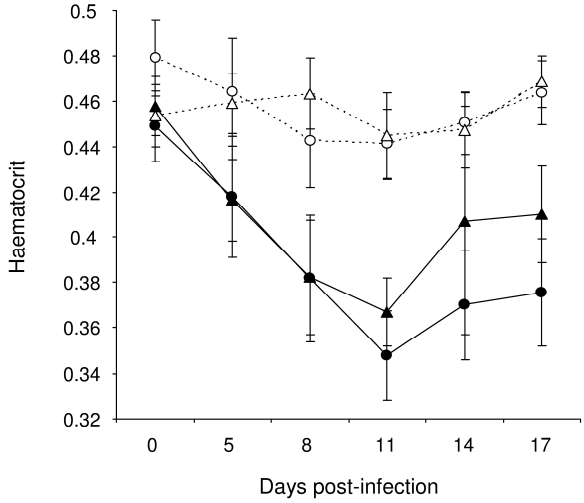


Fig. 3. Variation in haematocrit (mean ± SE) during *Plasmodium relictum* infection. Solid lines and black triangles represent birds in the $AG^-/P^+$ group, dashed lines and white triangles birds in the $AG^-/P^-$ group, solid lines and black dots birds in the $AG^+/P^+$ group, and dashed lines and white dots birds in the $AG^+/P^-$ group.

Table 2. Generalized linear mixed model showing the effect of the iNOS inhibitor aminoguanidine and the infection status with *Plasmodium relictum* on haematocrit. Time refers to days post-infection. Bird identity was declared as a random factor. Table A reports the full model; table B reports the model after removal of non-significant interactions.

A.

| Source of variation | DF | F | P |
|---|---|---|---|
| Time | 1,263.4 | 32.98 | < 0.001 |
| Squared time | 1,261.9 | 20.16 | < 0.001 |
| Infection | 1,149.3 | 0.19 | 0.668 |
| Aminoguanidine treatment (AG) | 1,149.3 | 0.03 | 0.866 |
| Time * AG | 1,263.4 | 0.10 | 0.756 |
| Time * infection | 1,263.4 | 11.72 | 0.001 |
| Squared time * infection | 1,261.9 | 5.37 | 0.021 |
| Squared time * AG | 1,261.9 | 0.00 | 0.954 |
| Infection * AG | 1,149.3 | 0.64 | 0.426 |
| Time * infection * AG | 1,263.4 | 1.28 | 0.260 |
| Squared time * infection * AG | 1,261.9 | 1.72 | 0.191 |

B.

| Source of variation | DF | F | P |
|---|---|---|---|
| Time | 1,267.3 | 32.96 | < 0.001 |
| Squared time | 1,265.8 | 20.06 | < 0.001 |
| Infection | 1,152 | 0.19 | 0.664 |
| Aminoguanidine treatment (AG) | 1,57.08 | 0.14 | 0.710 |
| Time * infection | 1,267.4 | 12.03 | 0.001 |
| Squared time * infection | 1,266 | 5.55 | 0.019 |

## 4. **Discussion**

The aim of this study was to experimentally assess the contribution of a specific immunological pathway (the NO response) to the control of *Plasmodium relictum* (lineage SGS1) parasitaemia in domestic canaries. Even though aminoguanidine has already been shown to be an effective inhibitor of iNOS in chickens (Wideman et al., 2006), we first wished to check whether its inhibitory function was preserved in domestic canaries. In agreement with the results reported for chickens, we found that LPS injected birds had a smaller NO response when simultaneously injected with AG. It is worthwhile to note that the inhibitory effect was not total and AG-

treated birds did produce some NO upon stimulation with LPS.

AG-treated canaries were less able to control the acute phase of infection with *Plasmodium relictum* compared to control animals, suggesting that NO contributes to the immunological defences deployed during the infection with avian malaria. This is in agreement with previous results involving other malaria parasites infecting mammalian hosts (see Taylor-Robinson, 2010 for a recent review). Our results therefore corroborate the well-established idea that NO has important anti-parasitic properties. Several studies have used similar experimental approaches to manipulate the NO response in insects and vertebrates. For instance, in the mosquito *Anopheles gambiae*, the inhibition of nitric oxide with inert L-arginine leads to a decrease in the ability of mosquitos to kill *Escherichia coli* bacteria (Hillyer and Estevez-Lao, 2010). In another mosquito species, *Anopheles stephensi*, a provision of the NOS substrate, L-ARGININE, reduced *Plasmodium* infections, whereas a dietary provision of the NO inhibitor L-NAME significantly increased parasite burden (Luckhart et al., 1998). Moreover, nitric oxide is associated with *Plasmodium* ookinete lysis (Peterson et al., 2007). In vertebrates, the role of NO as an effective immune effector against malaria has only been explored in humans (Anstey et al., 1996; Hobbs et al., 2002) and mice (Taylor-Robinson, 2010). Wang et al. (2009) showed that mice experimentally infected with *Plasmodium yoelii* exhibit an increase in NO which coincides with a decrease in parasitaemia. Some authors have even suggested that supplementation with the nitric oxide synthetic metabolite (S-nitrate) could be used as a therapy against *Plasmodium* infection (Nahrevanian et al., 2008).

Table 3. Generalized linear mixed model showing the effect of the iNOS inhibitor aminoguanidine and the infection status with *Plasmodium relictum* on body mass. Time refers to days post-infection. Bird identity was declared as a random factor. Table A reports the full model; table B reports the model after removal of non-significant interactions.

A.

| Source of variation | DF | F | P |
|---|---|---|---|
| Time | 1,276.9 | 3.12 | 0.079 |
| Squared time | 1,276.6 | 0.59 | 0.441 |
| Infection | 1,62.41 | 0.00 | 0.988 |
| Aminoguanidine treatment (AG) | 1,62.41 | 0.13 | 0.721 |
| Time * AG | 1,276.9 | 0.38 | 0.540 |
| Time * infection | 1,276.9 | 2.68 | 0.103 |
| Squared time * infection | 1,276.6 | 2.42 | 0.121 |
| Squared time * AG | 1,276.6 | 0.24 | 0.627 |
| Infection * AG | 1,62.41 | 0.34 | 0.560 |
| Time * infection * AG | 1,276.9 | 1.21 | 0.271 |
| Squared time * infection * AG | 1,276.6 | 1.56 | 0.213 |

B.

| Source of variation | DF | F | P |
|---|---|---|---|
| Time | 1,282.9 | 3.17 | 0.076 |
| Squared time | 1,282.6 | 0.59 | 0.442 |
| Infection | 1,57.18 | 0.10 | 0.757 |
| Aminoguanidine treatment (AG) | 1,57.18 | 0.04 | 0.837 |

Although our finding that AG-treated birds suffered from increased parasitaemia strongly suggests a role for NO in the control of the acute phase of the infection with *Plasmodium relictum*, a definitive conclusion cannot be drawn without measuring NO production following the experimental infection. We did not have a clear prediction on when would be the best time to measure NO during the infection period. Ideally, we would have measured NO on a daily basis but daily blood sampling would have certainly induced too much stress. Future work should nevertheless be directed towards establishing the link between NO production and parasitaemia in this system.

In spite of their increased parasitaemia, AG-treated birds did not seem to pay a higher cost of infection compared to control individuals. Haematocrit level, body mass and mortality (only 4 birds died during the experiment, one in $AG^+/P^-$ group, one in $AG^+/P^+$ group, and two in $AG^-/P^+$ group) did not differ between AG-treated and control hosts. A visual inspection of figure 3 suggests that AG-treated birds have a lower haematocrit

especially at day 14 and 17 p.i., that is when parasitaemia reaches its maximum values. However, there is no statistical support to the idea that AG-treated birds had a lower haematocrit compared to control individuals. Even when restricting the analysis to infected birds, the difference in haematocrit between $AG^-$ and $AG^+$ individuals was very far from reaching the significance threshold (time p.i. * AG treatment, $F_{1,131} = 0.11$, $p = 0.740$; squared time p.i. * AG treatment, $F_{1,130} = 0.33$, $p = 0.564$). These results might appear somewhat puzzling because infection does incur costs in our model system, especially in terms of reduction in haematocrit level (Cellier-Holzem et al., 2010; the present study). A reduction in haematocrit is partly the direct consequence of the asexual reproduction of the parasites within the red blood cells and the subsequent lysis and release of merozoites in the blood stream. Since haematocrit levels and parasitaemia are usually negatively correlated (the more parasites, the more lysis of red blood cells takes place) we should have expected that AG-treated birds paid a higher cost of infection. Anaemia and haematocrit reduction could also partly arise as a consequence of immune responsiveness, with immune effectors targeting infected red blood cells. Indeed, in a rodent malaria system, it has been estimated that 10% of anaemia is due to an over-reacting immune response (Graham et al., 2005b). We might then speculate that inhibiting the NO response produced two counter-balancing effects: increased parasitaemia enhances the cost of infection, but reduced NO production also reduces the costs of the immune defence.

These results can feed the current debate on the relationship between parasite multiplication and virulence (the trade-off model for the evolution of parasite virulence) and the role played by immunopathology as a major determinant of virulence (Alizon et al., 2009; Day et al., 2007; Graham et al., 2005a; Long and Graham, 2011). Nevertheless, the idea that down-regulating the immune response decouples the cost of infection from parasitaemia undoubtedly requires further work to be fully established.

We found no effect of *Plasmodium* infection and AG treatment on body mass. Benign environmental conditions, with *ad libitum* food and water, and constant temperature, might contribute to explain this result. Interestingly, other studies based on experimental infection of European passerines in the lab have reported a similar lack of effect of infection on body mass (Palinauskas et al., 2008, 2011; Zehtindjiev et al., 2008). These results suggest that, in addition to the role plaid by favourable environmental conditions, a lost in body mass is not include in the physiological costs of infection with *Plasmodium relictum* for birds that have coevolved with the parasite. A different picture emerges for host species that did not coevolve with the parasite, as shown by the experimental infection of Hawaiian birds with *Plasmodium relictum*. Both *Myadestes obscurus* and *Hemignathus virens* have been shown to suffer from a substantial decrease in body mass following the infection with *Plasmodium relictum* (Atkinson et al., 2000, 2001).

Parasitaemia was highly variable among infected birds. Among-individual variation in parasite intensity is a common finding (Cellier-Holzem et al., 2010; Palinauskas et al., 2009; Zehtindjiev et al., 2008), and multiple sources may account for this Individual hosts vary in their genetic background and this can shape their susceptibility to infection (Bonneaud et al., 2006; Loiseau et al., 2008, 2011; Westerdahl, et al., 2005, 2012). In addition host age and sex might also contribute to generate among-individual variation in parasite intensity (McCurdy et al., 1998; Sol et al., 2000; Williams, 2005).

Vector borne parasites have to face and adapt to different environments (the vector and the host) to complete their life cycle. Shared immunological pathways between the vector and the host could therefore be the main target of immune-mediated selection acting on the parasite. Nitric oxide is one such shared immunological pathway, and we might expect parasites to evolve strategies to escape the NO produced in response to infection. Indeed, it has been suggested that some protozoa (*Trypanosoma cruzi* and *Leishmania major*) can deplete the substrate of the NOS (L-arginine) by activating arginases (Vincendeau et al., 2003). Whether *Plasmodium* parasites have evolved the same escape strategy remains an open question.

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# Manuscrit 2

## Environment does not modulate costs of infection in dominant male canaries

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En préparation

# Environment does not modulate costs of infection in dominant male canaries

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**Abstract:** Understanding the different factors that may influence parasite virulence is of fundamental interest to ecologists and evolutionary biology. It has recently been demonstrated that part of the virulence of a parasitic infection may occur through manipulation of host competitive ability. In vertebrate species with social hierarchies, we proposed that differences in competitive ability associated with the behaviour/social status of a host may determine the outcome of parasitic infection. We observed flocks of domestic canaries to determine dominant or subordinate birds, and modified competition by providing restricted food (high competition) or ad libitum (low competition). Entire flocks were then infected with Plasmodium relictum. Contrary to our predictions we found that diet had no effect on the outcome of infection for dominant or subordinate birds, although the reduced diet did result in lower body masses overall. We found that dominant birds appeared to suffer greater infection mediated morbidity in both dietary treatments, with a greater reduction in haematocrit and higher intensity of infection. Our results show that dominance status in birds can certainly alter parasite virulence, though differences between individual hosts are likely to be multifaceted.

**Keywords:** Avian malaria, competition, infection, group living, social rank, virulence, social stress

## Introduction

The ability to resist and recover from pathogenic infection is one of the major fitness-determining traits shared by all animals. However, often parasites will differ in their virulence, the degree of morbidity and mortality they inflict upon hosts. Understanding the factors that drive these differences in virulence is of fundamental interest. For a given host, extrinsic factors such as parasite genotype and environment may modulate parasite virulence. For example, it has been shown that parasite virulence may be altered when host environment differs in factors such as temperature (Blanford et al., 2003), host density (Yourth et al., 2002), and food availability (Bedhomme et al 2004). Similarly, intrinsic factors such as host genotype (Lefevre et al. 2007), sex (de Roode et al., 2006) or age (Gardner and Remmington, 1977) may affect parasite virulence. A further difference between hosts that may potentially shape the outcome of parasitic infection is the social status of the host, especially in vertebrate species with social hierarchies. Here, the behaviour of a host may determine the outcome of parasitic infection (ref). Indeed, there is growing interest into how some animals, including birds, develop stable and profound differences between individuals in their behavioural profiles (Sih and Bell 2008). How such differences in behaviour or social status translate into differences in parasite virulence following infection remains unclear.

Dominance is associated with a number of benefits in wild birds, for example access to the best feeding opportunities (Parisot et al. 2004), predator free foodsites (Schneider, 1984), roosting positions (Weatherhead and Hoysak, 1984), or mating success (Post 1992). Despite these benefits there is increasing understanding of the costs of dominance. Social stress, the physiological stress

associated with attaining or maintaining a dominant social position, is a topic of current investigation (Creel et al., 1996). Several studies have demonstrated chronic elevated levels of potentially damaging hormones in dominant birds, compared with subordinates (e.g. Goymann et al. 2004; Goymann and Wingfield 2004). In addition, some evidence suggests that dominant individuals may have reduced immune function compared to subordinates (Li et al. 2007), although in other cases the reverse is true (Ungerfeld and Correa 2007). In a recent experiment, we showed that parasite-mediated morbidity and mortality in canaries was dependent on the social status of the host, when receiving a limited diet (Larcombe et al. submitted). Throughout this study, all birds received a reduced quantity of seeds, which may have altered the patterns of parasite virulence we observed in subordinate and dominant birds. Firstly, the energetic costs of obtaining and protecting food resources are likely to be higher for dominants than subordinates, especially since there is some evidence that dominant birds may have higher metabolic rates (Hogstad 1987). These costs of food gathering and food site protection will be increased when less food is available. Competition-mediated differences in parasite virulence may therefore be more severe for dominants than subordinates, when food is scarce, compared to food rich environments. For socially tolerant subordinates, the influence of food availability on competition and parasite virulence, is likely to be less severe. In this study, we tested whether mortality or morbidity of canaries infected with *Plasmodium relictum* differed between dominants and subordinates, receiving either a reduced or ad libitum diet.

The goal of this study was to assess the interactive effects between social status, and food availbility, on parasite virulence using domestic canaries as hosts and the avian malarial parasite *Plasmodium relictum* as a parasite. By keeping canaries in flocks of 5 birds, and scoring for consistent feeding behaviours, we divided birds into 2 categories: dominant (D) and subordinate (S) within each flock. Half of the flocks received an ad libitum diet, and the other half received a limited though adequate diet. Following infection, we measured morbidity (change in mass and haematocrit) and mortality of hosts, in addition to parasite intensity. In line with our previous experiment we predicted the following outcomes:

5. Infected dominant birds to have higher morbidity/mortality than infected subordinates in limited food groups
6. Infected dominant birds receiving an ad libitum diet would have lower morbidity/mortality, than infected dominants receiving a reduced diet.
7. The difference in morbidity/mortality between ad libitum and reduced diet infected birds should be greater in dominant than subordinate birds.

**Material and methods**

We used 30 adult male canaries during the experiment, and prior to commencement each bird was molecularly sexed following a standard PCR technique (Fridolffson and Ellegren 1999). We only used male canaries in the experiment as we did not wish to confound the experiment with differences between sexes, or by interactions in- and between pairs of birds. After confirming the sex of each bird, we divided the birds between 6 aviaries (2.5 * 1.5 * 2.2 m), 5 birds per aviary. Each bird was weighed, and had its tarsus length measured prior to re-housing in a new flock.

*Husbandry and Diet Manipulation*

Before commencing the diet manipulations, all cages were provided with ad libitum food (a commercial seed mix, lettuce, apple and hard-boiled egg) for 7 days. Since we were interested in determining costs of dominance and infection under different environmental conditions, we divided the flocks between two different feeding regimes. Following the 7 days of acclimation, the birds were provided with either ad libitum food or reduced food. Ad libitum diet consisted of 3 large round feeding dishes, each full of seeds. Reduced diet consisted of just one dish per cage, with 12g of seeds per bird per day. We had previously found that 12g of seeds is the maximum amount a single bird would eat per day (Larcombe et al.in review). This amount of seed was thus sufficient to nourish each bird, though allowed competition between birds (pers obs). During the course of the experiment, the cages were monitored daily, and if a bird died the amount of seed was reduced accordingly (in Reduced food flocks).

*Behavioural observation*

We performed behavioural observations to assess the social status and dominance related behaviours of each the birds in each flock. The procedure was similar to that outlined in Larcombe et al. (in review), but with some modifications. The first phase of observations was carried out 3 days before the start of the experimental diets, when all birds received an identical diet. The second phase of observations took place 11 days after being placed in their flocks. We performed behavioural observations for 3 consecutive days in both phases. Each morning at 09.00 we removed the remaining seed from the day before, and left cages for 1 hour without seeds. Following the 30 minute food deprivation, we placed a seed feeder in each cage that allowed only a single bird to feed at a time. We also placed a video camera in each cage and filmed the interactions between birds at the feeder for 20 minutes, starting when the feeder was first entered. Birds were marked with non-toxic colored pen on the back of the head or wings for identification on the video tapes.

In order to score the bird's behaviour, when the video was re-watched the 20 minute time period was divided into 10 two minute blocks. Birds were scored for the presence or absence of certain behaviours in each block: Primary Access (PA) to the feeder, where a bird successfully fed directly from the hole in the feeder. Secondary Access (SA), when a bird was motivated to feed, and appeared at the feeder, either attempting to feed, or pecking at discarded seeds, but did not achieve Primary Access. Aggression (AGG), where a bird aggressively postured towards another, typically by lowering its head and fanning and trembling its wings, or by pecking out at the other bird, sometimes escalating into a physical fight. All of these behaviours are repeatable across days for canaries (Larcombe et al. in review)

In this experiment we were interested in associating costs of infection and competition with differences in social behaviour. Rather than categorizing birds based on an assumption of linear hierarchies in each cage, here we scored birds as dominant or subordinate depending on the ratio of primary to secondary access to the feeder. Both these scores indicate a motivation to feed and so comparing the occasions spent as the primary bird, to a secondary bird (waiting near the feeder), offers a good approximation of the relative dominance status. We calculated this dominance ratio based on data from the second phase of observations as (PA day 9 + PA day 10 + PA day 11 +1) / (SA day 9 + SA day 10 + SA day 11 +1). Where the ratio was $\geq 1$ a bird was categorized as dominant, where it was $<1$ the bird was classified as subordinate. We did not use the data from the first phase of observations, since at that time the bird's seed diet was augmented with other food items (see above), and overall the birds were less motivated to feed. However, it is important to note that even allowing for this, the dominance ratio pre experiment (phase 1) was significantly positively correlated with the dominance ratio during the experiment (phase 2) (spearman's $\rho = 0.787$, $p < 0.0001$).

*Experimental Infection*

In order to assess the costs of infection determined by social status and food treatment, we performed a series of experimental infections. Infected group canaries were intraperitoneally inoculated with $5x10^5$ *Plasmodium relictum* parasites, strain SGS1. Parasites were initially obtained from a natural population of house sparrows (*Passer domesticus*), and transferred to domestic canaries. Parasites were subsequently maintained as a cryopreserved stock.

On the day of infection, we captured all birds within a flock. Each bird was weighed, and a small volume of blood was taken in a capillary tube for subsequent haematocrit assessment. Finally, the bird was injected with *Plasmodium* infected canary blood. We had four treatment schemes which were divided randomly within the aviary.

*Post-infection monitoring*

Following the experimental infection (day 0), birds were left in their flocks, and were monitored at regular intervals. We re-caught all birds on days 5, 8, 11, 14 and 17 post-infection. On each of these sampling days, we took a small blood sample for haematocrit measurement and qPCR, and weighed each bird. The measurement of haematocrit offered a good indication of the specific cost of infection, since a negative change in

haematocrit (the proportion of red cells in a given sample of blood) can be directly representative of damage caused by malarial parasites in canaries (Spencer et al. 2005, Cellier-Holzem et al. 2011).

**Table 1** Output from GLMM testing effects of experimental treatments and dominance on haematocrit in canaries.

| Random factor | Estimate | | |
|---|---|---|---|
| Bird (cage) | 0.005600 +/- 0.006732 | | |
| Time*bird (cage) | 0.2270 +/- 0.1488 | | |
| Residual | 0.008184+/- 0.001656 | | |
| | | | |
| *Main Effects* | *Estimate* | $F_{d.f.}$ | *P* |
| Baseline Haematocrit | 2.6243 +/- 0.5636 | $21.68_{1, 21.68}$ | 0.0001 |
| Time | -0.1181 +/- 0.02093 | $30.64_{1, 90.61}$ | <0.0001 |
| $Time^2$ | 0.004461 +/- 0.000856 | $27.16_{1, 89.45}$ | <0.0001 |
| Dominance | -0.09273 +/- 0.1120 | $0.69_{1, 63.76}$ | 0.4109 |
| Dominance*time | 0.01527+/- 0.008532 | $3.21_{1, 40.17}$ | 0.0809 |

**Table 2** Output from GLMM testing effects of experimental treatments and dominance on mass in canaries.

| Random factor | Estimate | | |
|---|---|---|---|
| Bird (cage) | $4.24^{e-17}$+/- . | | |
| Time*bird (cage) | 0.7764+/- 0.05357 | | |
| Residual | 1.2505+/- 0.2607 | | |
| | | | |
| *Main Effects* | *Estimate* | $F_{d.f.}$ | *P* |
| Baseline mass | 1.0075 +/- 0.06870 | $215.12_{1, 24.7}$ | 0.0001 |
| Time | -0.1839 +/- 0.06943 | $7.02_{1, 99.96}$ | 0.0094 |
| $Time^2$ | 0.006629 +/- 0.003016 | $4.83_{1, 100.1}$ | 0.0302 |
| Diet | -0.9456 +/- 0.3540 | $7.14_{1,25.24}$ | 0.0130 |

**Table 3** Output from GLMM testing effects of experimental treatments and dominance on intensity of infection in canaries.

| Random factor | Estimate | | |
|---|---|---|---|
| Bird (cage) | $7.63^{e-22}$+/- . | | |
| Time*bird (cage) | -0.09055+/- 0.08059 | | |
| Residual | 0.002775 +/- 0.00034 | | |
| | | | |
| *Main Effects* | *Estimate* | $F_{d.f.}$ | *P* |
| Time | 0.03802 +/- 0.01147 | $14.20_{1,130.8}$ | 0.0002 |
| $Time^2$ | -0.00166 +/- 0.00049 | $14.58_{1, 130.7}$ | 0.0002 |
| Dominance | 0.1002 +/- 0.06940 | $2.08_{1, 131}$ | 0.1513 |
| Dominance*time | -0.02608 +/- 0.01326 | $3.87_{1,130.8}$ | 0.0513 |
| | | | |
| $Dominance*time^2$ | 0.001141+/- 0.00057 | $4.01_{1,130.7}$ | 0.0472 |

*Assessing parasite intensity*

Parasite intensity was assessed using the quantitative PCR assay developed by Esparza-Salas *et al.* (submitted). For each individual we conducted two qPCR reactions in the same run: one targeting the nuclear 18s rDNA gene of *Plasmodium* (Primers 18sPlasm7 (5'-AGC CTG AGA AAT AGC TAC CAC ATC TA-3'), 18sPlasm8 (5'-TGT TAT TTC TTG TCA CTA CCT CTC TTC TTT-3'), and fluorescent probe Plasm Hyb2 (5'-6FAM-CAG CAG GCG CGT AAA TTA CCC AAT TC-BHQ1-3')) and the other targeting the 18s rDNA gene of bird (Primers 18sAv7 (5'-GAA ACT CGC AAT GGC TCA TTA AAT C-3'), 18sAv8 (5'-TAT TAG CTC TAG AAT TAC CAC AGT TAT CCA-3') and fluorescent probe 18sAv Hyb (5'-VIC-TAT GGT TCC TTT GGT CGC TC-BHQ1-3')).

Parasite intensities were calculated as relative quantification values (RQ) as $2^{-(Ct\ 18s\ Plasmodium\ -\ Ct\ 18s\ Bird)}$ using the software SDS 2.2 (Applied Biosystem). Ct represents the number of PCR cycles at which fluorescence is first

detected as statistically significant above the baseline and RQ can be interpreted as the fold-amount of target gene (*Plasmodium* 18s rDNA) with respect to the amount of the reference gene (host 18s rDNA). All qPCR reactions were carried out in an ABI Prism 7900 cycler (Applied Biosystem).

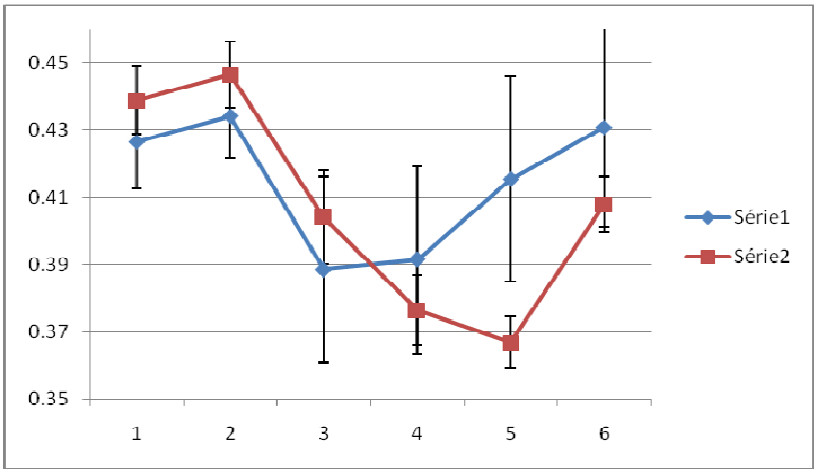


Fig. 1: Haematocrit change in days following infection in dominant and subordinate birds. (Dominant = red, subordinate = blue) (GLMM interaction dominance*infection, F = ??, p = ??).

*Statistic*

For body mass, haematocrit, and intensity of infection we constructed an identical GLMM using SAS (9.1.3). This approach allows for missing values caused by mortality and/or sampling problems. Body mass and intensity of infection were modelled with a normal distribution, and haematocrit was modelled with a binomial distribution. The models were fully factorial and included fixed factors dominance status (dominant/subordinate) and diet (reduced/ad lib), in addition to time and time$^2$ as continuous fixed effects to examine mean changes over time. We also included all possible two and three way interactions between these terms. Additionally we had two random factors. Bird identity nested within cage (bird(cage)) was added, as this allows the model to control for non-independence of birds housed in the same cage over the course of the experiment, and permits the variance between birds to be estimated. We also used time as a random factor with bird(cage) as a subject, using an autoregressive type 1 covariance matrix to estimate within individual variation, controlling for correlations taken closer in time. Baseline measures prior to the experiment were included for models of haematocrit and body mass. For our models explaining intensity of infection we did not a baseline, since this is always zero pre-infection. We also analyzed mortality using a simpler model. We tested the probability of mortality using a binary distribution (event = death), with diet and dominance and their interactions as fixed factors, and including cage as a random factor to control for the non-independence of birds grouped together. This model did not assess time, as very few birds died during the experiment. Non-significant terms were dropped from the models starting with higher-order interactions, until only significant terms remained. Throughout the results relevant statistics are reported from the final model, though statistics for non-significant terms of interest are reported from the point they were dropped from models. Degrees of freedom were corrected using the satterthwaite method.

**Results**

There were no differences in mass (means: dominants =23.49 +/- 0.54, subordinates = 25.05 +/- 0.89, F=2.19, p =0.15) or haematocrit (means: dominants =0.427 +/- 0.009, subordinates = 0.444 +/- 0.015, F=0.93, p =0.34) prior to the experiment.

Diet had no effect on change in haematocrit (Table 1), however there was a

trend for subordinate and dominant birds to differ in their haematocrit changes, where dominant birds seemed to suffer a greater decrease in haematocrit than subordinates (Figure 1).

We found a significant interaction between dominance and time on the intensity of infection for all birds (Table 3), and again, diet had no effect. As for haematocrit, our data show that dominant birds had a greater intensity of infection than subordinate birds (Figure 2).

Body mass did not differ between subordinate and dominant birds (Table 2), however, there was a significant effect of diet. Since this effect was independent of diet, we computed least-squares means for the different diets. Mean body mass following infection was 22.28 +/- 0.254 in reduced food groups, and 22.29 +/- 0.249 in ad lib food groups suggesting the restricted diet did reduce body mass.

We found no evidence that mortality was affected by either dominant status ($p>0.9$) or diet ($p=0.1245$).

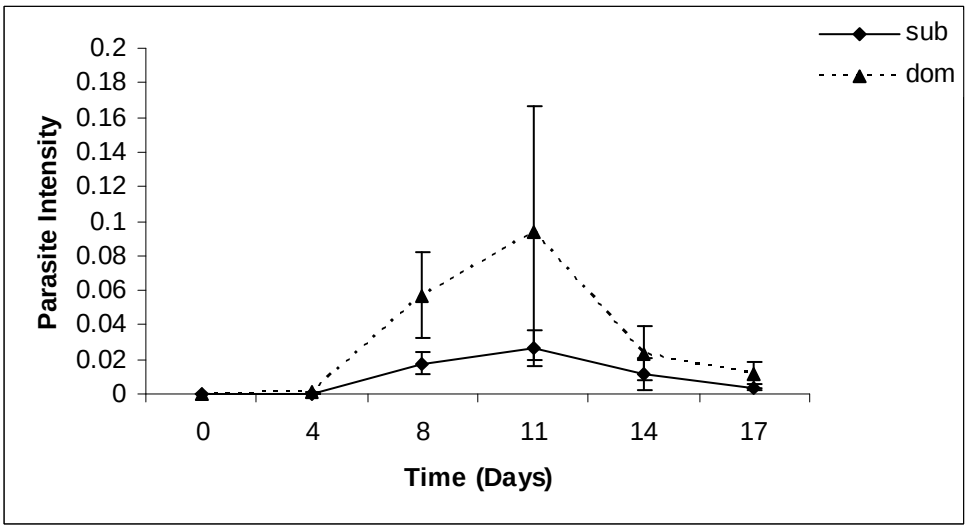


Fig. 2: Change in parasite intensity for infected birds. The legend describes the dominance status of individuals (GLMM time*infection, F = , p = ??)

## Discussion

Our aim in this experiment was to assess whether or not malarial virulence in adult canaries was dependent on host food availability or dominance status. As in a previous experiment, we found that dominant birds consistently paid higher costs of infection, however, we found no evidence that this was altered by the diet the birds received. This is a surprising result which we discuss in terms of host behaviour and physiology.

Firstly, we found a marginally non-significant effect of dominance on the change in haematocrit across the experiment. Dominant birds had greater decreases in haematocrit than subordinate birds, following the infectection. In keeping with our previous experiment, we initially predicted that following infection dominant birds would show greater morbidity and mortality than subordinates in reduced food groups, though we expected this difference to be ameliorated in *ad libitum* groups. In fact, there was no effect of the diet treatment on haematocrit change.

Haematocrit readings can be used as an effective measure of the destruction of red blood cells by malarial parasites, and monitoring the change in haematocrit in birds over the course of this experiment offered an estimation parasite-mediated morbidity. The biological relevance of increased intensity of infection, that is greater parasitaemia of host blood, is less clear (Zehtindjiev et al. 2008), though using experimental infections allowed

us to assess patterns of intensity of infection during the primary infection phase. We found that our patterns of parasite intensity closely matched those of haematocrit. Again, we found that diet did not significantly affect parasite virulence. However, dominant birds showed a greater, sharper, and more variable peak in parasitaemia than subordinates, further suggesting a greater virulence of the malaria parasite in dominant birds. This in spite of the fact that we expected dominants would have a higher competitive ability in our experimental flocks. Why then, do dominant birds fare worse than we expected? Firstly, our predictions were based on the idea that differences in parasite virulence may be mediated by costs of competition between birds. Perhaps, rather than competition, fundamental differences in physiology between dominants and subordinates determine the outcome of infection. It has been noted elsewhere that in captivity subordinate birds cannot escape their dominant competitors leading to unnaturally increased stress levels (Kotrschal, date). By the same token, it is possible that in our (deliberately large) cages dominant birds could never *fully* exclude other birds from the feeding territory, as they might in the wild. Chronic elevation of hormones associated with this unnatural conflict (e.g. Goymann 2009) may result in increased costs of dominance that would otherwise go unpaid. It is noteworthy that here mortality was identical for dominants and subordinates post-infection. Nonetheless, our results show that the ability of hosts to monopolise food resources, may be associated with higher parasite virulence.

Neither haematocrit nor intensity of infection were affected by diet, or interactions between diet and dominance status as predicted. This is a surprising finding. We are confident that there was indeed a difference between the two diets, mainly because we found that diet had a significant effect on body mass, with birds receiving a restricted diet having lower body mass than those fed an ad-lib diet. There are several reasons to expect that diet should influence malarial virulence. Simply, we predicted that increased food availability, or energy availability, should ameliorate the energetic costs of infection and competition. Secondly, some key nutrients, such as antioxidants, found within the diet can aid the immune response (e.g. Hartley and Kennedy 2004). However, interactions between diet and malarial virulence may be more complicated than initially expected. The ability to resist blood borne parasites may depend in part on circulating levels of free radicals. Since a greater concentration of dietary antioxidants in plasma will intercept and impede these radicals, a better quality (or at least antioxidant rich) diet may increase malarial virulence and deficiencies in antioxidants such as selenium and vitamin E may protect against malaria in non-avian species (e.g. Levander and Ager 1993). Indeed, the assumption that generally better nutritional state in hosts will benefit resistance to parasites is far from clear cut. In humans, for example, evidence that Protein Energy Malnutrition (PEM) can actually result in *decreased* malarial virulence is widespread, though disputed (reviewed Shankar 2000). Despite this, we found no evidence that our reduced diet actually helped reduce malarial virulence.

In this study we set out to investigate whether the virulence of malarial infection in canaries was modified by social status and/or food availability. As expected, we showed that dominant birds appeared to suffer greater infection mediated morbidity in reduced food flocks, however, contrary to our expectations this difference was not ameliorated by diet. Indeed, we found little evidence that greater food availability had any effect on traits specifically related to parasite virulence. Our results show that dominance status in birds can certainly alter parasite virulence, though differences between individual hosts are likely to be multifaceted. Further experiments are required to disentangle the different effects of environment and host behaviour and physiology on the costs of parasitic infection.

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# Manuscrit 3

## Impact of host condition on infection dynamics and parasite virulence in a bird-malaria system

Stéphane Cornet, Coraline Bichet, Stephen Larcombe, Bruno Faivre and Gabriele Sorci

Soumis à *Journal of Animal Ecology*

# IMPACT OF HOST CONDITION ON INFECTION DYNAMICS AND PARASITE VIRULENCE IN A BIRD-MALARIA SYSTEM

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*Running headline (45)*
Host nutrition alters *Plasmodium* virulence

**ABSTRACT**

1. Most host-parasite interactions exist in heterogeneous environments, which can affect host and parasite evolution and disease dynamics. However, it is unclear how pathogens respond and adapt to environmental changes.
2. Parasite traits such as development, reproduction and transmission are altered by within-host environmental factors such as host nutritional resources. Recent work with invertebrates shows that past conditions experienced by parasites mediate future levels of infectivity and virulence.
3. We studied the importance of host condition as a source of phenotypic variation in host exploitation by parasites. Using the malaria parasite *Plasmodium relictum* (SGS1) and domestic canaries we investigated (i) how the nutritional condition of the bird hosts altered the *Plasmodium* within-host dynamics of and (ii) how the interaction between past and current environments of the parasite could explain variation in the dynamics of infection. Birds raised under control- or enriched-food diets were experimentally infected. Parasites created in these two environment were subsequently transmitted in a full-factorial design to new hosts reared under similar control or supplemented diets.
4. Host nutrition had strong effects on infection dynamics and virulence and we found complex interactions between past and current environments of the parasite on disease dynamics and costs to the hosts (loss of body weight and anaemia). Food supplementation led to better body condition and resulted in lower parasite density and earlier parasite clearance. Overall *P. relictum* parasites were more successful in control birds, reaching larger population sizes and parasites originating from these hosts (maintained on control-food diet) were found to be more virulent to subsequent hosts compared to parasites originating from supplemented hosts.
5. Our study highlights that parasite virulence is affected by a cross-generational effect of the environment (host condition) and that both current host environment and parasite infection history account for variation in the dynamics of parasite infection.

**KEY-WORDS (10)**
Avian malaria, environmental variation, host-parasite interaction, nutrition, pathogen, *Plasmodium relictum*, virulence

**INTRODUCTION**

Environmental conditions have been recognized as a key determinant of host-parasite interactions in various systems, influencing the variation in the outcome of infection, disease severity and virulence (Lazzaro & Little 2009; Wolinska & King 2009). Because most host-parasite interactions exist in heterogeneous environments, the spatial and temporal variation in environmental conditions is likely to alter important characteristics of host-parasite dynamics, and to contribute to maintaining variation in both in host defences and parasite strategies to exploit hosts.

Nutritional resources show natural spatial and temporal fluctuations in the wild and such variation is likely to affect the evolution of host-parasite interactions and disease epidemiology (Pulkkinen & Ebert 2004; Hall *et al.* 2009). Previous work has already shown that quantity and quality of resources (host nutritional status) directly shape parasite virulence (Brown, Loosli & Schmid-Hempel 2000; Krist *et al.* 2004; Seppälä *et al.* 2008), reproduction and transmission (Bedhomme *et al.* 2004; Restif & Kaltz 2006; Seppälä *et al.* 2008). Parasites are often assumed to perform better in hosts in poor body condition, probably because of reduced immune competence (Krasnov *et al.* 2005). However, poor-quality hosts also provide less resource for parasites and parasite reproductive success is often decreased in poor quality (starved) hosts (Bedhomme *et al.* 2004; Tseng 2006; Tschirren *et al.* 2007; Seppälä *et al.* 2008). Parasite strategy of host exploitation should therefore balance the benefits of resource acquisition and the threat of host's immune defences. Accordingly, parasite fitness should be maximized in hosts of intermediate body condition (Bedhomme *et al.* 2004; Bize *et al.* 2008).

Whether parasite virulence may be altered under current environmental conditions does not provide much information regarding how the environment a parasite experiences at one point will affect its future virulence in the same (or in another) environment. Recent experimental work on invertebrate hosts showed cross-generational effects of host nutrition level on parasite virulence, with past diet conditions experienced by parasites affecting current infections (Tseng 2006; Little *et al.* 2007). For example, *Ascogregarina* parasites (Apicomplexa) produced more offspring (oocysts) and were more detrimental to host fitness when their hosts *Aedes albopictus* mosquitoes, were reared in high-food environments compared to parasites reared in hosts in low-food treatment. Moreover, parasites originating from well-fed hosts were more virulent to subsequent hosts compared to parasites that had propagated on poorly-fed hosts, especially if new hosts were currently reared with low level of food (Tseng 2006). Tseng's paper nicely underlines that parasite may become rapidly adapted, or at least acclimated, to their environment and that past environments experienced by parasites can influence the magnitude of harm they cause to their hosts.

Avian malaria (in particular, *Plasmodium sp.* and *Haemoproteus sp.*) has emerged as a promising model to investigate host-parasite interactions in the wild, coevolutionary processes and parasite-mediated effects on host life histories (for some examples see (Ricklefs, Fallon & Bermingham 2004; Bonneaud *et al.* 2006; Knowles, Palinauskas & Sheldon 2009; Marzal *et al.* 2011; Christe *et al.* 2012; Westerdahl *et al.* 2012). Bird malaria is extremely diverse (Valkiūnas 2005; Bensch, Hellgren & Perez-Tris 2009), highly prevalent in wild passerines (Cosgrove *et al.* 2008; Loiseau *et al.* 2011; Glaizot *et al.* 2012) and natural strains can easily be isolated making avian malaria parasites a convenient model for experiments under laboratory conditions (Palinauskas *et al.* 2008; Cellier-Holzem *et al.* 2010; Bichet *et al.* subm.).

Detrimental effects on host survival have been reported for both natural and experimental infections (Van Riper III *et al.* 1986; Atkinson *et al.* 2000; Williams 2005), and even for chronic infections (Knowles *et al.* 2009; Lachish *et al.* 2011). However, as for many non-model diseases, fundamental knowledge of parasite characteristics such as variation in parasite infectivity or virulence remains limited. Although genetic differences in host susceptibility may explain some

variation in the outcome of infection (Loiseau *et al.* 2011; Westerdahl *et al.* 2012), it is highly likely that there exists a variation among parasite 'strains' in their host exploitative strategies. The latter point has, to our knowledge, not been investigated yet in avian malaria parasites (for clone-specific infection dynamics using the rodent malaria parasite *Plasmodium chabaudi*, see for example Bell *et al.* 2006). In addition, host nutritional status (a part of the environment of the parasite) may account for variation in parasite exploitation. Overall, food quantity and quality are known to affect the level of immune competence in birds (Gonzalez *et al.* 1999; Smith *et al.* 2007). Resource availability and host immune response are also likely to affect parasite traits as found in *P. chabaudi* for parasite growth rate, virulence and transmission rate (Buckling & Read 2001; Mackinnon & Read 2003). *Plasmodium* parasites are thus expected to plastically adjust both in-host (asexual) replication and between-host transmission (sexual reproduction) according to changing environments (Paul, Ariey & Robert 2003; Reece, Ramiro & Nussey 2009).

Our study aims at evaluating the role of host condition as a source of phenotypic variation in host exploitation by malaria parasites. In the present work we used experimental infections of the avian malaria parasite *Plasmodium relictum* (lineage SGS1) in domestic canaries to investigate (i) how the manipulation of the parasite environment (imposing a rich-food diet or a control diet to the host) can alter the dynamics of infection and parasite traits and (ii) how the interaction between past and current environments of the parasite can explain variation in the dynamics of the infection. Briefly, we used the following experimental design: an initial inoculum of *P. relictum* parasites was used to infect hosts that were maintained either under a control or supplemented food diets (two food treatments differing in the quality of food, mainly dietary proteins); the parasites raised in these two host environments were collected and inoculated according to a full-factorial design into new hosts reared under similar control or supplemented diets. In the two sets of experiments, we monitored the dynamics of parasite infection as well as changes in bird body mass and haematocrit (two proxys of cost of infection and virulence, Mackinnon & Read 2003; Cellier-Holzem *et al.* 2010), and investigated how the variation in these traits (parasitaemia, body mass and anaemia) was affected by the past and current parasite environments.

## MATERIALS AND METHODS

*Bird maintenance and food treatments*

Domestic canaries (*Serinus canaria*), originating from a bird breeder, were kept in individual cages (0.6 x 0.4 x 0.4 m) at constant room temperature (21 ± 1°C) and under a controlled daily light cycle (LD 13:11 h).

Birds in the control food group received a commercial mixture of seeds for canaries (Versele-Laga, Belgium) provided *ad libitum*. Birds assigned to the supplemented food group received the same mixture of seeds plus, every 2 days, a supplement consisting of a quarter of hard-boiled egg and a small piece of apple and lettuce. All birds had water provided *ad libitum*. Birds were maintained under their food regime (control or supplemented) from 15 days prior to the parasite infection until the end of the experiment (17 days post infection).

The experiment was conducted during autumn 2009 and performed under the licence # 21-CAE-085 delivered by the departmental veterinary service.

*Parasites and experimental infections*

Experimental infections were carried out using parasites *Plasmodium relictum* (lineage SGS1) originally obtained from a natural population of house sparrows, *Passer domesticus*, and cross-transferred to naive canaries. Infected blood was cryopreserved and stored at -80°C (see details in Bichet *et al.* subm.). For the purpose of the present experiment, cryopreserved blood was thawed (Bichet *et al.* subm.) and transferred intraperitoneally to 5 domestic canaries. Eleven days post-infection (dpi), bird parasitaemia was evaluated from thin blood smears (absolute methanol fixation, 10% Giemsa staining, observation of 10,000

erythrocytes). Blood was collected from donors to prepare a stock solution diluted in PBS containing the desired number of parasites per inoculum (1 x $10^6$ asexual parasites) that served to infect birds of the experiment 1 (see below). A similar procedure was used to infect the birds in the experiment 2.

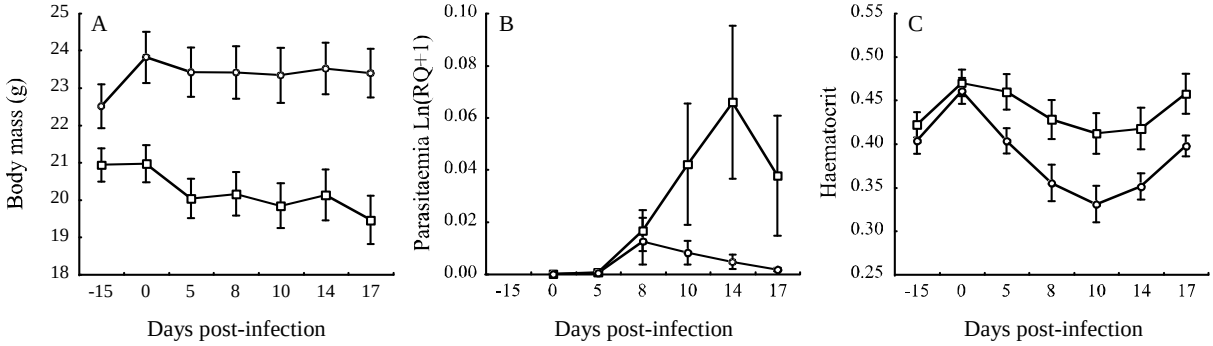


**Figure 1.** Variation (means ± s.e.) in body mass (A), blood parasitaemia (B) and haematocrit (C) in birds infected by the avian malaria *Plasmodium relictum* (SGS1) and maintained under control (squares) and supplemented diets (circles). Diets were provided from 15 days before parasite infection until the end of the experiment (17 dpi).

*Experimental design*

A full-factorial design with host condition (control or supplemented diets) and parasite origin (previously reared in control or supplemented birds) was used to test the effects of previous and current environments on parasite virulence.

In experiment 1, two groups of birds under control (n = 14 birds) or supplemented (n = 15 birds) diets were inoculated intraperitoneally with 1 x $10^6$ *P. relictum* parasites.

In experiment 2, parasites originating from control (C) and supplemented (S) birds were used to infect birds raised under similar control and supplemented diets. The parasitaemia at 10 dpi of infected birds in experiment 1 was estimated from blood smears. Infected blood solutions were prepared from the donor birds of the control ($P^C$) and supplemented ($P^S$) groups to infect a new set of birds raised under control ($B^C$) and supplemented ($B^S$) diets. The same inoculum solution of infected blood was used to infect all the birds in each treatment. To summarize, on one hand, birds raised under the control diet ($B^C$) received either parasites originated from control hosts ($B^CP^C$, n = 15 birds) or supplemented hosts ($B^CP^S$, n = 15 birds). On the other hand, birds raised under the supplemented diet ($B^S$) received either parasites originated from control hosts ($B^SP^C$, n = 15 birds) or the supplemented hosts ($B^SP^S$, n = 15 birds).

All birds were monitored at 5, 8, 10, 14 and 17 dpi by recording the body mass to the nearest 0.1 g and collecting a small amount of blood by puncturing the left brachial vein for haematocrit measurement (around 20 µL, centrifugation 10,000 rpm for 5 min) and molecular analysis (around 20 µL flushed with 500 µL Queen Lysis Buffer).

*Estimation of infection intensity by quantitative PCR*

Infection intensity was assessed using the quantitative PCR assay as described by Cellier-Holzem *et al.* (2010). For each individual, two qPCR reactions in the same run were conducted: one targeting the nuclear 18s rDNA gene of *Plasmodium* (Primers 18sPlasm7 5'-AGC CTG AGA AAT AGC TAC CAC ATC TA-3', 18sPlasm8 5'-TGT TAT TTC TTG TCA CTA CCT CTC TTC TTT-3', and fluorescent probe Plasm Hyb2 5'-6FAM-CAG CAG GCG CGT AAA TTA CCC AAT TC-BHQ1-3') and the other targeting the 18s rDNA gene of bird (Primers 18sAv7 5'-GAA ACT CGC AAT GGC TCA TTA AAT C-3', 18sAv8 5'-TAT TAG CTC TAG AAT TAC CAC AGT TAT CCA-3' and fluorescent probe 18sAv Hyb 5'-VIC-TAT GGT TCC TTT GGT CGC TC-BHQ1-3'). Parasite intensity was calculated as a relative quantification value RQ ($2^{-(Ct\ 18s\ Plasmodium\ -\ Ct\ 18s\ Bird)}$) using the software SDS 2.2 (Applied Biosystem). Ct is the number of PCR cycles at which fluorescence is first detected as statistically significant above

the baseline. RQ can be seen as the fold-amount of the target gene (*Plasmodium* 18s rDNA) with respect to the amount of the reference gene (host 18s rDNA). All qPCR reactions were carried out using an ABI Prism 7900 cycler (Applied Biosystem).

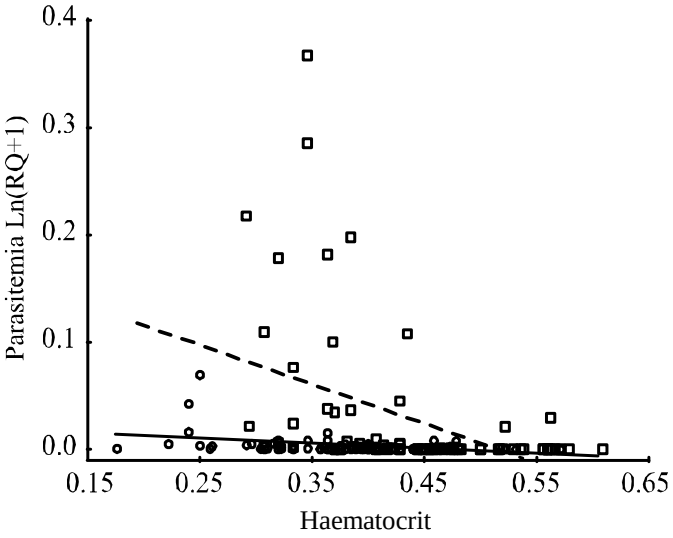


**Figure 2.** Covariation between haematocrit values and blood parasitaemia [Ln(RQ+1)] of *Plasmodium relictum* (SGS1) in infected birds reared under control (squares) and supplemented (circles) diets (experiment 1).

*Parasite transmission*

In experiment 1 we investigated the effect of food diet on the production of parasite sexual stages (i.e. gametocytes). From the blood smears, we estimated the proportion of parasite cells that were gametocytes, which is a proxy of parasite transmission rate (Mackinnon & Read 1999; Paul *et al.* 2007). Ideally, a hundred of parasites were observed and sorted as asexual (trophozoites and merozoites) or sexual (gametocytes) stages, according to their morphology as described in Valkiūnas (2005). The proportion of gametocytes (number of gametocytes over the total number of parasites) was arcsin-transformed to meet the statistical test assumptions. The birds for which less than 10 parasites per smear were observed were omitted from the analysis.

*Statistical analyses*

Variation in body mass, parasite intensity and haematocrit were analyzed with mixed linear models. Given the repeated nature of the data, bird individuals were treated as a random factor. A square time effect (time$^2$) was included to account for non-linear effects in analyses involving haematocrit and parasite intensity (RQ, log-transformed) variables. Degrees of freedom were corrected using the Satterthwaite method. Mortality was analyzed using a logistic regression.

All the two-way and three-way interactions were first included into the models and subsequently removed if not significant ($P > 0.05$). For convenience and clarity, test values and probabilities associated to non-significant terms were not explicitly mentioned in the manuscript. Statistical analyses were performed with JMP 7.0.

**RESULTS**

During the course of the experiment four birds died during or soon after manipulation. These were not included in the further analyses. Neither food nor parasite origin treatments accounted for differences in mortality among groups (experiment 1: diet $\chi^2_1 = 3.07$, $P = 0.0792$; experiment 2: diet $\chi^2_1 = 0.04$, $P = 0.8443$; parasite origin $\chi^2_1 = 2.28$, $P = 0.1313$).

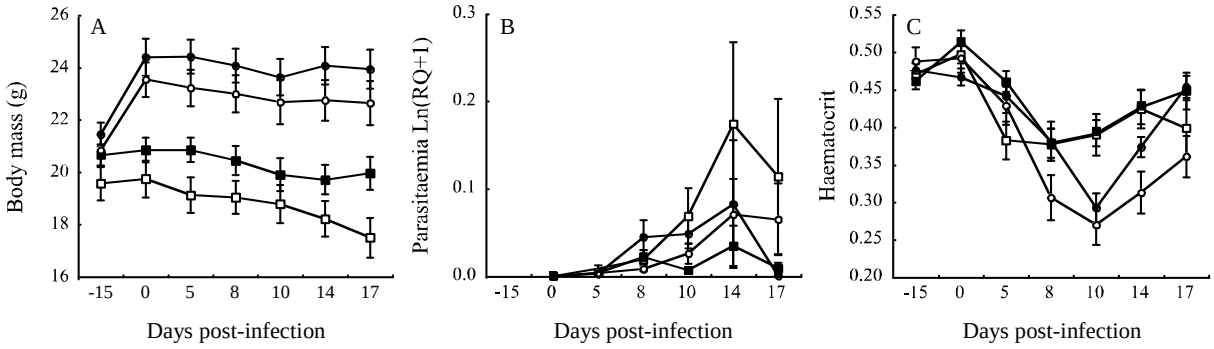


**Figure 3.** Variation (means ± s.e.) in body mass (A), blood parasitaemia (B) and haematocrit (C) in birds maintained under control ($B^C$, squares) and supplemented ($B^S$, circles) diets and infected by *Plasmodium relictum* (SGS1) parasites previously reared in control ($P^C$, open symbols) and supplemented hosts ($P^S$, filled symbols). Diets were provided from 15 days before parasite infection until the end of the experiment (17 dpi). Legend: $B^CP^C$: open squares, $B^CP^S$: filled squares, $B^SP^C$: open circles, $B^SP^S$: filled circles.

*Experiment 1 (first parasite environment)*

Although birds were randomly assigned to the diet treatment, supplemented birds were initially slightly bigger than control birds ($F_{1, 28}$ = 4.54, $P$ = 0.0421). Nevertheless, supplemented birds gained in body mass over the 15-day period of supplementation whereas body mass of control remained constant (time $F_{1, 28}$ = 9.43, $P$ = 0.0047; diet $F_{1, 28}$ = 8.45, $P$ = 0.0071; time*diet $F_{1, 28}$ = 8.52, $P$ = 0.0069; Fig. 1A). Haematocrit was also higher after 15 days of maintenance prior to infection (time $F_{1, 20.71}$ = 32.28, $P$ < 0.0001; Fig. 1C) but this was unrelated to the diet treatment (diet $F_{1, 27.69}$ = 0.31, $P$ = 0.5843).

Further analyses will consider the infection period (0-17 dpi).

Following infection, only birds that had received the control food diet experienced a reduction in body mass, whereas supplemented hosts maintained similar body mass along the experiment (time $F_{1, 141.4} = 31.66, P < 0.0001,$ diet $F_{1, 28.06} = 10.51, P = 0.0006,$ time* diet $F_{1, 141.4}$ = 11.98, $P$ = 0.0007; Fig 1A).

Parasitaemia followed the typical bell-shape variation of an acute *Plasmodium* infection (time $F_{1, 141.5}$ = 4.17, $P$ = 0.430; time$^2$ $F_{1, 141.1}$ = 0.89, $P$ = 0.3466; Fig. 1B). However, the dynamics of parasitaemia markedly differed between the two food diets (food $F_{1, 28.41}$ = 3.54, $P$ = 0.0703; time* diet $F_{1, 141.9}$ = 11.38, $P$ = 0.0010): supplemented birds had overall lower infection intensity and seemed to control the infection earlier than control birds. Peak parasitaemia was, indeed, reached at 8 and 14 dpi for supplemented and control bird, respectively (see Fig. 1B).

Infected birds were anaemic and haematocrit declined as the infection progressed, reaching the minimum values around 10-14 dpi before recovering to values similar to the pre-infection ones (time $F_{1, 130.8}$ = 45.36, $P$ < 0.0001; time$^2$ $F_{1, 129.9}$ = 36.70, $P$ < 0.0001; Fig. 1C). Whereas infected birds fed with control diet showed a moderate reduction in haematocrit, haematocrit of supplemented birds was critically reduced across the course of infection (diet $F_{1, 36.68}$ = 18.55, $P$ < 0.0001; time*diet $F_{1, 130.8}$ = 7.79, $P$ = 0.0060; time$^2$*diet $F_{1, 129.9}$ = 4.53, $P$ = 0.0352; Fig 1C). In addition, variation in haematocrit was affected by a diet*parasitaemia interaction (parasitaemia $F_{1, 141.7} = 14.04, P = 0.0003,$ diet*parasitaemia $F_{1, 141.7}$ = 6.66, $P$ = 0.0109). Whereas control birds were more heavily infected than supplemented birds, the latter paid a higher cost of infection in terms of haematocrit reduction (Fig. 2).

The diet treatment altered parasite dynamics but also the within-host trade-off parasites face between growth and reproduction. Ten days post-infection, the proportion of gametocytes among parasite cells was lower in birds fed with the supplemented diet (arcsin-transformed proportion of gametocytes, mean ± se = 0.298 ± 0.029, n = 12) compared to birds fed with the control diet (0.439 ± 0.034, n = 9) ($F_{1, 19}$ = 9.62, $P$ = 0.0059). It is worth noting that the

proportion of parasite sexual stages was unrelated to the parasitaemia values ($F_{1, 18}$ = 2.49, $P$ = 0.1321).

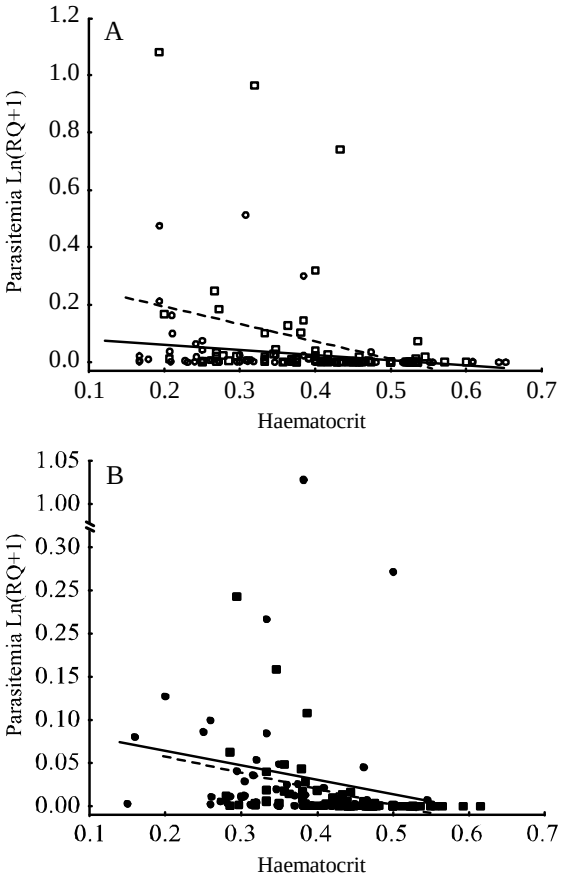


**Figure 4.** Covariation between haematocrit values and blood parasitaemia [Ln(RQ+1)] of *Plasmodium relictum* (SGS1) in birds reared under control (squares) and supplemented (circles) diets and infected with parasites collected from previously (A) control or (B) supplemented hosts (experiment 2).

*Experiment 2 (interaction between past and current environments of the parasite)*

Again, birds fed with the supplemented diet achieved higher body mass after the pre-infection maintenance, contrary to control birds (time $F_{1, 56.85}$ = 75.90, $P$ < 0.0001, diet $F_{1, 57.69}$ = 18.40, $P$ < 0.0001, time*diet $F_{1, 56.85}$ = 56.73, $P$ < 0.0001; Fig. 3A).

Parasites sampled from control or supplemented donors in experiment 1 at day 10 post-infection were inoculated into new birds that were also assigned to a control or supplemented food diet. The reduction in body mass during the infection (time $F_{1, 269}$ = 99.88, $P$ < 0.0001) was influenced by both current diet and parasite origin (see below). The 3-way diet*parasite*time interaction term was non significant, $F_{1, 268}$ = 2.44, $P$ = 0.1195). However, both of these factors (current diet and parasite origin treatments) had separately a strong effect in interaction with time. Overall, as in experiment 1, control birds lost more weight than did supplemented birds (diet $F_{1, 55.98}$ = 38.60, $P$ < 0.0001; diet *time $F_{1, 269}$ = 8.51, $P$ = 0.0038), and parasites originating from supplemented hosts (independently of the current diet in experiment 2) induced only a mild reduction in body mass (parasite $F_{1, 55.98}$ = 4.94, $P$ = 0.0302; time*parasite $F_{1, 269}$ = 11.22, P = 0.0009; Fig. 3A).

Surprisingly, current diet did not affect parasitaemia dynamics (diet $F_{1, 55.39}$ = 0.13, $P$ = 0.7199; Fig. 3B). However, parasitaemia varied between parasite treatments (parasite*time$^2$ $F_{1, 271.1}$ = 6.96, $P$ = 0.0088; Fig. 3B) and birds suffered lower parasite load when infected by parasites previously grown on food-supplemented hosts ($P^S$).

Haematocrit temporal variation (time $F_{1, 269.7} = 127.07$, $P < 0.0001$; time$^2$ $F_{1, 268.9} = 92.01$, $P < 0.0001$; Fig. 3C) was influenced by both the current diet and the parasite origin (significant 3-way interaction term, time*diet*parasite $F_{1, 275} = 5.20$, $P = 0.0233$). The reduction in haematocrit was more pronounced when (i) birds were raised under a supplemented diet ($B^S$ birds) (diet $F_{1, 55.12} = 9.88$, $P = 0.0027$; time*diet $F_{1, 275} = 2.97$, $P = 0.0860$; Fig. 3C) and (ii) when birds were infected by parasite originating from birds raised under a control diet ($P^C$ birds) (parasite $F_{1, 55.12} = 5.19$, $P = 0.0266$; time*parasite $F_{1, 275} = 4.01$, $P = 0.0464$). In addition, maximal reduction in haematocrit was reached 3 days earlier in birds with current control diet ($B^C$) than those under current supplemented diet ($B^S$) (Fig. 3C). It is worth mentioning that similar results (not shown) were obtained when the analysis was performed on the 0-10 dpi period. Hence, the remaining significant 3-way interaction suggests an important effect of current food regime and parasite origin in the early phase of infection only, the recovery of haematocrit in late infection being negligible.

Parasitaemia negatively affected haematocrit ($F_{1, 298.4} = 11.31$, $P = 0.0009$) but the relationship was influenced by the current host diet and parasite origin treatments (parasitaemia*diet*parasite $F_{1,296.9} = 3.94$, $P = 0.0482$) (Fig. 4). The slope of the covariation between haematocrit and parasitaemia did not differ between food diets when birds were infected with parasites originating from supplemented birds ($B^CP^S$ and $B^SP^S$ not different, Fig. 4B). However, when birds were infected by parasites that evolved in control hosts ($P^C$), birds currently fed with supplemented diet ($B^SP^C$) had lower parasitaemia but were more anaemic than birds fed with control diet ($B^CP^C$). Similar results were obtained in experiment 1 (Fig. 2).

## DISCUSSION

Our goal was to understand how environmental variation, in particular host nutritional condition, impacts parasite growth (parasitaemia) and infection severity (host anaemia and weight loss in a *Plasmodium*-bird system. To this purpose, *Plasmodium relictum* (SGS1) parasites were grown in two environments, either hosts experiencing enriched diet or control diet. Food supplementation led to better body condition in birds and resulted in lower parasite density. Similar effects on parasite dynamics have been observed in immunized mice infected by the malaria parasite *Plasmodium chabaudi* (Buckling & Read 2001; Mackinnon & Read 2003).

Immunity is a costly physiological function that requires a substantial resource budget. As such, body mass (a proxy for host condition) usually mirrors the level of immune competence (i.e. the ability to cope with diseases). Supplemented birds, mainly provided with a protein-enriched diet (egg albumen and yolk), reached higher body mass. They were also expected to have higher immune competence than control birds. We did not perform any measures of the immune response *per se*, because the assessment of immune competence is delicate and requires the measurement of several and diverse branches of the immune system (Adamo 2004). In addition, measuring the immune responses would have had required extra large amount of blood, which was incompatible with the repeated sampling design of our experiment. However, previous work showed that birds reared under protein diet similar to our supplemented diet maintain higher level of immune competence and mount more efficient immune responses (Gonzalez *et al.* 1999; Smith *et al.* 2007). Hence, the lower parasitaemia and earlier clearance observed in the supplemented group are in accordance with a higher level of host immunity and an efficient immune response against *Plasmodium*.

Available food quality of the hosts impacts the dynamics of *Plasmodium* infection; in addition, our results suggest that in-host environment experienced by parasite can have cross-generational effects and impact on future infection. Indeed, both current food treatment and parasite origin (parasites previously propagated in normal or supplemented hosts) affected the dynamics of *P. relictum* in secondary infections. In particular, higher parasitaemia and anaemia

were recorded in birds infected by parasites originating from birds fed with the control diet. Overall, these parasites were more harmful to hosts. This is somewhat contradictory to recent studies on invertebrates, where parasite virulence was found to be greater when hosts were infected by parasites that previously infected good-condition hosts (Tseng 2006; Little *et al.* 2007). It is likely that cross-generational effects are due to selection in the parental generation of different strains of *P. relictum*. Alternatively, given that parasite dynamics markedly differed between diet treatments in experiment 1 at 10 dpi, we may have transmitted different parasite populations.

Parasites reproductive success has been shown to be higher in good quality hosts in various host-parasite systems (Bedhomme *et al.* 2004; Tseng 2006; Tschirren *et al.* 2007; Seppälä *et al.* 2008). By contrast, we found overall that *P. relictum* parasites were more successful in control birds, reaching larger population sizes and causing longer infections. Krasnov *et al.* (2005) showed that fleas parasitizing underfed (and immune incompetent) rodents laid more eggs and that the larvae arising from these eggs had a higher probability of survival. In this system, host immunity rather than resources is more likely to explain why parasites perform better on poor-quality hosts (Krasnov *et al.* 2005). Similarly, parasites infecting hosts with enhanced immunity performed less well (Bize *et al.* 2008). Parasites seem to face a trade-off between infecting less well defended but lower quality *vs.* more well defended but higher quality hosts.

*Plasmodium* parasites are responsive organisms able to alter their traits to maximize their transmission (and ultimately their fitness) to changes occurring in their within-host environments including resource availability (erythrocytes), competitors, drugs and host immunity (Reece *et al.* 2009; Pollitt *et al.* 2011; Mideo & Reece 2012). In our study, the lower parasite density and altered dynamics observed in hosts with supplemented diet could suggest that parasites are more constrained, which is likely to result from the pressure of host immunity (see also Buckling & Read 2001; Mackinnon & Read 2003). Indeed, the pattern is similar to the infection dynamics in birds that were reexposed to the parasite and which developed an acquired immune response (Cellier-Holzem *et al.* 2010). Here, birds fed with the supplemented diet may have developed stronger innate immune response. It is worth mentioning that the lower proportion of gametocyte stages estimated at day 10 post-infection in supplemented birds suggests a decreased allocation to reproduction (transmission) compared to the control group (unless there were differences in the immune response in a way that targeted gametocytes). *Plasmodium* parasites are expected to escape hostile within-host environments by allocating more to transmission stages. Alternatively, parasites could adopt a 'reproductive restraint' strategy and reduce investment in gametocytes and favour within-host survival by maintaining low but persistent infection (asexual stages) to ensure future transmission (Reece *et al.* 2009). This is what our gametocyte data suggest. Unfortunately we had no information on the long-term persistence of the infection (after 17 dpi) to investigate this issue in further details.

In our study system, supplemented birds had lower parasitaemia, controlled the infection earlier and did not suffer from body mass reduction. Surprisingly though, they suffered from a stronger anaemia. Anaemia and weight loss are often described as a proxy for virulence in infection by *Plasmodium* (Mackinnon & Read 2003). Here we found diverging effects, suggesting that these two traits may be unrelated. Anaemia is primarily the consequence of the disruption of infected red blood cells and it often correlates with parasitaemia. Anaemia might also be a host response to control an infection by increasing the clearance and the destruction of both parasitized and non-parasitized red blood cells (Lamikanra *et al.* 2007). However, infected birds of control and supplemented diets should not behave differently. Why did supplemented birds suffer from a stronger reduction in haematocrit, while exhibiting lower parasitaemia compared to control birds? An explanation may arise from the host immune responsiveness *per se*. There is increasing evidence that the host immune

system overreacts to an immune challenge and has immunopathological consequences (Graham, Allen & Read 2005; Day, Graham & Read 2007; Long & Graham 2011). Given that supplemented host can afford allocating more resources to the immune function, this hyper immune responsiveness might account for an extra-loss of cells leading to a more severe anaemia. This would be consistent with the fact that inhibiting the nitric oxide response in canaries infected by *P. relictum* led to higher parasitaemia but dampened the immunopathological cost as seen by the mild haematocrit reduction compared to non-manipulated birds (Bichet *et al.* subm.).

Our study demonstrates that (i) variation in the host diet can significantly impact growth and transmission of avian malaria parasite *Plasmodium relictum* and (ii) parasite previous environment can help explain variation in disease outcome of current infections. *Plasmodium relictum* and other Haemosporidia are highly prevalent in passerine populations (Cosgrove *et al.* 2008; Loiseau *et al.* 2011; Glaizot *et al.* 2012) and significantly affecting bird fitness (Knowles *et al.* 2009; Lachish *et al.* 2011). However, we still know little about how the variation in the parasite's environment in its broad sense affects infection dynamics and virulence in wild populations. Because most host-parasite interactions exist in heterogeneous environments (Wolinska & King 2009), more work on how pathogens respond and adapt to environmental changes is clearly needed to understand host and parasite evolution, as well as predicting disease dynamics.

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# Manuscrit 4

## Epidemiology of *Plasmodium relictum* infection in the house sparrow

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Soumis à *Journal of Parasitology*

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**ABSTRACT:** The epidemiology of infectious diseases depends on host and parasite traits, as well as on the environment where the interaction takes place. Numerous host traits directly or indirectly determine the risk of becoming infected with a given parasite as well as its multiplication within the host. For instance, males and females differ with many respects (behavior, physiology, immunity) which might make one sex more prone to harbor infectious diseases than the other. Similarly, age can affect both prevalence and parasitemia because the immune response varies with age or because older individuals have a longer exposure time to pathogens. Here, we investigated the prevalence and parasitemia variability of *Plasmodium relictum* in the house sparrow (*Passer domesticus*), across a large number of rural and urban populations (n = 16). We found that prevalence was not predicted by any of the host traits (age, sex or body condition). However, parasitemia was higher in females than in males and in 1-yr-old compared to older individuals. Neither prevalence nor parasitemia differed according to the habitat type (urban vs. rural). These results suggest that inter-population variation in parasitemia depends on host intrinsic factors, whereas variation in prevalence could be more due to environmental differences between populations unrelated to urbanization, such as the availability of vectors or climatic variables.

## INTRODUCTION

The epidemiology of infectious diseases depends on the interactions between biotic and abiotic factors. Both host and parasite characteristics can shape the transmission rate of the parasite, with environmental conditions further affecting the dynamics of the interaction. For vector-borne diseases, the presence and abundance of vectors add another layer of complexity to the epidemiology of the disease. Because of the major impact of infectious diseases on the ecology and evolution of hosts, a great deal of work has been devoted to understand the factors shaping parasite transmission and infectivity (Poulin, 2006, Wood et al., 2007; Monaghan, 2008; Rogers and Randolph, 2006; Loiseau et al., 2008; Palinauskas et al., 2008).

Among host characteristics, sex, age and body condition are known to affect infection dynamics (Weatherhead and Bennett, 1991; McCurdy et al., 1998). Sex and age are associated with differences in both behavioral and physiological traits making individuals of a given sex or a given age more susceptible to infectious diseases. For instance, males and females may differ in their susceptibility to parasites because of differential exposure (McCurdy et al., 1998). More importantly, males and females have different production of steroid hormones (i.e. testosterone and corticosterone) especially during the reproductive season and they have been suggested to have immunosuppressive properties (McCurdy et al., 1998; Rolff, 2002). Studies found that males were more vulnerable to infectious diseases (Poulin, 1996; Schalk and Forbes, 1997; van Oers et al., 2010). However, females usually

engage a large reproductive effort (producing large gametes, incubating eggs, feeding young). The energetic requirements of reproduction may reduce the amount of resources available for immune defences and therefore females should be more susceptible than males (Chernin, 1952; Peirce and Marquiss, 1983; McCurdy et al., 1998).

Host age is also an important factor shaping immune functioning and therefore parasite resistance. Immune functioning is usually related with age in a non-linear manner (Miller, 1996; Adamo et al., 2001; Cichon et al., 2003; Saino et al., 2003; Lavoie, 2005; Palacios et al., 2007). Young and old individuals are the most likely to suffer from infectious diseases because young hosts have an inexperienced immune system whereas old hosts usually suffer from a decline in immune surveillance due to senescence (Hudson and Dobson, 1997). One might expect old individuals being especially vulnerable to infection (long exposure time and poor immune defences; Allander and Bennett, 1994; Stjernman et al., 2004; Wood et al., 2007).

Finally, host body condition can be linked in a complex way to parasite transmission and dynamics (Atkinson et al., 1995, 2000; Karell et al., 2011; Palacios et al., 2012; Shurulinkov et al., 2012). On one hand, hosts in poor body condition may provide few resources to parasites and therefore slow down its multiplication and transmission. On the other hand, poor host condition might result from an overexploitation of the host by the parasite and therefore correlate with higher parasite multiplication and transmission (unless overexploitation induces host mortality thus stopping transmission). Finally, poor condition can predispose hosts to infectious diseases, further reducing condition (Beldomenico et al. 2010).

Avian blood parasites have been extensively studied for more than 100 years, initially as a model system for human disease and more recently, in the past few decades, as models for studies in evolutionary ecology (Anderson and May, 1982; Poulin et al., 2000; Combes, 2001). Recently, Knowles et al. (2011) investigated fine-scale environmental and host predictors of malaria infection status and parasitemia in 1 population of blue tits (*Cyanistes caeruleus*). Prevalence and parasitemia both increased with host age, and parasitemia was higher in individuals investing more in reproduction.

Here, we explored further these findings and took advantage of our large-scale survey carried out in France of the host-parasite system *Plasmodium relictum*-house sparrow (*Passer domesticus*) (Loiseau et al., 2009, 2011). In 16 urban and rural populations, we determined prevalence, quantified parasitemia and examined the inter-population and inter-individual variations according to host characteristics (age, sex and body condition), seasonality and habitat type (urban or rural).

We predicted that prevalence and parasitemia should be higher during the spring and summer because in temperate latitudes, mosquitos are only available during this period. Outside the transmission period, the parasite is maintained in the vertebrate host as chronic infection. Predicting the effect of urbanization on prevalence and parasitemia is more challenging since urbanization may have complex effects on disease transmission (Bradley and Altizer 2006). Urban sites might on one hand have lower availability of vectors, resulting in a reduced risk of infection; on the other hand, pollutants released in urbanized habitats might weaken the immune system of the host making it more susceptible to infectious diseases.

## MATERIALS AND METHODS

### Study sites and sampling

We sampled 16 populations of house sparrows (*Passer domesticus*) in France, from 2004 to 2010, during two different seasons (autumn-winter: October to March and spring-summer: April to September) (Fig. 1). The house sparrow is tightly associated to humans and only occurs within human settlements. Nevertheless, as humans, house sparrows can occur in highly urbanized areas as well as in more rural environments. In order to assess the role played by urbanization on the risk of malaria infection, we scored each of the 16 populations studied here as either urban or rural based on the size of the village/city where sparrows were sampled and the surrounding habitat. The sample size for each population is summarized in Table I. Each bird was caught using mist-nets and was banded. We measured wing length ($\pm$ 1 mm) and body mass ($\pm$ 0.5 g). Blood samples (ca 20 µl) were collected by brachial vein puncture and stored in 500µl of Queen's Lysis Buffer (Seutin et al., 1991) for subsequent molecular analyses.

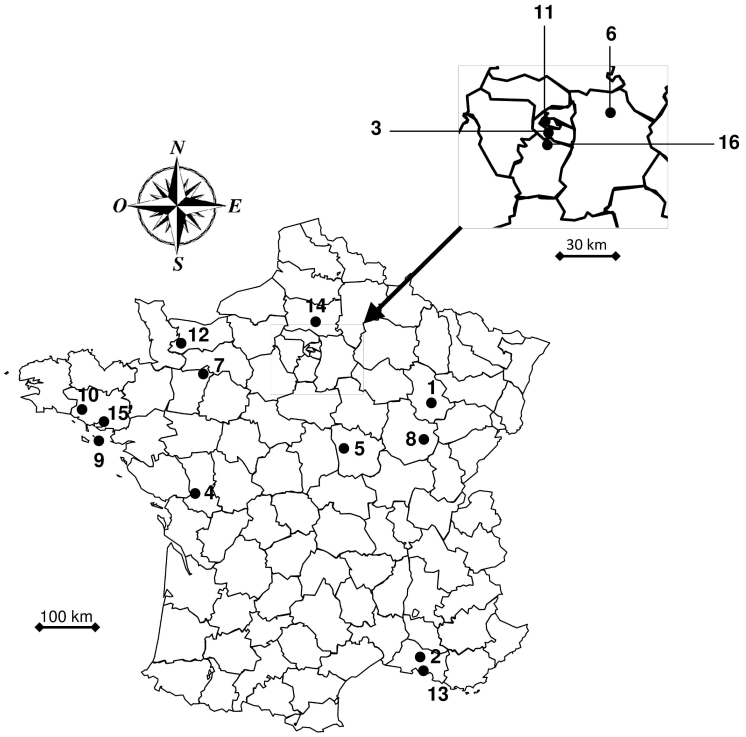


FIGURE 1. Geographical localization of the 16 house sparrow populations: 1 – Anglus, 2 – Arles, 3 – Cachan, 4 – Chizé, 5 – Cosnes-Cours sur Loire, 6 – Crégy les Meaux, 7 – Crennes-sur-Fraubee, 8 – Dijon, 9 – Hoedic, 10 – Languidic, 11 – Paris (Jardin des Plantes), 12 – Rully, 13 – Saintes-Maries-de-la-Mer, 14 – Thieux, 15 – Vannes and 16 – Wissous. The zoomed region, in the upper right corner, corresponds to the region Ile de France. Scale bar, 100 km for France and 30 km for the region Ile de France.

Each adult bird was sexed based on sexually dimorphic plumage. Each bird was also assigned to one of two age classes: 1-yr-old (1Y, birds born in the year of sampling), more than 1-yr-old (>1Y). Because of field constraints, we obtained the age of individuals for 14 populations and morphological measures for 13 populations (Table I).

**Parasite screening**

DNA was extracted using standard phenol/chloroform protocol (modified from (Hilis et al., 1996). In order to detect the presence of malaria parasites, we used a nested polymerase chain reaction (PCR) (Waldenstrom et al., 2004) to amplify a fragment of the parasite mitochondrial cytochrome *b* gene. We sequenced the positive PCR products and identified lineages using the NCBI nucleotide Blast search. For this study, we focused on *Plasmodium relictum* (SGS1 and GRW11 lineages) the most predominant parasite species in all populations (Loiseau et al., 2011).

For each positive PCR product, we also performed a relative quantitative PCR to obtain parasitemia following the protocol described in Cellier-Holzem et al. (2010). For each individual we conducted 2 qPCR reactions in the same run: one targeting the nuclear 18s rDNA gene of *Plasmodium* (Primers 18sPlasm7 (5'-AGC CTG AGA AAT AGC TAC CAC ATC TA-3'), 18sPlasm8 (5'-TGT TAT TTC TTG TCA CTA CCT CTC TTC TTT-3'), and fluorescent probe Plasm Hyb2 (5'-6FAM-CAG CAG GCG CGT AAA TTA CCC AAT TC-BHQ1-3')); and the other targeting the 18s rDNA gene of birds (Primers 18sAv7 (5'-GAA ACT CGC AAT GGC TCA TTA AAT C-3'), 18sAv8 (5'-TAT TAG CTC TAG AAT TAC CAC AGT TAT CCA-3') and fluorescent probe 18sAv Hyb (5'-VIC-TAT GGT TCC TTT GGT CGC TC-BHQ1-3')). Parasitemia were calculated as relative quantification values (RQ) as $2^{-(Ct\ 18s\textit{Plasmodium} - Ct\ 18s\ Bird)}$ using the software SDS 2.2 (Applied Biosystem, Carlsbad, California). Ct represents the number of PCR cycles at which fluorescence is first detected as statistically significant above the baseline and RQ can be interpreted as the fold-amount of target gene (*Plasmodium* 18s

rDNA) with respect to the amount of the reference gene (host 18s rDNA). All qPCR reactions were carried out in an ABI Prism 7900 cycler (Applied Biosystem, Carlsbad, California).

**Statistical analyses**

We performed Spearman correlation coefficient to investigate the correlation between mean prevalence and mean parasitemia (log transformed) across our sixteen populations.

We investigated the relationships between patterns of infection and host traits using a combination of multiple regression models. In statistical models, the dependent variable was either the infection status of sampled individuals (infected or not, hereafter referred to as *prevalence*, n = 775) or the parasitemia obtained from quantitative PCR (n = 126). The *parasitemia* variable (log-transformed) was analysed using linear mixed-effects models (LMM) while the *status* was examined through generalised linear mixed-effects models (GLMM) assuming binomial distribution of errors and logit link function. Fixed-effect explanatory variables were the season (binary variable, either spring-summer or autumn-winter), urbanization (binary variable, either urban or rural population), sex (binary), age (binary, either 1Y or >1Y), body mass and wing length (continuous variables). The population and year factors were treated as random variables. We also examined correlations between explanatory variables, which revealed moderate positive correlation between body weight and wing length (Pearson's r = 0.20, $P < 10^{-4}$) and strong variations in wing length among sexes, year and populations (these three variables together explained 50% of wing length variation in linear multiple regression, $P < 10^{-4}$). The effects of first order interactions between explanatory variables on prevalence and parasitemia were also investigated. In order to avoid model over-parametrization, we first compared all models including 1 single interaction term to the model without interaction on the basis of the Akaike Information Criterion (AIC). All interaction terms significantly improving the AIC score (i.e., reducing the AIC by ≥2 as compared with the model without interaction) were then included to the final model.

All models presented in the main results are based on GLLM (for status) and LLM (for intensity). In order to test the robustness of our conclusions to other modelling frameworks and to accurately compute the proportions of independent variance explained by each explanatory variable, we also performed hierarchical partitioning (HP, Chevan and Sutherland, 1991). HP uses all models in a regression hierarchy to distinguish those variables that have high independent correlations with the dependent variable (Mac Nally, 2002). Results are expressed in terms of percentage of total independent effect of each explanatory variable on the dependent variable ($R^2$). A randomization test (1000 iterations) was performed for testing the significance of each explanatory variable in each analysis (Walsh and Mac Nally, 2003). Interactions were not considered in HP models.

All statistical analyses were conducted with R 2.10.1 (R Development Core Team, 2009).

**RESULTS**

*Plasmodium* prevalence varied from 11% (Hoedic) to 79% (Arles; Table I). Parasitemia was very low as expected for chronic malaria infections, and varied from $4.55.10^{-6} \pm 3.53.10^{-6}$ (Paris, Jardin des Plantes) to $4.84.10^{-4} \pm 4.18.10^{-4}$ (Arles; Table I). Among the 16 populations of house sparrow, there was no statistically significant correlation between prevalence and parasitemia ($R_S = 0.06$, $P = 0.84$).

Prevalence was 18.97% in 1Y birds and 31.73% for older individuals (>1Y). Twenty-two per cent of females were infected versus 27% in males. In the GLLM model with no interactions fitted, no fixed-effect explanatory variable was significantly correlated with the infection status of individuals, but HP models indicated that there was significant among population variation in prevalence ($Z=7.7$, $P<10^{-4}$), with population explaining 11.3% of the variance in status among individuals. No interaction significantly improved model fit (Table II).

Among infected individuals, host characteristics had significant effects on parasitemia. Although no fixed effect predictor was significantly correlated with parasitemia in mixed-effects models without interactions, the sex × age and season × wing length interaction terms improved model fit (Table II) and revealed sex and age variations in parasitemia (Table III).

Overall the model indicated that (a) although parasitemia tended to be lower for males than for females, the way sex affects parasitemia in individuals differed between ages (Table III, Fig.2); (b) the wing length was negatively correlated with parasitemia in the spring-summer season and positively correlated in the autumn-winter season (Table III).

TABLE III. Linear mixed-effects model on parasitemia (log-transformed). Fixed-effect variables were the season (either spring-summer or autumn-winter), sex, age (either 1Y or >1Y), urbanization (either urban or rural), body mass and wing length (continuous variables). The population and year factors were treated as random variables. SE: Standard error of the estimate; t: Student statistics.

| | Estimate | SE | t | P-value |
|---|---|---|---|---|
| season | 34.477 | 12.488 | 2.761 | 0.006 |
| sex | -4.099 | 1.323 | -3.098 | 0.002 |
| age | -2.183 | 0.927 | -2.355 | 0.019 |
| weight | -0.125 | 0.114 | -1.100 | 0.271 |
| wing lenght | 0.290 | 0.137 | 2.120 | 0.034 |
| urbanization | 0.510 | 0.606 | 0.842 | 0.400 |
| sex:age | 3.265 | 1.416 | 2.306 | 0.021 |
| season:wing lenght | -0.446 | 0.161 | -2.772 | 0.006 |

## DISCUSSION

Parasite prevalence and parasitemia are known to vary in space and time and several abiotic or biotic parameters are responsible for these variations. To evaluate which factors can contribute to parasite infection is a timely topic in order to predict disease dynamics. To our knowledge, few studies have measured parasitemia and prevalence of the same parasite species across populations of the same host. Here, we showed in a large number of natural house sparrow populations that host characteristics, age, sex, and body condition, as well as seasonality were correlated with *Plasmodium* parasitemia but not prevalence.

We found differences in parasitemia between sexes, in interaction with age, with females generally suffering higher parasitemia than males. Previous work on sexual differences in parasite infection has mostly stressed a higher susceptibility of males because the testosterone and corticosterone produced by males during the reproductive season can substantially weaken the immune response (Poulin, 1996; Schalk and Forbes, 1997; McCurdy et al., 1998; van Oers et al., 2010). Alternatively, differences in parasitemia between males and females might reflect different “habitat” use. For instance, females might be an easy target for vectors during the extended time spent in the nest to incubate eggs and brood hatchlings (Chernin, 1952; Peirce and Marquiss, 1983; Korpimaki et al., 1993; Norris et al., 1994). However, in addition to higher parasitemia, this should also result in a higher prevalence in females than in males. Our finding of statistically similar prevalence between sexes does not support the differential exposure hypothesis. Tentatively, one might speculate that the cost of reproduction is higher in females than in males, as already shown in several bird species. This differential reproductive effort would make female immune response less competent when facing a malaria infection (Richner et al., 1995; Williams, 2005).

House sparrow age also had an effect on *Plasmodium* parasitemia. One year old birds generally harboured a higher parasitemia than older individuals, although quantitative difference between individuals of different ages was sex-dependent. This is a rather common finding that has been reported in a variety of systems (Graves et al., 1988; Gregory et al., 1992; Allander and Bennett, 1994; Merilä et al., 1995; Dawson and Bortolotti, 2000; Sol et al., 2000, 2003; Amo et al., 2005; Hasselquist et al., 2007; Syafruddin et al., 2009). As for the sex differences, the observed age effect could be due to either a differential exposure to vectors or differences in immune functioning (Hasselquist et al., 2007). A differential exposure to vectors should nevertheless also produce a difference in prevalence which was not found in the present study. We therefore believe that the observed

age-dependent variation in parasitemia is likely due to the development of a competent immune response as long as birds become exposed and re-exposed to the parasite (Graczyk et al., 1994; Hudson and Dobson, 1997; Atkinson et al., 2001; Sol et al., 2003). In agreement with this hypothesis, immunologically naïve canaries experimentally infected with *Plasmodium relictum* (SGS1 lineage) suffer from higher parasitemia compared to individuals that have already been exposed to the parasite (Cellier-Holzem et al., 2010). Hasselquist et al. (2007) found a similar result in the great reed warbler (*Acrocephalus arundinaceus*), where parasitemia was higher in females than in males and in 1 year old than in older individuals. Similarly to our study, they found no differences in prevalence between sexes and age classes.

We did not find strong support to the prediction that seasonality should explain variation in prevalence and parasitemia. We only found a statistically significant two-way interaction between a morphometric measurement and season on parasitemia. These results suggest that although transmission only occurs during the season when mosquitos are available, the parasite manages to maintain a quite stable prevalence and parasitemia even when transmission does not occur. Similarly, we did not find support to the hypothesis that urbanization affects the risk of being infected with malaria parasites in the house sparrow. Even though, in most cases animals living in urban habitats may be protected from infectious diseases, there have been reports of increased transmission of pathogens in urban areas (Bradley and Altizer 2006). With respect to malaria parasites, Evans et al. (2009) have shown that urban populations of blackbirds (*Turdus merula*) have lower prevalence of malaria infection. It is worth noting that house sparrows live only in close proximity to human settlements. The lack of difference in prevalence and parasitemia between rural and urban populations might reflect similar vector abundance across the environmental gradient considered here.

To conclude, our large scale survey of house sparrow populations provided evidence in support to the hypothesis that parasitemia does depend on host traits, such as age and sex, while prevalence may depend more on abiotic factors (unrelated with the degree of urbanization, such as temperature) that are driving host and vector densities and interactions between them (Wood et al., 2007; Fokidis et al., 2008; Geue and Partecke, 2008; Evans et al., 2009).

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TABLE I. Prevalence (%), parasitemia (log-transformed), body mass (g) and wing length (mm) with sample sizes associated to each of these variables are given for each of the sixteen house sparrow populations (U and R refer to urban and rural populations, respectively), in each year and in each season. A-W: autumn-winter, S-S: spring-summer, N1: number of individuals sampled for infection status, N2: number of individuals sampled for parasitemia, N3: number of individuals sampled for body condition.

| n° | Population | Year | Season | N1 | Prevalence [CI] (%) | N2 | Parasitemia (±SE) | N3 | Body mass (±SE) (g) | Wing length (±SE) (mm) |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Anglus (R) | 2004 | A-W | 29 | 37.93 [20.6-57.7] | 11 | 1.02E-4 (±8.61E-5) | 28 | 29.86 (±0.45) | 77.98 (±0.45) |
| | | 2005 | A-W | 27 | 33.33 [16.4-54.0] | 9 | 1.89E-5 (±1.08E-5) | 21 | 28.05 (±0.41) | 78.33 (±0.39) |
| | | Total | | 56 | 35.71 [23.2-49.7] | 20 | 6.47E-5 (±4.75E-5) | 49 | 29.04 (±0.33) | 78.17 (±0.30) |
| 2 | Arles (U) | 2005 | A-W | 27 | 85.19 [66.1-95.8] | 23 | 7.19E-5 (±2.36E-5) | 23 | 28.54 (±0.37) | 77.93 (±0.48) |
| | | 2005 | S-S | 15 | 66.67 [38.2-88.2] | 10 | 1.30E-3 (±1.31E-3) | 14 | 27.5 (±0.51) | 76.11 (±0.89) |
| | | Total | | 42 | 78.57 [63.1-89.7] | 33 | 4.84E-4 (±4.18E-4) | 44 | 28.11 (±0.26) | 77.14 (±0.43) |
| 3 | Cachan (U) | 2004 | S-S | 15 | 20.00 [4.31-48.1] | 3 | 2.00E-7 | 15 | 25.23 (±0.49) | 76.33 (±0.63) |
| | | 2005 | S-S | 5 | 60.00 [14.6-94.8] | 3 | 6.88E-5 (±6.34E-5) | 5 | 26.10 (±0.70) | 76.20 (±1.81) |
| | | Total | | 20 | 30.00 [11.8-54.3] | 6 | 3.45E-5 (±3.22E-5) | 20 | 25.45 (±0.41) | 76.30 (±0.62) |
| 4 | Chizé (R) | 2005 | S-S | 48 | 66.67 [51.4-79.6] | 31 | 8.20E-6 (±3.40E-6) | _ | _ | _ |
| 5 | Cosne-Cours sur Loire (R) | 2004 | A-W | 45 | 48.89 [33.6-64.3] | 22 | 9.45E-5 (±3.37E-5) | 45 | 28.21 (±0.32) | 76.25 (±0.14) |
| | | 2005 | A-W | 13 | 53.84 [25.0-80.7] | 7 | 8.12E-4(±3.58E-4) | 13 | 27.62 (±0.29) | 79.12 (±0.80) |
| | | Total | | 58 | 50.00 [36.4-63.5] | 29 | 2.92E-4 (±1.54E-4) | 58 | 28.10 (±0.26) | 76.68 (±0.21) |
| 6 | Cregy (R) | 2004 | A-W | 21 | 28.57 [11.2-52.2] | 6 | 6.35E-6 (±5.87E-6) | 21 | 28.76 (±0.39) | 78.83 (±0.35) |
| | | 2005 | A-W | 27 | 44.44 [25.4-64.7] | 1 | 2.00E-7 | 26 | 28.94 (±0.30) | 79.81 (±0.35) |
| | | 2005 | S-S | 4 | 25.00 [0.6-80.6] | 1 | 3.29E-06 | 4 | 28.13 (±0.22) | 79.75 (±0.44) |
| | | 2006 | A-W | 17 | 47.06 [22.9-72.2] | _ | _ | 17 | 28.03 (±0.26) | 79.03 (±0.40) |
| | | 2006 | S-S | 4 | 75.00 [19.3-99.3] | _ | _ | 4 | 27.88 (±0.23) | 78.63 (±0.29) |
| | | Total | | 73 | 41.10 [29.6-53.3] | 8 | 5.20E-6 (±4.37E-6) | 83 | 28.44 (±0.22) | 79.12 (±0.22) |
| 7 | Crennes (R) | 2004 | A-W | 7 | 28.57 [3.7-71.0] | 2 | 8.5E-5 (±7.80E-5) | 7 | 26.29 (±0.56) | 76.71 (±0.81) |
| | | 2005 | A-W | 45 | 33.33 [20.0-49.0] | 15 | 1.08E-4 (±8.40E-5) | 17 | 27.29 (±0.54) | 77.57 (±0.56) |
| | | Total | | 52 | 32.69 [20.2-47.2] | 17 | 1.05E-4 (±7.41E-5) | 26 | 26.94 (±0.40) | 77.23 (±0.44) |
| 8 | Dijon (U) | 2009 | S-S | 20 | 55.00 [31.4-76.9] | 11 | 2.14E-4 (±1.05E-4) | _ | _ | _ |
| 9 | Hoedic (R) | 2006 | S-S | 44 | 6.82 [1.4-18.7] | 3 | 6.90E-5 (±4.07E-5) | _ | _ | _ |
| | | 2007 | A-W | 24 | 8.33 [1.0-27.0] | 2 | 7.67E-5 (±1.5E-7) | 13 | 28.65 (±0.84) | 76 (±0.78) |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 2007 | S-S | 92 | 4.35 [1.2-10.8] | 4 | 4.58E-4 (±4.15E-4) | _ | _ | _ |
| | | 2008 | A-W | 37 | 2.70 [0.0-14.1] | 1 | 3.81E-6 | 24 | 28.38 (±0.29) | 76.65 (±0.37) |
| | | 2008 | S-S | 81 | 4.94 [1.4-12.3] | 3 | 2.00E-7 | 14 | 27.71 (±0.43) | 75.86 (±071) |
| | | 2009 | A-W | 53 | 1.89 [0.0-10.1] | 1 | 1.80E-5 | 52 | 28.27 (±0.28) | 74.87 (±0.31) |
| | | 2009 | S-S | 143 | 0.70 [0.00-3.87] | 1 | 5.98E-5 | 20 | 27.84 (±0.39) | 75.00 (±0.49) |
| | | 2010 | A-W | 11 | 0.00 | _ | _ | 11 | 30.29 (±0.31) | 76.41 (±0.43) |
| | | 2010 | S-S | 200 | 30.00 [23.7-37.0] | 4 | 6.69E-6 (±6.49E-6) | 33 | 27.63 (±0.29) | 74.89 (±0.36) |
| | | Total | | 685 | 11.09 [8.8-13.6] | 19 | 1.21E-4 (±8.83E-5) | 168 | 28.17 (±0.14) | 75.33 (±0.17) |
| 10 | Languidic (R) | 2008 | A-W | 56 | 16.07 [7.6-28.4] | 9 | 7.66E-5 (±3.53E-5) | 56 | 27.65 (±0.25) | 75.01 (±0.29) |
| 11 | Paris, Jardin des Plantes (U) | 2004 | A-W | 11 | 18.18 [2.3-51.8] | 2 | 7.47E-7 (±5.47E-7) | 10 | 26.95 (±0.53) | 78.95 (±0.63) |
| | | 2004 | S-S | 27 | 29.63 [13.7-50.3] | 8 | 8.52E-6 (±6.70E-6) | 26 | 25.58 (±0.39) | 78.77 (±0.35) |
| | | 2005 | A-W | 1 | 100.00 | 1 | 2.00E-7 | 1 | 27.00 | 79.00 |
| | | 2005 | S-S | 13 | 38.46 [13.8-68.4] | 5 | 2.22E-7 (±2.54E-7) | 5 | 27.70 (±1.66) | 79.40 (±0.75) |
| | | Total | | 52 | 30.77 [18.7-45.1] | 16 | 4.55E-6 (±3.53E-6) | 42 | 26.19 (±0.35) | 78.89 (±0.27) |
| 12 | Rully (R) | 2005 | A-W | 18 | 50.00 [25.9-74.0] | 9 | 1.37E-4 (±1.17E-4) | 18 | 26.39 (±0.41) | 78.11 (±0.53) |
| | | 2005 | S-S | 38 | 31.58 [17.4-48.6] | 10 | 3.48E-4 (±1.80E-4) | 37 | 26.76 (±0.36) | 77.00 (±0.47) |
| | | Total | | 54 | 35.19 [22.6-49.4] | 19 | 2.48E-4 (±1.10E-4) | 55 | 26.60 (±0.27) | 77.29 (±0.36) |
| 13 | Saintes-Marie-de-la-Mer (R) | 2007 | A-W | 20 | 70.00 [45.6-88.1] | 14 | 1.63E-5 (±7.48E-6) | _ | _ | _ |
| | | 2008 | A-W | 9 | 77.78 [39.9-97.2] | 7 | 2.34E-5 (±8.35E-6) | _ | _ | _ |
| | | 2009 | S-S | 83 | 72.29 [61.3-81.6] | 36 | 2.61E-5 (±5.65E-6) | _ | _ | _ |
| | | Total | | 112 | 72.32 [61.9-79.5] | 57 | 2.33E-5 (±4.16E-6) | _ | _ | _ |
| 14 | Thieux (R) | 2004 | S-S | 45 | 33.33 [20.0-49.0] | 15 | 1.05E-4 (±8.38E-5) | 35 | 27.86 (±0.35) | 78.09 (±0.51) |
| 15 | Vannes (U) | 2008 | A-W | 41 | 24.39 [12.3-40.4] | 10 | 7.39E-5 (±2.17E-5) | 40 | 26.67 (±0.26) | 74.86 (±0.30) |
| 16 | Wissous (U) | 2004 | A-W | 12 | 58.33 [27.6-84.9] | 7 | 5.94E-6 (±3.17E-6) | 12 | 26.75 (±0.51) | 78.21 (±0.79) |
| | | 2004 | S-S | 27 | 40.74 [22.3-61.2] | 11 | 3.07E-5 (±1.11E-5) | 27 | 27.06 (±0.58) | 78.22 (±0.72) |
| | | Total | | 39 | 48.72 [32.3-65.2] | 19 | 2.02E-5 (±7.02E-6) | 37 | 26.96 (±0.30) | 78.22 (±0.41) |

TABLE II. AIC values for the mixed models conducted for prevalence and parasitemia. Missing values indicate that the model did not converge. Interactions that improved model fit (reducing the AIC by ≥2 as compared to the model without interaction) were included in the final model.

| Dependant variable | Prevalence | Parasitemia |
|---|---|---|
| Sample size | 775 | 126 |
| no interaction | 454.1317 | 350.9338 |
| season×sex | 453.3917 | 349.8699 |
| season×age | 455.8444 | 349.2505 |
| season×weight | 455.9944 | 353.7176 |
| season×wing lenght | 454.3304 | 348.1664 |
| season×population | 456.1317 | 352.9338 |
| sex×age | 455.5478 | 346.1485 |
| sex×weight | 456.1135 | 353.9513 |
| sex×wing lenght | 455.0481 | 354.2791 |
| sex×population | 456.1317 | 352.9338 |
| age×weight | 456.1036 | 352.9289 |
| age×wing lenght | 454.9425 | 352.9304 |
| age×population | | |
| weight×wing lenght | 466.3952 | 357.5665 |
| weight×population | | |
| aile×population | | |
| year×season | 456.1317 | 352.3697 |
| year×sex | 456.1041 | 352.9274 |
| year×age | | |
| year×weight | | |
| year×wing lenght | | |
| year×population | 456.1317 | 352.9338 |
| urbanization×season | 456.1314 | 351.0125 |
| urbanization×sex | 455.9468 | 350.9587 |
| urbanization×age | 455.9468 | 350.9587 |
| urbanization×weight | 453.7248 | 353.8833 |
| urbanization×wing lenght | 455.8103 | 353.6134 |
| urbanization×population | | |
| urbanization×year | | |
| Final model | 454.1317 | 342.5857 |

# CHAPITRE 2

# Effets des facteurs environnementaux sur les interactions hôtes-parasites

*En plus des caractéristiques intrinsèques aux hôtes et aux parasites, l'environnement dans lequel ils évoluent peut exercer des pressions de sélection importantes, modifiant la dynamique des interactions Dans le cadre de la malaria aviaire, les études en populations naturelles prenant en compte les facteurs environnementaux sont, à l'heure actuelle, de plus en plus fréquentes. En effet, les changements globaux, et l'anthropisation de l'environnement sont autant de nouvelles pressions de sélection extrinsèques qu'il faut prendre en compte. Dans ce chapitre, nous présenterons plusieurs études tentant de montrer l'influence de certaines caractéristiques de l'environnement et leurs impacts, et ce, à plusieurs échelles géographiques.*

## 1. Le climat

La malaria humaine est une pandémie responsable de la mort de 700 000 à 2.7 millions de personnes chaque année (World Health Organization, 2011 ; www.cdc.gov/malaria). Les facteurs climatiques permettant d'expliquer l'aire de distribution de cette maladie sont aujourd'hui très étudiés. En effet, les problématiques de réchauffement climatique rendent ces études encore plus importantes pour tenter de prédire l'évolution de la distribution géographique de la maladie. Pourtant, la controverse demeure. Les prédictions sont difficiles à confirmer selon les régions étudiées car l'effet confondant des facteurs économiques et sociaux avec les facteurs environnementaux ajoute un degré de complexité (Bouma *et al.*, 2011; Gething *et al.*, 2010).

Les maladies à vecteurs comme la malaria apparaissent particulièrement sensibles aux changements climatiques. En effet, le parasite passe la moitié de sa vie au sein d'un hôte invertébré, et donc ectotherme, dont le cycle de vie est particulièrement dépendant des conditions climatiques, telle que la température (Harvell *et al.*, 2002; Lafferty, 2009; Paaijmans *et al.*, 2010; Patz, Reisen, 2001). De plus, le parasite lui-même dépend fortement de la température. Par exemple, le virus SLE (St Louis encephalitis) a besoin d'une température supérieure à 17°C pour assurer sa réplication au sein du moustique (Reisen, Chiles, 1997). Concernant la malaria humaine, les températures minimales de développement de *Plasmodium falciparum* et *Plasmodium vivax* sont respectivement de 18 et 15°C (Patz, Reisen, 2001). Il apparaît donc clairement qu'une augmentation globale de la température peut augmenter l'aire de distribution du parasite (Lindsay, Birley, 1996; Lindsay, Martens, 1998; Patz, Olson, 2006; Patz, Reisen, 2001).

Une étude sur la malaria humaine, et une autre chez le rongeur, concluent qu'une augmentation des fluctuations journalières de température augmente les risques d'infection (Paaijmans *et al.*, 2010; Paaijmans *et al.*, 2009). Ces études montrent aussi que des fluctuations à des températures basses (16-18°C) augmentent le risque, tandis qu'il est diminué à des températures hautes (24-26°C). Les fluctuations et l'imprévisibilité des facteurs climatiques semblent également augmenter les risques d'infections par la malaria humaine, et ce dans différentes régions du globe (Bouma *et al.*, 1996; Lindblade *et al.*, 1999; Poveda *et al.*, 2001; Thomson *et al.*, 2006).

Dans différentes études, il apparaît que l'augmentation de température affectera très peu les zones où la température est déjà élevée. Ces zones sont déjà touchées par la malaria humaine et l'augmentation du risque sera limitée, principalement à cause de l'immunité de ces populations non-naïves (Patz, Reisen, 2001). Au contraire, dans les zones où la malaria n'est pas présente, une faible augmentation de la température pourrait avoir des effets dévastateurs sur ces populations naïves (Patz, Reisen, 2001). Sur un gradient altitudinal, la température diminue d'environ 6°C tous les 1000m. Les populations africaines vivant en altitude sont actuellement protégées car le parasite ne peut pas se développer. Mais, avec le réchauffement climatique, ces populations seront particulièrement menacées (Bouma *et al.*, 2011; Patz, Lindsay, 1999).

Concernant la malaria aviaire, une étude sur le développement de *Plasmodium* chez le moustique a permis de montrer qu'une augmentation de la température faciliterait l'implantation du parasite dans les zones présentant des conditions favorables à son développement (LaPointe *et al.*, 2010). Une méta-analyse récente, menée sur plus de 3000 espèces d'oiseaux, a permis de montrer que la prévalence de *Plasmodium* avait augmenté en parallèle avec les changements climatiques (Garamszegi, 2011). Beaucoup d'aspects restent encore à explorer dans ce domaine, notamment en ce qui concerne l'épidémiologie de l'hôte intermédiaire.

Le **Manuscrit 5** explore les variations spatiales de prévalence et de parasitémie de *Plasmodium relictum,* dans 24 populations de moineaux domestiques à l'échelle de la France. Dix neuf variables bioclimatiques ont été extraites de chacun des sites et ont été analysées pour évaluer les corrélations avec les prévalences et les intensités parasitaires. Les résultats montrent que la prévalence est fortement corrélée à la température en général, et aux variations journalières de température en particulier. Par contre, aucune variable bioclimatique ne semble expliquer les variations spatiales de parasitémies entre les

populations. Si la prévalence de *Plasmodium relictum* chez le moineau est liée à des variables climatiques, la parasitémie dépendrait plutôt de facteurs intrinsèques à l'hôte, comme semble le confirmer nos études présentées dans le chapitre 1. Des modèles climatiques ont été réalisés afin de prédire la prévalence de *Plasmodium relictum* pour les années 2050 et 2080. Ces modèles montrent que l'aire de distribution de la malaria se déplace vers le nord de la France, et que les zones déjà concernées par des prévalences moyennes verront ces dernières augmenter. Cette étude est une des premières du genre sur ce modèle et illustre clairement l'importance du climat et des futurs changements climatiques sur l'épidémiologie de la malaria aviaire.

## 2. Les facteurs anthropiques

### 2.1 Fragmentation de l'habitat et déforestation

Les activités humaines ont de plus en plus d'impacts sur les milieux naturels. Ces transformations rapides peuvent avoir une forte influence sur la répartition des espèces, y compris sur les pathogènes et les parasites. En particulier, les changements dans l'utilisation des terres, beaucoup plus rapides que les changements climatiques, peuvent affecter des conditions microclimatiques telles que la température et la présence d'eau (Foley *et al.*, 2005; Patz *et al.*, 2005; Suwonkerd *et al.*, 2002), dont les vecteurs de la malaria sont fortement dépendants. Chez la malaria humaine, une étude menée en Thaïlande a montré que les moustiques étaient plus présents en zone forestière qu'en zone agricole (Overgaard *et al.*, 2003). De plus, la fragmentation des habitats forestiers provoque une diminution des densités de moustiques, et donc potentiellement une diminution de la transmission de la malaria aux populations (Overgaard *et al.*, 2003). Pourtant, au Kenya, il a été montré que la déforestation augmentait la température des zones concernées par rapport aux zones forestières, provoquant un raccourcissement de 2 à 3 jours du cycle gonotrophique des femelles moustiques (Afrane *et al.*, 2005). Un résultat similaire a été trouvé en Ouganda en comparant les densités de moustiques trouvés dans les habitations, entre des populations humaines vivant en bordure de zones agricoles et d'autres vivants près de zones peu impactées par les activités humaines (Lindblade *et al.*, 2000). Un changement dans l'utilisation des terres peut aussi affecter les milieux aquatiques, indispensables à la reproduction du moustique. Ainsi, en Afrique, la

présence de larves de moustiques est négativement corrélée avec la présence d'une couverture végétale au-dessus des plans d'eau (Minakawa *et al.*, 2002; Tuno *et al.*, 2005).

Les effets des facteurs anthropiques sur les maladies à vecteurs chez les oiseaux sont peu connus (Sehgal, 2010) et se limitent à quelques études récentes. En 2007, Wood et collaborateurs ont montré que, à l'échelle d'une seule population d'oiseaux, des associations existaient entre la malaria aviaire et la nature de l'environnement. Sebaio *et al.* (2010) ont étudié l'effet de la fragmentation sur la prévalence en *Plasmodium*, *Haemoproteus,* et *Trypanosomes* pour 109 espèces d'oiseaux, et n'ont pas trouvé de différences significatives entre petits et grands patchs forestiers. Au Cameroun, il a été montré une plus forte prévalence en parasites aviaires du genre *Plasmodium* dans les zones perturbées (Bonneaud *et al.*, 2009), contrairement à ce qui avait été montré pour la malaria humaine. Une étude menée, également au Cameroun, sur deux espèces d'oiseaux, a confirmé ce résultat, montrant toutefois que l'effet des perturbations pouvait être bien différent selon la lignée de *Plasmodium* et l'espèce hôte considérée (Chasar *et al.*, 2009). Par contre, sur les îles Hawaï, la présence de zones agricoles et la fragmentation des forêts augmentent la probabilité de capture du moustique vecteur de *Plasmodium relictum* (Reiter, Lapointe, 2007).

Globalement, les activités humaines qui modifient les paysages affectent de façon significative les vecteurs de la malaria, et donc potentiellement l'épidémiologie de cette maladie. Ces modifications peuvent toutefois dépendre de la région du globe, du vecteur, de l'hôte et du parasite concerné. Les résultats parfois contrastés de ces différentes études posent la question de la pertinence des études globales et des méta-analyses (plusieurs hôtes, plusieurs vecteur, plusieurs parasites, plusieurs régions) sur ce type de sujet (Sehgal, 2010).

## 2.2 L'urbanisation et la pollution

L'urbanisation et l'agriculture intensive, en plus de fragmenter les habitats, rejettent un grand nombre de substances toxiques et de polluants capables de dégrader la viabilité des populations naturelles. Des substances, telles que les pesticides, polychlorobiphényles (PCBs) et perturbateurs endocriniens (EDCs), sont connus pour affecter le succès reproducteur, le comportement et le développement de nombreuses espèces (De Luca-Abbott *et al.*, 2001; Gibbs, Bryan, 1986; Markman *et al.*, 2011; Walker, 2003). La pollution aux métaux lourds constitue aussi une menace importante sur la qualité de l'environnement (Dauwe *et al.*, 2000). La présence de ces métaux dans l'environnement est positivement corrélée avec

l'urbanisation et les activités agricoles (Orlowski *et al.*, 2010; Roux, Marra, 2007; Scheifler *et al.*, 2006).

Les oiseaux peuvent être contaminés par ces métaux via l'air, l'eau et la nourriture (Dauwe *et al.*, 2000; Hahn *et al.*, 1993; Scheifler *et al.*, 2006; Veerle *et al.*, 2004). Ces métaux sont connus pour avoir des effets négatifs sur des paramètres physiologiques, tels que l'hématocrite et le statut oxydatif (Baos *et al.*, 2006; Geens *et al.*, 2010; Hoffman, Heinz, 1998; Isaksson *et al.*, 2009), ainsi que sur le succès reproducteur de nombreuses espèces d'oiseaux (De Luca-Abbott *et al.*, 2001). Les métaux lourds semblent aussi affecter la condition corporelle des oiseaux, mais ces effets semblent dépendre du sexe des individus, de la population et de l'espèce étudiée (Dauwe *et al.*, 2006; Janssens *et al.*, 2003; Roux, Marra, 2007; Scheifler *et al.*, 2006; Snoeijs *et al.*, 2005; Snoeijs *et al.*, 2004). La contamination aux métaux lourds est suspectée être une cause du déclin du moineau domestique en Finlande (Kekkonen, 2011).

Si les métaux lourds sont capables d'affaiblir les populations d'oiseaux contaminés, on peut émettre l'hypothèse que cette pollution aura un impact sur la dynamique des maladies infectieuses. En particulier, les maladies à vecteurs seront doublement impactées par la pollution, de part son effet sur l'hôte et sur le vecteur. Un hôte affaibli sera plus facilement contaminé, mais pourra être moins exploité par le parasite (voir chapitre 1). De plus, une contamination aux métaux lourds peut avoir un impact direct sur les fonctions immunitaires, mais les études sur le sujet restent limitées (Snoeijs *et al.*, 2005; Snoeijs *et al.*, 2004).

Pour citer quelques exemples, chez l'harle boréale (*Clangula hyemalis*), il a été montré que les individus contaminés par le choléra étaient aussi ceux qui présentaient la plus forte concentration en cadmium (Mashima *et al.*, 1998). Par ailleurs, le plomb a un effet négatif sur les fonctions immunitaires des oiseaux d'eau (Rocke, Samuel, 1991), tout comme le cadmium chez les mammifères et le poisson chat (Bozelka, 1985; Saxena *et al.*, 1992). La réponse humorale semble également être affectée par la pollution chez la mésange charbonnière (*Parus major*) (Snoeijs *et al.*, 2004). Dans des conditions expérimentales, chez la mésange bleue (*Cyanistes caeruleus*), la réponse immunitaire contre un virus diminue lors d'une contamination au plomb (Fair, Myers, 2002), tandis qu'aucun effet du plomb sur la réponse immunitaire n'a été détecté chez la caille du Japon (*Coturnix japonica*) (Fair, Ricklefs, 2002). Aussi, chez le gobe-mouche noir (*Ficedula hypoleuca*), la réponse humorale augmente dans les zones polluées (Eeva *et al.*, 2005a; Eeva *et al.*, 2005b). Chez l'homme, le zinc augmente également les capacités immunitaires (Shankar, Prasad, 1998), conduisant à une meilleure résistance à la malaria (Good *et al.*, 1998).

Toutes ces études, parfois contradictoires, illustrent la complexité des interactions entre système immunitaire et environnement. Ces interactions semblent dépendre d'une multitude de facteurs, tels que les espèces hôtes et parasites considérées, le polluant étudié et les voies immunitaires mesurées. De plus, les métaux lourds peuvent augmenter le stress oxydatif (Hoffman, Heinz, 1998; Isaksson *et al.*, 2009), ce qui peut interférer avec les voies immunitaires liées à l'inflammation (Sorci, Faivre, 2009). Le temps entre la contamination par les métaux lourds et l'infection apparaît également primordial (Galloway, Depledge, 2001). En effet, il a été montré, chez la souris de laboratoire, que la réponse immunitaire augmentait quand le challenge immunitaire (infection par *Klebsiella pneumoniae*) était précédé d'une contamination au plomb et au nickel, tandis qu'elle diminuait quand la contamination intervenait après le challenge (Laschiloquerie *et al.*, 1987).

La plupart des études en immunotoxicologie concerne les invertébrés et les vertébrés modèles, tels que poulets et rongeurs, le plus souvent en conditions expérimentales (Bridger, Thaxton, 1983; Cook *et al.*, 1975; Galloway, Depledge, 2001; Gardner *et al.*, 1977). L'impact de la pollution sur les maladies infectieuses, comme la malaria, dans les populations naturelles de vertébrés est aujourd'hui largement négligé. Aussi, dans le **Manuscrit 6**, nous avons tenté d'expliquer les causes des concentrations en métaux lourds (plomb, cadmium et zinc) dans 16 populations naturelles de moineaux domestiques. Cette étude est l'une des premières illustrant l'effet de la pollution environnementale sur un parasite relativement répandu dans des populations naturelles d'oiseaux. Les concentrations en plomb ont directement été mesurées chez les individus, via des prélèvements de plumes. Pour 5 de ces 16 populations, nous avons aussi étudié le lien potentiel entre métaux lourds, prévalence, parasitémie de *Plasmodium relictum* et la condition corporelle des oiseaux. En accord avec nos prédictions, les concentrations en plomb sont associées aux zones urbaines. Par contre, les plus fortes concentrations en cadmium et en zinc sont trouvées dans les zones les plus boisées. Nos résultats suggèrent aussi que la concentration en plomb dans les plumes est positivement corrélée avec la prévalence de *Plasmodium relictum*. La relation avec les autres métaux, le statut infectieux et la condition corporelle apparaît plus complexe, et semble notamment dépendre de la population. Quant à la parasitémie, aucune des variables étudiées n'explique les variations observées entre populations. Comme le montrent nos autres études, il apparaît encore ici que l'intensité parasitaire est peu dépendante des facteurs environnementaux.

# 3. Les barrières géographiques

Comme nous venons de le voir, la fragmentation des habitats peut avoir des conséquences sur l'épidémiologie de la malaria aviaire, et en particulier sur les vecteurs. Cette fragmentation, conduisant à la formation de barrières géographiques, peut également avoir un effet sur les populations hôtes, notamment au niveau génétique. En particulier, les populations se trouvant dans des milieux isolés (on parle alors d'îles écologiques), souvent de petites tailles, ont généralement une faible variabilité génétique et une différenciation génétique forte avec les autres populations (Frankham, 1996; Frankham, 1997; Frankham, 1998; Hinten *et al.*, 2003; Luikart, Cornuet, 1998; Miller, Lambert, 2004; Sommer, 2005; van Tienderen *et al.*, 2002; White, Searle, 2007). Ce phénomène est expliqué en grande partie par l'action de facteurs stochastiques et démographiques. La dérive génétique, d'autant plus forte que la taille de la population est faible, fixe certains allèles dans la population et en élimine d'autres. Les flux de gènes permettent d'augmenter la variabilité génétique des populations en apportant de nouveaux allèles, venus d'autres populations. Mais, ces flux sont d'autant plus faibles que les populations sont éloignées et que le milieu est fragmenté. Pourtant, la variabilité génétique est indispensable aux changements évolutifs dans les populations naturelles (Frankham, 1996).

## 3.1 Importance de la variabilité génétique

De nombreuses études ont montré que les populations présentant une variabilité génétique plus faible possédaient un potentiel adaptatif aux variations environnementales réduit, et un risque d'extinction plus élevé (Allendorf, Luikart, 2007; Altizer *et al.*, 2003). Ainsi, l'étude d'une métapopulation de papillon (*Melitaea cinxia*) a révélé qu'une diminution de variabilité génétique intra-population augmentait le risque d'extinction (Saccheri *et al.*, 1998). Une perte drastique de variabilité génétique conduit en général à une augmentation de la consanguinité et à la fixation d'allèles délétères (Luikart, Cornuet, 1998). En effet, une diminution de la taille de la population et des échanges avec les autres populations augmente la probabilité des reproductions entre individus apparentés (Keller, Waller, 2002). Ces appariements consanguins diminuent souvent l'aptitude phénotypique de la descendance, via notamment l'expression de mutations récessives délétères (*e.g.* Leberg, 1993; Ralls *et al.*, 1988). Ces effets extrêmes sont souvent visibles dans les populations d'espèces très menacées. Par

exemple, une étude menée chez une sous-espèce très menacée de panthère (*Puma concolor*) a mis en évidence des traits morphologiques délétères (cryptochordisme, malformation des vertèbres, faible qualité spermatique) liés à la fixation de mutations par dérive génétique (Culver *et al.*, 2000).

Dans la plupart des études citées précédemment, et dans la majorité des études sur la variabilité génétique, cette dernière a été évaluée en utilisant des marqueurs dits neutres, des loci microsatellites. Ces marqueurs sont aujourd'hui bien développés et accessibles, même pour les organismes non-modèles. Si cette variabilité génétique neutre reflète l'action des processus démographiques, tels que dérive et flux de gènes, elle n'apporte aucune information sur la variabilité génétique adaptative (McKay, Latta, 2002; Sommer, 2005). Le potentiel adaptatif des populations naturelles est plus difficilement mesurable. Une possibilité est d'étudier le polymorphisme de gènes soumis à la sélection, c'est-à-dire des gènes codants pour des fonctions de l'organisme. Mais, ce type de gènes polymorphes est d'une part, peu répandu, et d'autre part, difficilement accessible pour les organismes non-modèles. Les gènes sélectionnés, s'ils sont soumis à l'action de la sélection naturelle, n'échappent pas aux effets des facteurs démographiques. Afin de pouvoir dégager l'effet de la sélection sur ces gènes, il est nécessaire d'étudier en parallèle la variabilité obtenue avec des marqueurs neutres (van Tienderen *et al.*, 2002).

Les exemples de marqueurs sélectionnés restent limités chez les organismes non-modèles. Nous pouvons tout de même citer les gènes de résistance aux insecticides chez le moustique (Pasteur, Raymond, 1996), et le gène TAP (Transporteur associated with Antigen Processing), codant pour un transporteur associé à la réponse immunitaire, chez le saumon (Jensen *et al.*, 2008). Les gènes les plus souvent utilisés dans ce domaine sont les gènes du Complexe Majeur d'Histocompatibilité (CMH).

### 3.2 Les gènes du Complexe Majeur d'Histocompatibilité

L'utilisation des gènes du CMH dans des problématiques d'adaptation et de variabilité génétique est de plus en plus importante. Leur fonction connue, leur génotypage possible chez des espèces non-modèles, leur polymorphisme et les preuves que la sélection agit sur ces gènes, expliquent leur succès (Bernatchez, Landry, 2003).

Ces gènes codent pour des protéines de surface cellulaire situées à la base de la réponse immunitaire chez les vertébrés (Hedrick, 1994 ; voir la partie modèles biologiques et méthodologies pour plus d'informations). Les gènes du CMH constituent la famille de gènes

la plus polymorphe du génome des vertébrés. Cet important polymorphisme peut paraître contradictoire avec l'action supposée de la sélection naturelle sur ces gènes. Pourtant, de nombreuses preuves de sélection ont été mises en évidence sur ces gènes (Encadré 1) et un type de sélection particulier, la sélection balancée, permettrait de maintenir le polymorphisme de ce type de gènes (Bernatchez, Landry, 2003). Deux grandes hypothèses non-exclusives de mécanismes sélectifs permettant d'expliquer le maintien du polymorphisme des gènes du CMH sont généralement avancées : la sélection sexuelle (voir chapitre 3), et la sélection exercée par les parasites (PMS, pour parasite-mediated selection) (Figure 16). En effet, les parasites sont de forts agents de sélection pour leurs populations hôtes car ils ont un impact énorme sur la survie, la reproduction et la structure génétique des populations hôtes (Little, 2002). Des études ont notamment mis en évidence une association entre la présence d'allèle CMH et une résistance/susceptibilité à certains parasites (Bonneaud *et al.*, 2005; Loiseau *et al.*, 2008; Plachy *et al.*, 1992; Tollenaere *et al.*, 2008) ; ainsi qu'une corrélation positive entre diversité CMH et diversité parasitaire au sein des populations (Wegner *et al.*, 2003b). La PMS pourrait maintenir le polymorphisme des gènes du CMH selon trois mécanismes : *(i) l'avantage de l'hétérozygote*, *(ii) l'avantage du rare*, et *(iii) les variations spatio-temporelles*.

*(i) L'avantage de l'hétérozygote* a été proposé pour la première fois par Doherty, et Zinkernagel (1975). Ce mécanisme implique qu'un individu hétérozygote pour un locus CMH pourra, en théorie, reconnaître plus de pathogènes qu'un individu homozygote, et ainsi augmenter sa fitness (Doherty, Zinkernagel, 1975; Hedrick, 1998; Hughes, Nei, 1992). On parle de dominance quand l'hétérozygote est aussi résistant que l'homozygote le plus résistant, et de super-dominance quand l'hétérozygote est plus performant que les deux homozygotes. La super-dominance est plus efficace que la dominance pour maintenir le polymorphisme du CMH (Sommer, 2005). Ce mécanisme repose sur des modèles théoriques (Hedrick, 2002; Hughes, Nei, 1992) et sur des tests empiriques. Chez l'homme, une homozygotie pour deux loci CMH (HLA-A et HLA-B) a été associée à une progression plus rapide du VIH (Tang *et al.*, 1999). Chez un rongeur (*Rhabdomys pumilio*), l'hétérozygotie à un locus (DRB) est associée à une diminution de la prévalence et de la parasitémie de la communauté de nématodes (Froeschke, Sommer, 2005). Au contraire, d'autres études n'ont pu mettre en évidence d'avantages aux individus hétérozygotes, et ont même montré que certains génotypes hétérozygotes étaient plus susceptibles aux infections que certains génotypes homozygotes (Woelfing *et al.*, 2009). Une hétérozygotie maximale n'est peut-être pas toujours optimale (Woelfing *et al.*, 2009), comme le montre une étude chez l'épinoche où

les individus les moins parasités ont un nombre d'allèles CMH intermédiaire (Wegner *et al.*, 2003a).

*(ii) L'avantage du rare* est un mécanisme basé sur la coévolution hôte-parasite qui prédit une adaptation locale du parasite au génotype hôte le plus commun (Lively, Dybdahl, 2000; Sasaki, 2000). Ce mécanisme est aussi appelé sélection fréquence-dépendante. Un hôte possédant un allèle CMH rare va être avantagé dans la population, la fréquence de cet allèle va augmenter jusqu'à ce que le parasite s'y adapte, de nouveaux allèles rares étant alors sélectionnés. Ces fluctuations temporelles des fréquences alléliques permettent ainsi le maintien du polymorphisme (Golding, 1992; Takahata, Nei, 1990). Ce modèle a été validé par quelques études en populations naturelles. Chez le microcèbe *Microcebus murinus*, l'allèle CMH le plus commun est porté par les individus les plus infectés, contrairement aux individus porteurs d'allèles rares (Schad *et al.*, 2005). De même, les jeunes moutons porteurs d'allèles CMH fréquents ont une survie plus faible et une résistance aux parasites moins efficace (Paterson, 1998).

**Encadré 1 : Les signes de sélection sur les gènes du CMH**

**Preuve de sélection à l'échelle des temps évolutifs**

**dn/ds ratio :** C'est la méthode la plus courante pour détecter une sélection positive (Hill, Hastie, 1987; Hughes, Nei, 1988). Elle est basée sur le fait que les mutations synonymes (ds) sont neutres, car elles ne changent pas la nature de l'acide aminé. Les mutations non-synonymes (dn) peuvent être maintenues dans les populations par une sélection positive. Dans ce cas, le ratio dn/ds sera significativement supérieur à 1. Sur 48 études ayant utilisé cette méthode, seulement une n'a pas obtenu ce résultat sur les gènes du CMH (Bernatchez, Landry, 2003). Cette méthode implique d'avoir un accès aux séquences des gènes du CMH.

**Le polymorphisme trans-espèces :** La théorie de la coalescence prédit que le polymorphisme neutre n'est pas maintenu longtemps après un événement de spéciation (Figueroa *et al.*, 1988). En revanche, ce polymorphisme peut persister sous sélection balancée, et donner lieu à des relations généalogiques très longues entre allèles d'espèces différentes (Takahata, Nei, 1990). La persistance de ces lignées alléliques sur les gènes du CMH a été détectée dans 40 études sur 42 (Bernatchez, Landry, 2003), et en particulier entre saumon royal et saumon du Pacifique (Garrigan, Hedrick, 2001).

**Preuves de sélection dans les populations contemporaines**

**L'homozygotie :** Sous une hypothèse de neutralité, l'homozygotie attendue d'une population dépend de sa taille et du nombre d'allèles (Ewens, 1972; Watterson, 1978). Sous sélection balancée, quand les allèles rares sont avantagés, on s'attend à avoir significativement moins d'homozygotes pour les gènes du CMH, qu'attendu sous hypothèse de neutralité (Hambuch, Lacey, 2002; Landry, Bernatchez, 2001; Miller *et al.*, 2001; Paterson, 1998).

**La distribution des fréquences alléliques :** Sous hypothèse de neutralité, on observe en général quelques allèles fréquents et de nombreux allèles rares. Pour les gènes du CMH, on trouve généralement de nombreux allèles à des fréquences relativement homogènes. Cette distribution des fréquences allélique peut être déterminée par l'action de la sélection balancée (Bernatchez, Landry, 2003; Slatkin, Muirhead, 2000).

**Structure des populations pour les gènes du CMH :** Cette méthode nécessite l'analyse parallèle de la structure génétique neutre et de la structure génétique obtenue avec les gènes du CMH, dans les mêmes populations. Sous neutralité, les niveaux de différenciation entre populations pour marqueurs neutres et gènes du CMH devraient être similaires (Aguilar, Garza, 2006; Aguilar *et al.*, 2004; Piertney, 2003). Sous sélection balancée, les niveaux de différenciation CMH devraient être plus faibles qu'avec les marqueurs neutres (Bernatchez, Landry, 2003; Boyce *et al.*, 1997; Hedrick *et al.*, 2001; Huang, Yu, 2003; Schierup *et al.*, 2000). Par contre, si les pressions de sélection diffèrent entre populations, un niveau de différenciation plus élevé est attendu pour les gènes du CMH que pour les marqueurs neutres (Charbonnel, Pemberton, 2005; Ekblom *et al.*, 2007; Loiseau *et al.*, 2009). Cette méthode implique toutefois de pouvoir comparer les patrons de différenciation obtenus avec deux types de marqueurs différents, n'ayant pas les mêmes taux de mutations. Parfois, la différenciation n'est pas exactement calculée de la même manière entre les deux types de marqueurs.

*(iii)* Des *variations spatio-temporelles* dans l'abondance et la composition des communautés parasitaires sont courantes, même à des échelles de temps et d'espace très courtes (Freeman-Gallant *et al.*, 2001; Wegner *et al.*, 2003b). Ces variations peuvent générer des fluctuations dans les pressions de sélection sur les gènes du CMH et contribuer au maintien de leur diversité, comme l'a montré le modèle proposé par Hedrick (2002). Ce troisième type de mécanisme est de loin le moins bien étudié, quelques études le suggèrent, mais les preuves de son existence restent relativement indirectes. Une étude menée sur les moutons de l'archipel de Saint Kilda a mesuré des niveaux de différenciation entre populations plus élevés pour les gènes du CMH que pour les marqueurs neutres (Charbonnel, Pemberton, 2005). Ce résultat est imputé aux fluctuations spatiales de la prévalence d'un nématode (*Teladorsagia circumcincta*). Les niveaux de différenciation génétique temporelle sont aussi plus élevés pour les gènes du CMH, suggérant également des fluctuations temporelles dans les cortèges parasitaires (Charbonnel, Pemberton, 2005). Cette hypothèse reste cependant à étayer par des preuves empiriques. En effet, si les parasites sont généralement évoqués comme exerçant de fortes pressions de sélection sur les gènes du CMH, relativement peu d'études ont mesuré à la fois la structure génétique des hôtes et la structure des communautés de parasites.

Dans le **Manuscrit 7**, nous avons cherché à savoir si les parasites sanguins des genres *Plasmodium* et *Haemoproteus*, pouvaient exercer des pressions de sélection capables d'avoir une influence sur la structure génétique des hôtes. Cette étude est une des premières à tenter de prendre en compte à la fois l'hôte, le parasite et l'environnement (Figure 3). En effet, nous avons étudié douze populations de moineaux domestiques en Bretagne, comprenant 6 populations insulaires et 6 populations continentales. Ces deux types de populations se trouvent donc dans des contextes démographiques très différents, ce qui peut avoir une influence sur la variabilité et la structure génétique des populations hôtes et parasites (Blondel, 1995; Frankham, 1997; Frankham, 1998). Afin de pouvoir déterminer l'effet potentiel des parasites, nous avons étudié la variabilité et la structure génétique de nos populations à l'aide de deux types de marqueurs : des marqueurs neutres, avec 12 loci microsatellites, et des marqueurs sélectionnés, avec les gènes du CMH de classe I. Des études précédentes, chez le moineau domestique, ont déjà mis en évidence des signes de PMS sur les gènes du CMH. La présence de certains allèles CMH a été associée à une résistance/susceptibilité aux parasites sanguins (Bonneaud *et al.*, 2006b; Bonneaud *et al.*, 2005; Loiseau *et al.*, 2008; Loiseau *et al.*, 2011). De plus, il a été déjà mis en évidence

l'action d'une sélection diversifiante sur ces gènes, dans d'autres populations, à une échelle géographique plus large (Loiseau *et al.*, 2009 ; Loiseau *et al.*, 2011).

De par leur isolement plus important et leur plus faible surface, nous nous attendons à ce que les îles présentent une variabilité génétique neutre plus faible que sur le continent. Nous attendons également une différenciation génétique neutre plus forte au niveau des populations insulaires qu'au niveau des populations continentales. Concernant les gènes du CMH, les patrons de variabilité et les niveaux de différenciation dépendront de la force et du type de pression de sélection exercée. En effet, si la sélection est assez forte pour contrecarrer la dérive dans les petites populations, la variabilité génétique neutre sera faible, mais la variabilité CMH sera maintenue (Aguilar *et al.*, 2004; Garrigan, Hedrick, 2001; Gutierrez-Espeleta *et al.*, 2001). Par contre, si la dérive est trop forte, la variabilité des gènes du CMH sera faible elle aussi (Miller, Lambert, 2004; Munguia-Vega *et al.*, 2007; Wan *et al.*, 2006). Nous pouvons noter que les études citées précédemment ont été réalisées sur des populations et des espèces menacées. La variabilité génétique de ces populations a donc été comparée avec celle d'autres espèces proches ou d'autres populations, mais géographiquement éloignées. Notre étude a l'avantage de comparer petites et grandes populations pour la même espèce, à une échelle géographique relativement faible. Les pressions de sélection exercées par les parasites sanguins ont été évaluées par la prévalence dans chaque population. La différenciation des communautés de parasites à été évaluée via des indices de similarité entre chaque paire de populations.

Nos résultats montrent que la richesse allélique neutre est plus faible dans les populations insulaires que dans les populations continentales. Avec ces mêmes marqueurs, les populations insulaires apparaissent également plus différenciées. La différenciation est principalement expliquée par la présence de mer entre populations, qui semble constituer une barrière importante à la dispersion pour notre espèce.

Concernant les gènes du CMH, nous n'avons pas noté de diminution significative de la variabilité génétique entre populations insulaires et populations continentales. En général, ce maintien de variabilité génétique est imputé à l'action de la sélection sur ces gènes (Aguilar *et al.*, 2004; Garrigan, Hedrick, 2001; Gutierrez-Espeleta *et al.*, 2001; Hedrick, 2002). Les patrons de différenciation sur les gènes du CMH montrent aussi que les populations insulaires sont les plus différenciées. Cependant, cette différenciation CMH apparaît moins marquée que pour les marqueurs neutres, ce qui peut mettre en évidence l'effet d'une sélection balancée (Bernatchez, Landry, 2003; Boyce *et al.*, 1997; Hedrick *et al.*, 2001; Huang, Yu, 2003; Schierup *et al.*, 2000). A une faible échelle géographique, les pressions de sélection

peuvent être relativement homogènes entre nos différentes populations. Mais, une absence de sélection ou une sélection faible sur les gènes du CMH peuvent aussi expliquer la similarité entre nos patrons de différenciation neutre et sélectionnée (Aguilar, Garza, 2006; Aguilar *et al.*, 2004; Piertney, 2003). La forte corrélation qui existe dans nos populations entre différenciation neutre et différenciation sélectionnée indique d'ailleurs que les gènes du CMH sont également soumis à l'action des processus neutres.

La prévalence en parasites sanguins est très faible dans nos populations, et elle est encore plus faible dans les populations insulaires que dans les populations continentales. Ainsi, nous pouvons supposer que les pressions de sélection exercées par ces parasites seront relâchées dans les îles. Nous avons aussi vu que des variations spatiales dans les cortèges parasitaires pouvaient permettre le maintien du polymorphisme des gènes du CMH. Ces variations spatiales ont été mises en évidence dans d'autres études, même à de petites échelles géographiques (Loiseau *et al.*, 2011; Wood *et al.*, 2007).

Le résultat le plus marquant de notre étude est la mise en évidence d'une corrélation entre la similarité des cortèges de parasites sanguins et la différenciation génétique CMH entre populations. Plus les populations de moineaux possèdent des communautés de parasites sanguins différentes, plus elles sont différenciées pour leurs gènes du CMH, ce qui n'est pas le cas au niveau des marqueurs neutres.

*Pour résumer :*

| Manuscrit | Etude | Hypothèses | Résultats | Conclusion |
|---|---|---|---|---|
| 5 | Effet du climat sur la malaria aviaire et prédictions à long terme. | Des variables bioclimatiques telles que la température ont des conséquences sur les risques d'infection des hôtes.<br><br>Leurs modifications provoqueront des changements dans l'épidémiologie de la malaria et dans la distribution géographique de cette maladie. | Les fluctuations journalières de température expliquent très bien les prévalences mesurées. Aucune variable bioclimatique n'influence la parasitémie.<br><br>En 2050 et en 2080, une augmentation de la température élargira l'aire de répartition de la malaria vers le nord de la France et augmentera la prévalence des zones déjà infectées. | La température permet de déterminer la prévalence en *Plasmodium* des populations de moineaux. La parasitémie n'est pas liée au climat mais pourrait plutôt dépendre des caractéristiques intrinsèques de l'hôte.<br><br>Les variations journalières de température sont primordiales pour comprendre l'épidémiologie de la malaria aviaire et doivent être prise en compte dans un contexte de réchauffement climatique. |
| 6 | Effet de l'urbanisation sur les contaminations en métaux lourds et les conséquences sur l'épidémiologie de la malaria aviaire dans des populations naturelles de moineaux domestiques. | La présence de métaux lourds augmente avec le degré d'urbanisation.<br><br>De part l'effet immunosuppresseur des polluants, les individus contaminés auront plus de risques d'être infectés par *Plasmodium*. | Le plomb est plus élevé dans les populations urbaines. Cadmium et zinc sont associés à la présence de boisements.<br><br>La concentration en plomb est positivement corrélée à la prévalence. L'effet des autres métaux est plus complexe et dépend notamment de la population. | La pollution environnementale a un effet sur l'épidémiologie de *Plasmodium*. Les interactions hôtes-parasites sont susceptibles d'être modifiées par ces facteurs anthropiques dont les conséquences à long terme sont difficiles à évaluer.<br><br>Là encore, la parasitémie n'est pas influencée par les caractéristiques environnementales étudiées. |
| 7 | Effet des communautés de parasites sanguins et de la géographie sur la structure génétique des gènes du CMH de populations naturelles de moineaux domestiques. | La structure génétique du CMH dans les populations reflète l'action de la sélection exercée par les parasites.<br><br>La taille de la population et son isolement ont aussi un effet sur la structure génétique. | La variabilité génétique neutre est plus faible dans les îles. La variabilité des gènes du CMH est maintenue.<br><br>Les populations insulaires sont les plus différenciées, pour les deux types de marqueurs.<br><br>La différenciation CMH est négativement corrélée à la similarité des communautés de parasites sanguins entre populations. | La géographie a une influence sur la structure génétique des hôtes.<br><br>La structure des gènes du CMH est sous l'influence des pressions de sélection exercées par les parasites, même quand la prévalence et l'échelle géographique sont faibles.<br>Les gènes du CMH sont aussi soumis aux processus neutres. |

# Manuscrit 5

## Predictions of avian *Plasmodium* expansion under climate change

Claire Loiseau, Ryan J. Harrigan, Coraline Bichet, Romain Julliard, Stéphane Garnier, Ádám Z. Lendvai, Olivier Chastel and Gabriele Sorci

Soumis à *Scientific Report*

# Predictions of avian *Plasmodium* expansion under climate change

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**Abstract:** Vector-borne diseases are particularly responsive to environmental conditions, as climate can affect both vector population dynamics and disease transmission. Diurnal temperature variation has been identified as a particularly important factor for the development of malaria parasites within vectors. Here, we conducted a large survey across France, screening 24 populations of House sparrow (*Passer domesticus*) for malaria parasites (*Plasmodium relictum*). Both prevalence and parasitemia exhibited extensive spatial variability across populations. We therefore investigated whether variation in remotely-sensed environmental variables accounted for the spatial variation of prevalence and parasitemia. While prevalence was highly correlated to daytime temperature range and other measures of temperature variation, environmental conditions could not predict spatial variation in host parasitemia. Based on our empirical data, we mapped malaria distribution under climate change scenarios for 2050 and 2080 and predicted that *Plasmodium* occurrence will spread to regions in northern France, and that prevalence levels are likely to increase in locations where transmission already occurs, resulting in potential impacts to both house sparrows and other avian malaria hosts. Our findings, based on remote sensing tools coupled with empirical data, show that climatic change will significantly alter transmission of malaria parasites and reinforce the notion that diurnal temperature variation is a key factor for *Plasmodium* transmission in natural host populations.

## Introduction

Climate change and its resulting habitat alteration are expected to have major impacts on the dynamics of infectious diseases (Massad *et al.*, 2011; Paaijmans *et al.*, 2010; Harvell *et al.*, 2002). Vector-borne parasites can have complex responses to climate change since environmental modifications affect the developmental time and survival of the vector as well as of the parasite within its invertebrate host (Rohr *et al.*, 2011). Global warming effects on human vector-borne diseases, such as dengue or malaria, have been extensively investigated (Patz & Reisen, 2011; Patz & Olson, 2006); however, it can be extremely challenging to address the impacts of future climate change on human infectious diseases, as the influence of socioeconomic and environmental factors are often intertwined[7]. Although it is now well-documented that the malaria transmission cycle is sensitive to climate, there is a mismatch between the predicted expansion of

areas with endemic malaria based on climatic models and the observed reduction in endemicity over the last century (Gething *et al.*, 2010). Therefore, the assumed link between rising temperature and the spread of human malaria is heavily debated (Bouma *et al.*, 2011). A way to circumvent the problem of the antagonistic effects of socioeconomic and environmental factors is to focus on wildlife parasites, such as the avian malaria *Plasmodium relictum*. This avian model system offers the opportunity to examine and better understand how modifications of environmental conditions will affect the spatial distribution of vector-borne diseases.

**Table 1.**
GPS coordinates, sample size, prevalence (%) and parasitemia (relative quantification log+1 ± SE) are given for each of the 24 populations sampled from South to North. No SE is given for Quimper and Kerinou since only one individual was infected in each site.

| Site | N | Prevalence | Parasitemia | Latitude | Longitude |
|---|---|---|---|---|---|
| Saintes Maries de la Mer | 112 | 72.32 | $1.01^{E-05}$ ($\pm 1.81^{E-06}$) | 43°27'10" N | 4°25'43" E |
| Arles | 49 | 78.57 | $2.10^{E-04}$ ($\pm 1.80^{E-04}$) | 43˚40'35.90" N | 4˚37'40.12" E |
| Chizé | 54 | 56.52 | $3.83^{E-06}$ ($\pm 1.48^{E-06}$) | 46˚08'49.35" N | 0˚25'31.98" W |
| Dijon | 20 | 55 | $9.29^{E-05}$ ($\pm 4.57^{E-05}$) | 47°19'18" N | 5°02'29" E |
| Hoedic | 685 | 11.09 | $5.26^{E-05}$ ($\pm 3.83^{E-05}$) | 47˚20'23.46" N | 2˚52'40.56" W |
| Cosnes Cours sur Loire | 61 | 50 | $3.57^{E-06}$ ($\pm 6.67^{E-05}$) | 47˚23'15.05" N | 2˚54'27.88" E |
| Groix | 45 | 0 | 0 | 47°38'21.95" N | 3°27'13.04" W |
| Vannes | 42 | 23.81 | $3.21^{E-05}$ ($\pm 9.40^{E-06}$) | 47°39'21" N | 2°45'37" W |
| Ploemeur | 15 | 13.33 | $1.35^{E-05}$ ($\pm 3.51^{E-06}$) | 47°44'08.50" N | 3°25'38.18" W |
| Languidic | 56 | 16.07 | $3.33^{E-05}$ ($\pm 1.53^{E-05}$) | 47°50'03" N | 3°09'24" W |
| Quimper | 32 | 3.13 | $3.69^{E-04}$ | 47°59'51.58" N | 4°05'52.76" W |
| Sein | 32 | 0 | 0 | 48°02'13.94" N | 4°51'06.23" W |
| Kerinou | 26 | 3.85 | $1.58^{E-04}$ | 48°20'32.92" N | 4°45'21.69" W |
| Crennes | 55 | 30.77 | $4.57^{E-05}$ ($\pm 3.22^{E-05}$) | 48˚22'42.54" N | 0˚16'44.60" W |
| Molène | 30 | 0 | 0 | 48°23'46.51" N | 4°57'30.36" W |
| Anglus | 58 | 35.71 | $2.81^{E-05}$ ($\pm 1.97^{E-05}$) | 48˚23'54.54" N | 4˚44'23.47" E |
| Saint Elven | 15 | 0 | 0 | 48°27'41.15" N | 4°22'22.07" W |
| Ouessant | 64 | 0 | 0 | 48°27'48.15" N | 5°05'16.57" W |
| Wissous | 39 | 48.72 | $8.79^{E-06}$ ($\pm 3.05^{E-06}$) | 48˚43'51.48" N | 2˚19'38.69" E |
| Cachan | 20 | 30 | $1.50^{E-05}$ ($\pm 1.40^{E-05}$) | 48˚47'41.08" N | 2˚20'06.64" E |
| Rully | 58 | 35.19 | $1.08^{E-04}$ ($\pm 4.76^{E-05}$) | 48˚49'30.91" N | 0˚42'52.15" W |
| Paris | 52 | 30.77 | $1.97^{E-06}$ ($\pm 1.53^{E-06}$) | 48˚50'39.47" N | 2˚21'43.62" E |
| Crégy les Meaux | 85 | 41.1 | $2.26^{E-06}$ ($\pm 1.90^{E-06}$) | 48˚58'40.54" N | 2˚52'36.15" E |
| Thieux | 45 | 33.33 | $4.57^{E-05}$ ($\pm 3.64^{E-05}$) | 49˚32'36.74" N | 2˚19'01.13" E |

Recently, temperature constraints on the sporogonic development of the avian *Plasmodium* parasite have been investigated within the mosquito vector (LaPointe *et al.*, 2010), demonstrating that any increase of temperature could facilitate the spread of the parasite to areas that present suitable environmental conditions for its development. In addition, a literature survey from more than 3,000 avian species found that the prevalence of *Plasmodium* has increased in parallel with climate changes (Garamszegi, 2011). While literature surveys are informative, identifying the environmental drivers of local malaria prevalence and parasitemia is paramount to linking the disease to expected environmental changes. Here, we extensively sampled a ubiquitous host, the House sparrow (*Passer domesticus*) and its generalist parasite *Plasmodium relictum*, on a regional spatial scale. Generalist parasites are particularly interesting with respect to the potential consequences of climate change because they might easily switch to new hosts as long as they colonize new areas (Hellgren *et al.*, 2009). Generalist parasites are maintained by multiple host species and they can persist with higher virulence relative to specialist parasites (Hellgren *et al.*, 2009). They can also be devastating to immunologically naïve host populations. The example of the introduction of *Plasmodium relictum* (the lineage GRW4) and its competent vector, *Culex quinquefasciatus*, in Hawaii is well known to have decimated endemic avian species (VanRiper *et al.*, 1986).

From a physiological perspective, in addition to mean temperatures, diurnal fluctuations in temperature have been shown to affect the rate of parasite development, and

the essential elements of mosquito biology (Paaijmans *et al.*, 2010). With the recent advent of fine-scale remotely sensed variables for both current and future climate conditions (Hijmans *et al.*, 2005), an investigation of how such remote sensing layers are related to detailed empirical field data on host-parasite dynamics can add much to our understanding of the potential direction of evolutionary changes induced by environmental modifications (Sehgal *et al.*, 2010). Based on the sampling of a ubiquitous host species across a large region, we predict that prevalence (i.e. the likelihood to get infected) of *Plasmodium relictum* should be linked to climatic conditions and especially temperature variables, and that parasitemia (i.e. the multiplication of the parasite within the host) should not exhibit any relation to environmental characteristics since parasitemia is known to be rather linked to intrinsic host factors, such as its genetic makeup or its immune response (Knowles *et al.*, 2011; Kaslow *et al.*, 2008). In addition, we predict that under climate change scenarios, the occurrence of *Plasmodium* will experience significant geographical range changes.

**Materials and Methods**

Samples were collected across France at 24 sites between 2004 and 2008. In total, 1750 individuals were captured using mist-nets and nest boxes (Table 1). We banded them with a numbered metal ring and blood samples (20 µl) were collected from the brachial vein and stored in lysis buffer (10 mM Tris-HCL pH 8.0, 100 mM EDTA, 2% SDS).

DNA was extracted from whole blood using the Qiaquick 96 Purification Kit (QIAGEN) according to the manufacturer's instructions. For *Plasmodium* detection, we used a nested PCR to amplify a 600 bp fragment of cytochrome *b* with the primers HAEMF/HAEMR2 - HAEMNF/HAEMNR2 (Waldenström *et al.*, 2004). The PCR products were run out on a 2% agarose gel using 1×TBE, and visualized by an ethidium bromide stain under ultraviolet light to check for positive infections. We identified lineages by sequencing the fragments on an ABI3730XL, Applied Biosystems. For each positive PCR product, we also performed a quantitative PCR to obtain parasitemia (Cellier-Holzem *et al.*, 2010). Briefly, for each individual, we conducted two qPCR in the same run: one targeting the nuclear 18s rDNA gene of *Plasmodium* and the other targeting the 18s rDNA gene of bird (see Cellier-Holzem *et al.*, 2010 for primers and probe sequences). Parasite intensities were calculated as relative quantification values (RQ) as $2e - (C_t18s\ Plasmodium - C_t18s\ bird)$ using the software SDS 2.2 (Applied Biosystem). $C_t$ represents the number of PCR cycles at which fluorescence is first detected as statistically significant above the baseline, which is inversely correlated with the initial amount of DNA in a sample. RQ can be interpreted as the fold-amount of target gene (*Plasmodium* 18s rDNA) with respect to the amount of the reference gene (host 18s rDNA). All qPCR were run on an ABI 7900HT real-time PCR system (Applied Biosystem).

*Modeling Under Random Forest*

We modeled the ability of 19 bioclimatic variables, downloaded from the WorldClim database (Hijmans *et al.*, 2005; http://www.worldclim.org; see Supplemental Information) at 1km resolution, and elevation measure, downloaded from the Earth Remote Sensing Data Analysis Center (ERSDAC, http://www.gdem.aster.ersdac.or.jp) at ~30m resolution, to predict variation in two response variables in House sparrow hosts, parasitemia and prevalence of avian malaria (see Supplemental Information).

We ran 5000 regression trees in a Random Forest model (RandomForest; Liaw & Weiner, 2002), under the R framework (R development Core Team, 2004) to measure the percent variation explained in each response. We extracted two measures of variable importance for each model, the percent increase in mean square error when individual variables are randomly permutated, and the total decrease in node impurities from splitting each variable, as measured by the residual sum of squares. Due to the inability of our suite of variables to predict variation in parasitemia, we focused further analyses only on malaria prevalence.

We then created spatial predictions by applying the relationships determined by the models to 30,000 randomly-selected points within the host range, to predict prevalence in unsampled areas under current climatic conditions. These predictions were then used to create interpolations between points using an Ordinary Kriging (Oliver, 1990), in order to

generate a continuous spatial prediction map of current malaria prevalence (Fig. 3a).

Finally, using the current relationship between climate and malaria prevalence, we projected the spatial variation of prevalence under future climate conditions using environmental data downloaded from the 4th Assessment of the IPCC under an A1 scenario, http://www.worldclim.org). These projections were made for two decadal time periods, 2050 and 2080, in an attempt to understand how patterns of malaria prevalence are likely to change under future climate conditions (Fig. 3b).

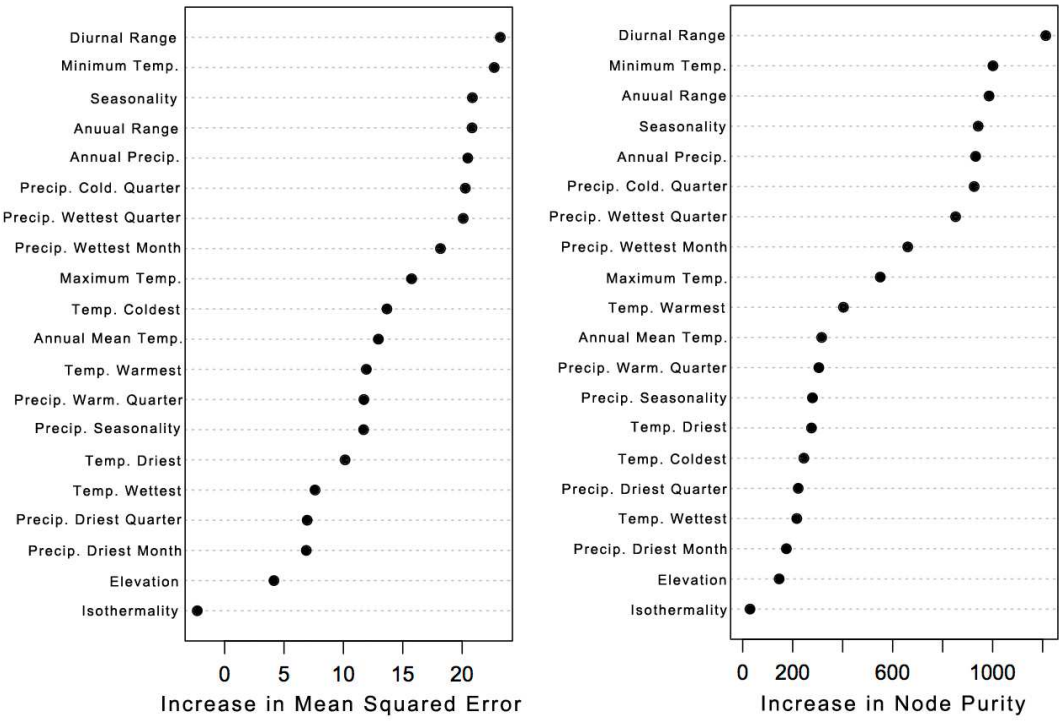


**Figure 1.**
Importance scores for each environmental variable used as input in random forest algorithm models. Increase in mean square error is calculated as the average increase in squared residuals of the test set when the explanatory variable is randomly permuted. When a given variable has little predictive power, its permutation will not cause substantial difference in model residuals, therefore a higher increase in mean square error is indicative of a more important variable. A larger increase in purity represents more homogeneous calls within partitions of the data (see Supplemental Information).

## Results

We determined the prevalence and parasitemia of *Plasmodium relictum* of 1750 individuals sampled in 24 different populations in France (Table 1). We found 24% of infected sparrows with the parasite *Plasmodium relictum* (lineages *Plasmodium* SGS1 and *Plasmodium* GRW11). Prevalence varied from 0% to 78% and parasitemia (relative quantification, see methods) from 0 to 3.7E-04 ± SE between populations (Table 1).

Bioclimatic variables and elevation did little to explain parasitemia, and optimized models never explained more than 2% of variation in parasitemia. In contrast, models attempting to explain variation in malaria prevalence performed well, with up to 83% of all variation explained by only bioclimatic and elevation variables. Of these variables, under both criteria used to evaluate performance (percentage of increase of mean squared error and increase of purity; Fig. 1 and see Supplemental Information), temperature variables were the most important in describing where difference in prevalence occurred (Fig. 1). Interestingly, sampled locations with higher diurnal temperature ranges (Fig. 2), higher temperature seasonality, and higher annual temperature ranges, were areas that had higher malaria prevalence in sparrow hosts. Under future climate scenarios (IPCC 4th Assessment A1 scenario for the year 2050 and 2080), we found a shift in regions suitable for *Plasmodium* occurrence. Sparrow populations from most of the geographical

area covered by this study are predicted to suffer from a substantial increase of malaria prevalence (Fig. 3). This is due to the fact that for much of the study region, while temperatures are predicted to increase, temperature diurnal and annual ranges are also predicted to increase. This is supported by evidence suggesting weather extreme events are likely to increase globally (Coumou & Rahmstorf, 2012).

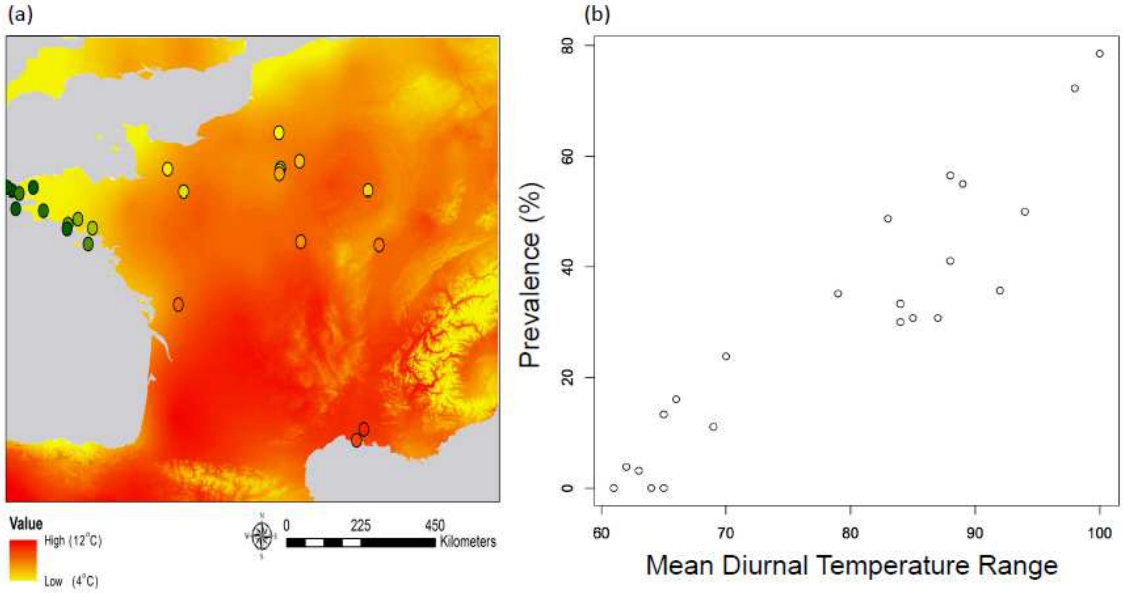


**Figure 2.**
(a) Map showing i) prevalence of *Plasmodium* (dots at sampled locations, warmer colors equal higher prevalence) and ii) the diurnal temperature range (mean of monthly (max temp − min temp); the most important variable in our models); warmer colors indicate higher diurnal temperature ranges; (b) Correlation between the prevalence (percentage of infected individuals per site) and the mean diurnal temperature range (Celsius degree x10). Map created in ArcMap 10 (Environmental Systems Resource Institute, ArcMap 10.0 ESRI, Redlands, California).

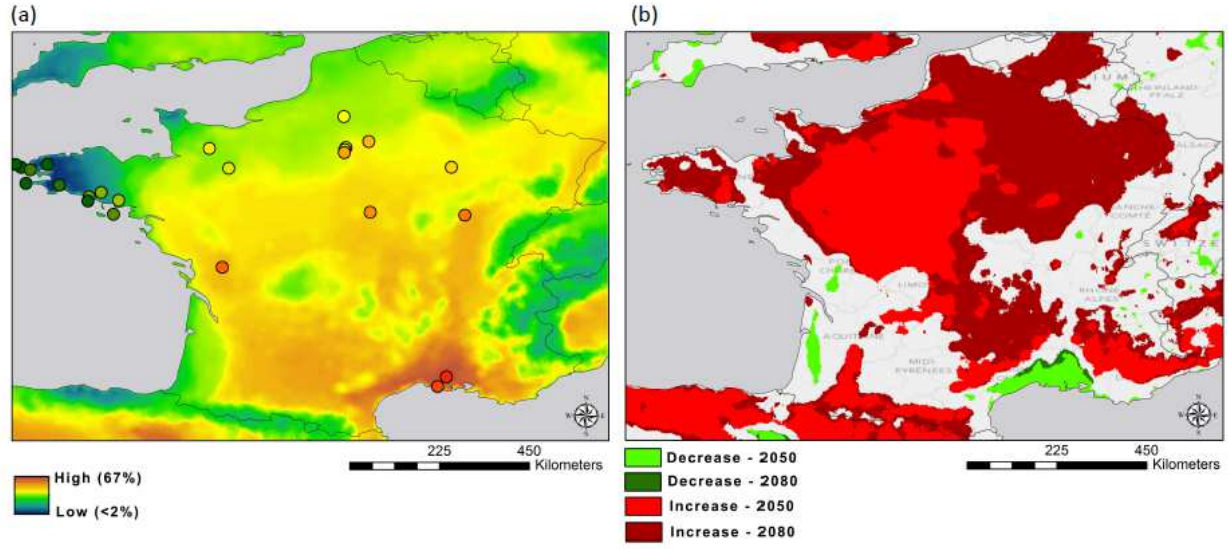


**Figure 3.**
Predictive map of malaria prevalence under (a) current environmental conditions and (b) future conditions in years 2050 and 2080. In green areas a decrease in prevalence is predicted as compared to current predictions, and red areas are those predicted to have an increase in prevalence by at least 10% over current predictions. Maps created in ArcMap 10 (Environmental Systems Resource Institute, ArcMap Release10.0, ESRI, Redlands, California).

## Discussion

Under the same suite of models, prevalence and parasitemia were each evaluated as to their relationship with environmental factors. To our knowledge, we have shown here, for the first time, that avian malaria prevalence is greatly affected by bioclimatic conditions, and that *Plasmodium* transmission will increase as warming trends continue. As expected, while prevalence should tightly depend on the availability of infected mosquitos vectors, parasitemia should be better predicted by intrinsic host factors.

Recent laboratory studies suggest differential development and transmission of malaria under varying temperature conditions (Paaijmans *et al.*, 2010). Using a lineage of rodent malaria, *Plasmodium chabaudi* and the vector *Anopheles stephensi*, it has been shown that daily temperature fluctuations around a cooler temperature (18°C) enhanced parasite transmission, whereas fluctuations around a warmer temperature (24°C) impaired transmission. Given the fact that our study region is thermally more analogous to the cooler temperature regime in this laboratory study (for our 24 sites, average mean temperature in the warmest quarter ranges from 15.6°C to 21.8°C, with a mean of 16.6°C), the effects of diurnal temperature range on host prevalence in our models is concordant with these laboratory findings. Our results suggest that temperature fluctuations can indeed substantially affect malaria transmission even in a natural setting where parasites, vectors, and hosts are exposed to a full range of environmentally variable conditions.

The House sparrow is a ubiquitous passerine host in Europe, and is used in this study as an indicator species to forecast the response of malaria prevalence with a larger avian community facing climate change and the spread of infectious diseases. We provide here valuable results regarding the future distribution of a generalist parasite that could threaten passerine species by host switching or by competing with other *Plasmodium* parasites. A great number of migratory species use mainland Europe as a stopover, and therefore are potential hosts for generalist parasites such as *Plasmodium relictum*. Of course, each host species is likely to experience differential levels of exposure and response, and as such, this study should only serve as a first prediction of the spatial extent of avian malaria under future conditions. Our study also provides evidence that with impending global warming, malaria will spread in the north of France and that populations already experiencing malaria infections will exhibit higher prevalences. The predictive maps also show that the northwest part of France (Brittany) remains relatively unaffected by malaria infection, as well as the southwest. It will be very interesting to apply these data as a testable spatial template for verification by future ground-truthing efforts.

We do not discount here other variables that may affect transmission, such as vector development and its life cycle; a survey of vector abundance and diversity will be performed in parallel with host populations. In this time of rapid global change, the goal of our study is to reveal and emphasize the necessity of studying the effects of ecological change on vector-borne parasites in wildlife, with obvious conservation implications, but also in consideration of larger management and policy decisions, as our methods may be useful when applied to other host-parasite systems.

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## Supplemental Information

### Bioclimatic variables description and elevation.

We used a set of moderately high-resolution climate and satellite remote sensing variables to characterize the environmental differences among our sampling areas. Variables were re-aggregated from their native resolutions to 1 km resolution.

We used 19 bioclimatic variables (representing both temperature and precipitation) from the WorldClim database (1) which are 50-year averages (1950-2000) of annual means, seasonal extremes and degrees of seasonality in temperature and precipitation, and represent biologically meaningful variables for characterizing species range (2, 3):
(http://biogeo.berkeley.edu/worldclim/bioclim.htm)

BIO1 = Annual mean temperature (Celsius degree x10)
BIO2 = Mean diurnal range [mean of monthly (max temp − min temp)]
BIO3 = Isothermality (Bio 2/Bio 7) (×100)
BIO4 = Temperature Seasonality (standard deviation *100)
BIO5 = Max Temperature of Warmest Month (degree Celsius, °C)
BIO6 = Min Temperature of Coldest Month (degree Celsius, °C)
BIO7 = Temperature annual range (Bio 5–Bio 6)
BIO8 = Mean temperature of wettest quarter
BIO9 = Mean temperature of driest quarter
BIO10 = Mean temperature of warmest quarter
BIO11 = Mean temperature of coldest quarter
BIO12 = Annual Precipitation
BIO13 = Precipitation of wettest month
BIO14 = Precipitation of driest month
BIO15 = Precipitation Seasonality (Coefficient of Variation)
BIO16 = Precipitation of wettest quarter
BIO17 = Precipitation of driest quarter
BIO18 = Precipitation of warmest quarter

BIO19 = Precipitation of Coldest Quarter

In addition, from the Shuttle Radar Topography Mission (SRTM), we acquired elevation data, with mean altitude and standard deviation as variables.

## Methods

Most statistical procedures and traditional data modeling technique (such as linear regression or ANOVA) measure variable importance indirectly by selecting variables using criteria such as statistical significance and Akaike's Information Criterion. The Random Forest has a different approach (4); it is a non-parametric algorithm method. We used this method because Random Forest procedures (i) do not require the use of any particular model (which might be difficult to assign given a complex response such as disease prevalence), (ii) do not require normalized data, and (iii) have consistently outperformed traditional regression procedures on a number of datasets (3, 5-7). The advantage of random forest models is their ability to predict a continuous (in this case, prevalence) rather than categorical (presence/absence) variable across a landscape, and their ability to model complex interactions among predictor variables. Also, autocorrelation between predictors is not an obstacle for these algorithms because if climatic variables are highly correlated, then removal of one variable does not affect the model, because an autocorrelated variable can just take its place in the model. In our study, for the case of prevalence, each of the first couple of variables explained unique variation in prevalence; this is not the case for the parasitemia data.

In details, for each tree in the forest, there is a misclassification rate for the out-of-bag observations. To assess the importance of a specific predictor variable, the values of the variable are randomly permuted for the out-of-bag observations, and then the modified out-of-bag data are passed down the tree to get new predictions. The difference between the misclassification rate for the modified and original out-of-bag data, divided by the standard error, is a measure of the importance of the variable[6]. The mean square error is an estimate of the full model's error rate, whereas the purity is a measure of how often an out-of-bag record from the set would be incorrectly called in the "child" leaves of the tree as compared to the "parent". It is a measure of homogeneity versus heterogeneity in each node of the branch. The purity index represented therefore the sum of how each variable contributes to the homogeneity of out-of-bag calls, and that a larger increase in purity represents more homogeneous calls within partitions of the data.

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# Manuscrit 6

## Urbanization, trace metal pollution and malaria prevalence in the house sparrow

Coraline Bichet, Renaud Scheifler, Michaël Cœurdassier, Romain Julliard, Gabriele Sorci and Claire Loiseau

Sous presse dans *PloS One*

# Urbanization, trace metal pollution, and malaria prevalence in the house sparrow

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**Abstract**: Anthropogenic pollution poses a threat for the environment and wildlife. Trace metals (TMs) are known to have negative effects on haematological status, oxidative balance, and reproductive success in birds. These pollutants particularly increase in concentration in industrialized, urbanized and intensive agricultural areas. Pollutants can also interfere with the normal functioning of the immune system and, as such, alter the dynamics of host-parasite interactions. Nevertheless, the impact of pollution on infectious diseases has been largely neglected in natural populations of vertebrates. Here, we used a large spatial scale monitoring of 16 house sparrow (*Passer domesticus*) populations to identify environmental variables likely to explain variation in TMs (lead, cadmium, zinc) concentrations in the feathers. In five of these populations, we also studied the potential link between TMs, prevalence of infection with one species of avian malaria, *Plasmodium relictum*, and body condition. Our results show that lead concentration is associated with heavily urbanized habitats and that areas with large woodland coverage have higher cadmium and zinc feather concentrations. Our results suggest that lead concentration in the feathers positively correlates with *P. relictum* prevalence, and that a complex relationship links TM concentrations, infection status, and body condition. This is one of the first studies showing that environmental pollutants are associated with prevalence of an infectious disease in wildlife. The mechanisms underlying this effect are still unknown even though it is tempting to suggest that lead could interfere with the normal functioning of the immune system, as shown in other species. We suggest that more effort should be devoted to elucidate the link between pollution and the dynamics of infectious diseases.

**Keywords**: Avian malaria, ecotoxicology, house sparrow, *Plasmodium relictum*, trace metals, urbanization.

## Introduction

Urbanization and intensive agriculture can drastically affect wildlife populations. Anthropogenic activities produce pollutants known to negatively affect population viability [1]. For instance, organochlorines (OCl) and trace metals (TMs) are known to severely affect breeding success, behavior, and development in birds [2-4]. Trace metals particularly increase in concentration in industrialized, urbanized and intensive agricultural areas [5-7] and contaminate organisms through air, water and food [8-10].

Several biomonitoring programs focused on TMs in birds and top predators since these pollutants may accumulate in organisms [11] and transfer through food chain [12-14]. Tissues, including feathers, are often sampled as proxies for total body TM concentrations (avoiding the need to use whole birds for analysis), and the TM levels in such tissues is assumed to be indicative of past TM exposure of the individual [12,15,16]. For instance, Scheifler et al. compared urban and rural populations of blackbirds (*Turdus merula*), and they found that lead (Pb) concentration in

feathers of birds in urban polluted areas and in their major food item (the community of anecic, epigeous and endogeous earthworms) was greater than in rural areas [7]. TMs are also known to affect physiological parameters such as haematological and oxidative status [17-20] potentially resulting in reduced reproductive success [2]. To date, effects of TMs on body condition remain to be fully understood and, in some species, depend on various factors such as sex and populations [21].

TMs are also known to have immunotoxic effects. Although studies on possible effects of pollutants on immune system function remain limited in birds [22,23], toxicants are know to interfere with immune receptor binding and trigger inappropriate and inhibited immune responses in model species [24-28].

In this study, we surveyed sixteen house sparrow (*Passer domesticus*) populations, inhabiting areas with different degrees of urbanization and we determined TM concentrations in feathers. The house sparrow is a widely distributed and sedentary species associated with human settlement, and therefore, is an interesting species in which we can investigate effects of anthropogenic pollution on wildlife. We measured the concentrations of three elements: i) zinc (Zn), which is an essential element, but regulated by organisms because high levels can be toxic, ii) lead (Pb) and cadmium (Cd), two non-essential elements, which have no biological functions identified in birds and can accumulate in organisms.

For a subset of populations ($n$ = 5), we also examined the relationship between TM concentrations and the prevalence and intensity of *Plasmodium* infection. *Plasmodium* parasites are the agent of a widespread vector-borne disease of wild birds [29]. House sparrows are infected with several lineages of haemosporidian parasites, including *Plasmodium relictum*. Upon infection, parasitemia usually increases to a peak between one and two weeks post-infection. This acute phase is followed by a chronic infection where parasitemia persists at low levels [29]. Avian malaria has been shown to be harmful to naïve populations and domestic species [30-34]. In addition, recent studies based on experimental infections and treatment with antimalarial drugs have also shown that avian malaria parasites can be costly and reduce host fitness in several passerine species [35-38].

## Materials and Methods

*Ethics statement*

This fieldwork study involved the sampling of feathers and a small amount of blood of free-ranging birds. The work has been conducted according to relevant national guidelines. The permit to sample birds was delivered by the French Ministère de l'Ecologie et du Développement Durable and the permit to band birds by the Centre de Recherche sur la Biologie des Populations d'Oiseaux at the National Museum of Natural History, Paris.

*Study sites and sampling*

We studied 16 populations of house sparrow in France along a gradient of urbanization (Fig. 1, Table 1). Habitat characteristics were obtained from the CORINE (Coordination of information on the environment) Land Cover database using the geographical information system package ArcView 3.2 (ESRI 2000). This is a European geo-referenced land-cover database, based on satellite digital images, that allows classifying landscape units, according to a list of environmental classes [39]. It provides consistent localized geographical information on the land cover of 12 Member States of the European Union. We used the data for year 2000 (http://www.eea.europa.eu/themes/landuse/interactive/clc-download). All the details about the program and the complete methodology can be found in the following internet site: http://www.eea.europa.eu/publications/COR0-landcover. To characterize the surface covered by the different habitat types, we used a circle with a radius of 10 km, centred at the site of capture. We extracted six variables that describe the major habitat characteristics: 1) urban areas, 2) surface covered by intensive agriculture, 3) surface covered by extensive agriculture, 4) woodland, 5) meadow, and 6) shrubland vegetation. These surfaces were expressed in $km^2$ and log-transformed for statistical analyses (Table 1).

Adult house sparrows were caught in 2004 and 2005 using mist-nets. The sample size for each population is summarized in Table 1. Each bird was ringed with a numbered metal ring and sexed visually. Two rectrices were collected in all populations for TM measurements. All individuals were, at least, one year old. In five out of the 16 populations (Fig. 1), we measured wing length ($\pm$ 1 mm)

and body mass (± 0.5 g), and collected a small volume of blood (ca. 20 µl) by brachial vein puncture. Blood was subsequently stored in 500µl of Queen's Lysis Buffer (QLB) [40]. Blood samples were not collected for all individuals captured which is the reason for the difference in sample size between the models exploring the environmental predictors of feather TMs and the association between feather TMs and malaria prevalence and parasitemia.

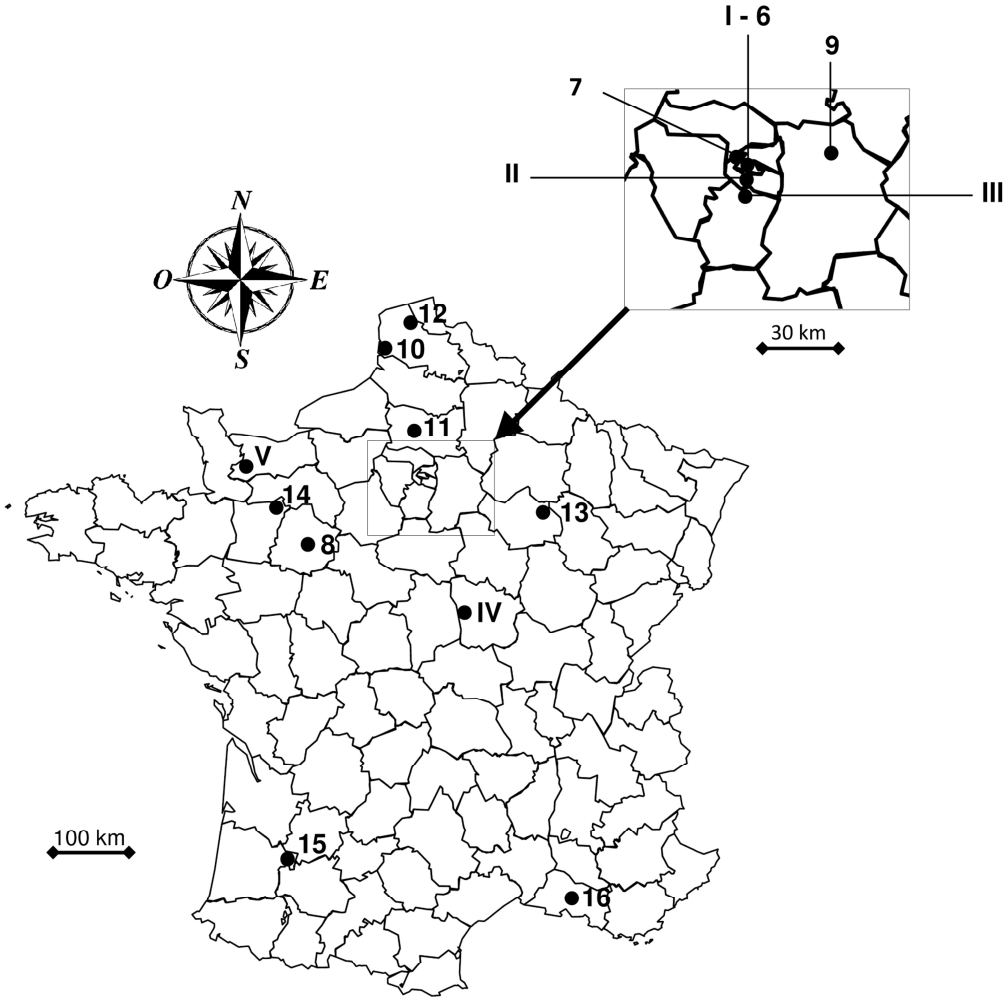


Fig. 1. Geographical localization of the 16 house sparrow populations sampled in this study: I - Paris (Jardin des Plantes), II - Cachan, III - Wissous, IV - Cosne-Cours sur Loire, V - Rully, 6 - Paris (Cité internationale universitaire), 7 - Gennevilliers, 8 - Le Mans, 9 - Crégy les Meaux, 10 - Berck, 11 - Thieux, 12 - Seninghem, 13 - Ceffonds, 14 - Crennes, 15 - Réaup-Lisse and 16 - Arles. The five populations in roman numbers were sampled for parasite prevalence and intensity. The zoomed region, in the upper right corner, corresponds to the region Ile de France. Scale bar, 100 km for France and 30 km for the region Ile de France.

*Parasite screening*

DNA was extracted from blood samples using standard phenol/chloroform protocol (modified from Hillis et al. [41]). In order to detect the presence of malaria parasites, we used a nested polymerase chain reaction (PCR) [42] to amplify a 500bp fragment of the parasite mitochondrial cytochrome *b* gene. This PCR detects parasites from *Haemoproteus* and *Plasmodium* genera. We sequenced the positive PCR products and identified lineages using the NCBI nucleotide Blast search. For this study, we focused on *Plasmodium relictum* (SGS1 and GRW11 lineages), the predominant parasite in all populations [43]. In addition, for each positive PCR product, we also performed a quantitative PCR to obtain parasitemia (relative quantification) following the protocol described in Cellier-Holzem et al. 2010 [34].

Table 1. Environmental characteristics and TM concentrations for 16 house sparrow populations in France.

| | | Environmental characteristics (km²) | | | | | | Mean TM concentrations (µg $g^{-1}$ dry mass) in house sparrow feathers ± standard error | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| N° | Population | Urban area | Intensive agriculture | Extensive agriculture | Woodland | Meadow | Shrubland | N | Cd | N | Pb | N | Zn |
| I | Paris, Jardin des Plantes | 293.63 | 0.37 | 0.61 | 10.27 | 0.11 | 1.32 | 15 | 1.51 ±0.98 | 15 | 19.54 ±2.06 | 15 | 188.34 ±36.56 |
| II | Cachan | 265.03 | 7.73 | 1.13 | 21.05 | 0.44 | 11.67 | 13 | 0.33 ±0.05 | 13 | 8.58 ±1.18 | 13 | 175.84 ±20.13 |
| III | Wissous | 241.66 | 22.87 | 1.57 | 24.57 | 1.43 | 15.54 | 14 | 0.47 ±0.15 | 14 | 27.06 ±9.01 | 14 | 627.49 ±88.66 |
| IV | Cosne-Cours sur Loire | 17.33 | 149.76 | 49.18 | 48.76 | 40.98 | 2.00 | 18 | 0.17 ±0.04 | 15 | 3.71 ±0.86 | 18 | 201.65 ±20.72 |
| V | Rully | 4.37 | 110.92 | 25.36 | 4.09 | 168.94 | 0.48 | 12 | 0.04 ±0.01 | 18 | 3.04 ±0.20 | 18 | 135.72 ±6.65 |
| 6 | Paris, Cité internationale universitaire | 293.63 | 0.37 | 0.61 | 10.27 | 0.11 | 1.32 | 10 | 0.20 ±0.01 | 10 | 15.89 ±2.03 | 10 | 167.81 ±10.18 |
| 7 | Gennevilliers | 289.61 | 0.88 | 9.72 | 4.77 | 1.02 | 0.40 | 18 | 0.20 ±0.04 | 18 | 18.70 ±1.67 | 18 | 178.33 ±15.10 |
| 8 | Le Mans | 70.88 | 67.43 | 25.68 | 53.21 | 93.18 | 0.35 | 10 | 0.65 ±0.41 | 10 | 1.87 ±0.61 | 10 | 161.15 ±34.64 |
| 9 | Crégy les Meaux | 46.64 | 192.21 | 0.87 | 43.90 | 7.47 | 14.54 | 3 | 0.63 ±0.22 | 3 | 10.61 ±4.35 | 3 | 503.52 ±103.08 |
| 10 | Berck | 27.19 | 57.49 | 5.49 | 17.81 | 32.70 | 14.34 | 10 | 0.26 ±0.05 | 10 | 6.71 ±2.55 | 10 | 188.61 ±23.99 |
| 11 | Thieux | 16.29 | 261.85 | 15.00 | 20.50 | 0.51 | 0 | 18 | 0.22 ±0.03 | 17 | 2.79 ±0.56 | 18 | 151.96 ±11.57 |
| 12 | Seninghem | 10.05 | 176.75 | 3.60 | 34.91 | 88.35 | 0.51 | 18 | 0.11 ±0.01 | 18 | 2.71 ±0.32 | 18 | 163.20 ±20.33 |
| 13 | Ceffonds | 6.28 | 127.87 | 8.71 | 101.79 | 64.29 | 4.08 | 18 | 0.68 ±0.11 | 18 | 3.77 ±0.71 | 18 | 190.47 ±18.60 |
| 14 | Crennes | 4.14 | 115.26 | 5.13 | 36.02 | 151.60 | 2.01 | 10 | 0.69 ±0.10 | 10 | 3.99 ±0.64 | 10 | 252.60 ±68.47 |
| 15 | Réaup-Lisse | 1.62 | 43.50 | 55.04 | 190.09 | 11.69 | 11.96 | 10 | 0.36 ±0.12 | 10 | 21.08 ±3.45 | 10 | 215.26 ±22.19 |
| 16 | Arles | 1.42 | 31.33 | 55.92 | 6.91 | 0 | 67.99 | 12 | 0.07 ±0.01 | 18 | 3.84 ±0.27 | 18 | 106.92 ±5.14 |

Among the 16 populations, we screened for malaria parasites in five populations along a gradient of urbanization. Paris, Cachan, and Wissous were the most urbanized sites. In Paris, individuals were captured in the botanical garden (*Jardin des Plantes*, hereafter Paris-Jdp). Cachan is located in the suburban area of Paris and individuals from Wissous were caught in an industrialized area near the Orly International Airport. In contrast to these highly urbanized sites, the other populations (Cosne-Cours sur Loire [hereafter Cosnes-C/L] and Rully) can be considered as rural, with less than 6% of urbanization (Table 1). All populations were sampled in spring/summer except Cosne-C/L, which was partly sampled in fall/winter.

Table 2. Pearson's correlation coefficient matrix among the environmental variables used to characterize 16 house sparrow populations in France. * and ** indicates P values ≤ 0.05 and ≤ 0.01, respectively.

| | Urban areas | Intensive agriculture | Woodland | Extensive agriculture | Meadow |
|---|---|---|---|---|---|
| Urban areas | | | | | |
| Intensive agriculture | -0.7016** | | | | |
| Woodland | -0.3292 | 0.4896* | | | |
| Extensive agriculture | -0.6895** | 0.4231 | 0.1710 | | |
| Meadow | -0.5285* | 0.6531** | 0.3919 | 0.3224 | |
| Shrubland | -0.1856 | 0.0211 | 0.1310 | 0.0175 | -0.2897 |

*TMs analysis*

In order to remove exogenous contamination, feathers were washed (1 min in acetone) and then rinsed (1 min with deionised water, 18.2 MΩ $cm^{-2}$) three times in an ultrasonic bath. Before analysis, washed feathers were dried in an oven at 60°C until constant dry mass was achieved. Dry mass was determined to the nearest 0.0001g using an electronic balance (Mettler Toledo AB 54). Samples were digested in a mixture of 2ml $HNO_3$ (68%) and 2ml $H_2O_2$ (30%) for 48 h in an oven at 60°C. Samples were diluted by adding 11ml deionised water and were stored at −20°C until analysis. All reagents were of analytical grade and obtained from Carlo Erba (Val de Rueil, France).

Total Cd and Pb concentrations were measured by furnace atomic absorption spectrophotometry (AAS, Varian 220Z, Les Ulis, France), Zn by flame AAS (Varian 220FS, Les Ulis, France). All concentrations are expressed in micrograms per gram on a dry mass basis (µg $g^{-1}$ dm).

Validity of analytical methods was checked by means of standard biological reference material (TORT-2, lobster hepatopancreas, and DOLT-3, dogfish liver, from the National Research Council of Canada–Institute for National Measurement Standard, Ottawa, ON, Canada). Recoveries for Cd, Pb, and Zn concentrations from the TORT-2 and DOLT-3 reference materials were 126 ± 24 and 103 ± 12%, 169 ± 32 and 175 ± 48%, and 98 ± 5 and 100 ± 8%, respectively. Detection limits (DL) calculated using blank values and average dry mass of feathers were 0.05 and 1.59 µg $g^{-1}$ for Cd and Pb, respectively. DL could not be calculated for Zn because blank measurements gave systematically negative values. For statistical analyses, values under DL were replaced by half of the DL [44].

One individual gave values below the DL for Cd and Pb and was excluded from the analyses.

*Statistical analyses*

First, we tested the correlations between TM concentrations and environmental variables using Pearson's correlations. Since some of these habitat variables were correlated among them (Table 2), and to avoid co-linearity in the statistical models, we summarized the information carried by these descriptors across major axes, using a principal component analysis (PCA). Axes 1 to 3 explained 49%, 20%, and 16% of the variance, respectively, and were used in the following statistical analyses. Axis 1 represented a major gradient of urbanization with a positive load for intensive (0.51), extensive agriculture

(0.40), woodland (0.34), and meadows (0.45), and a negative load for urban areas (-0.51). Axis 2 strongly loaded for shrubland vegetation (0.87), whereas axis 3 loaded for woodland (0.70), and negatively for extensive agriculture (-0.61). The predictive power of axes 1 to 3 to explain the amount of TMs incorporated into sparrow feathers was estimated using linear mixed models (LMM) with a normal distribution of errors. To this purpose, TM concentrations were log-transformed and the site was included in the models as a random variable.

For a subset of five populations, we also assessed the association between prevalence (defined as a binary variable, 0 for non-infected and 1 for infected birds) or parasitemia (using infected individuals only) of malaria parasites, and sex, site, and TMs, using a generalized linear model (distribution of errors: binomial, link: logit) and a general linear model (distribution of errors: Gaussian, link: identity), respectively. To perform these analyses, several models were constructed: the null model, five models each containing one of the five variables (sex, site, Cd, Pb, or Zn), a model containing all the variables, a model containing only sex and site, a model containing all TMs (Cd, Pb, Zn), and a model containing non-essential TMs (Cd, Pb).

Body condition was assessed as the residuals of a linear regression of body mass on wing length ($F_{1,64}$ = 9.34, $P$ = 0.003). Body condition was then modelled (linear model) using sex, site, infection status (as a binary variable), or TMs, as explanatory variables. First order interactions between the infection status and other variables were also added in the models. More complex interactions could not be tested due to the limited sample size. The various models were built as described above (null model, models with each one of the six variables, models with sex and site, models with all or non-essential TMs). In the models exploring the associations between TMs and malaria, site (n = 5) was included as a fixed factor because the comparisons of models with site as a fixed factor and those with site as a random factor showed that the former were more parsimonious.

We used the package lme4 [45], implemented in R 2.15.0 to run all LMMs. We used the information-theoretic (IT) approach as it has recently been suggested as being more appropriate for observational studies [46]. Model support was assessed using the corrected version of Akaike Information Criterion (AICc) for small sample sizes, and ΔAIC was used to infer support for models in the candidate set [47]. We calculated the Akaike weights ($\omega$) for each model, which is the probability that a model is selected as the best model in a model set [46]. We deemed that there was essentially no evidence in support to a model when its ΔAIC value was greater than 10 [46]. It is also worth noting that when a fitted parameter was added to a model, a penalty of 2 is added to the model's AIC value [46]. Thus, we considered that a variable improved the fit of the model only when the ΔAIC was lower than 2. The maximized log-likelihood (LL) and number of estimated parameters (K) were also calculated and reported in the text.

Selected linear models were checked graphically for homogeneity of variance, normality of error and linearity/additivity. The leverage was evaluated by looking at plots of the standardized residuals versus leverage. Model outputs were satisfactory, and transformations of the measured variables did not bring significant improvement. Non-transformed variables were therefore used in the statistical analyses.

Model coefficients and confidence intervals are given for the linear predictor for linear models and for the exponential of the linear predictor in linear/generalized models.

## Results

*Environmental variables associated with TM concentrations*

Average TM concentrations varied between 0.04 and 1.51 µg $g^{-1}$ for Cd, 1.87 and 27.06 for Pb, and 106.92 and 627.49 for Zn (Table 1).

Axis 1 of the PCA describes a gradient of urbanization/natural landscapes, with positive values indicating prevailing natural habitats, negative values urbanized areas. According to Akaike parameters, Pb concentrations were best explained by a model including the axis 1 and the axis 2 (LL: 4.94, K: 3, AICc: -12.21, ΔAICc: 0.00, ω: 0.35). A competitive model (ΔAICc: 0.28) included only the axis 1. Pb concentrations were negatively associated with this axis, showing that high Pb concentrations were tightly associated with heavily urbanized habitats (Fig. 2a).

Cd concentrations were best explained by the model including only the axis 3 (LL: 152, K: 2, AICc: -308.88, ΔAICc: 0.00, ω: 0.51). The result was similar for Zn, where the best

model also included the axis 3 (LL: 43.43, K: 2, AICc: -88.34, ΔAICc: 0.00, ω: 0.46). Feather concentrations of Cd and Zn were positively associated with axis 3 of the PCA. Axis 3 describes woodland areas, indicating that sparrows sampled in areas with a large woodland coverage tended to have higher concentrations of Cd and Zn in their feathers (Fig. 2b).

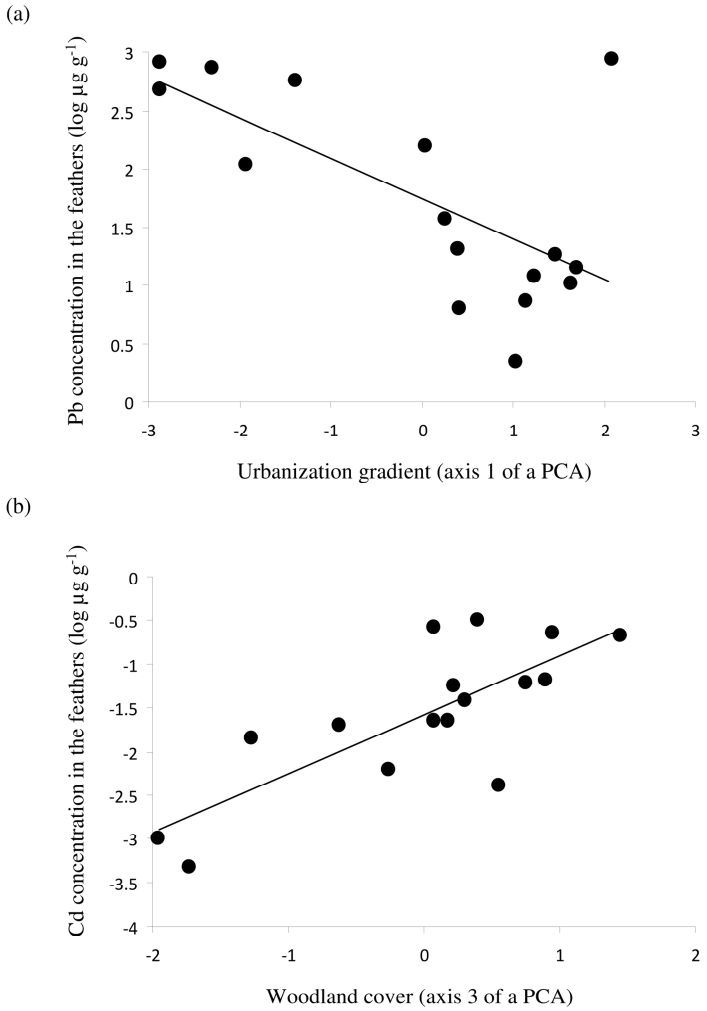


Fig. 2. (a) Negative correlation between Pb concentration in feathers and the axis 1 of the principal component analysis on environmental variables (see materials and methods for more details). (b) Positive correlation between Cd concentration in feathers and the axis 3 of the principal component analysis. Each point represents a house sparrow population.

*TMs and risk of malaria infection*

*Plasmodium* prevalence was 25% in Rully, 36% in Paris-JdP, 43% in Wissous, 44% in Cosne-C/L and 46% in Cachan. According to Akaike parameters, *Plasmodium* prevalence was best explained by a model including the two non-essential metals, Cd and Pb (LL: -39.83, K: 3, AICc: 86.05, ΔAICc: 0.00, wic: 0.44). According to the exponential of the linear predictor of the variables and their confidence intervals, prevalence is negatively associated to Cd concentrations (exponential value of the linear predictor: 0.14, confidence interval: 0.01 / 0.95) and positively associated with Pb concentrations (1.08, 1.01 / 1.18) (Fig. 3). In addition to parasite prevalence, we assessed parasitemia of infected birds. Parasitemia was very low as expected for chronic malaria infections. The null model was found to be the best (LL: 140.48, K: 2, AICc: -276.42, ΔAICc: 0.00, wic: 0.31), most likely because of limited statistical power and/or low variability in parasitemia.

*Body condition*

According to Akaike criterion, body condition was best fitted by a model including site, the infection status, and their interaction (LL: -

118.52, K: 11, AICc: 263.93, ΔAICc: 0.00, wic: 0.44). A competitive model (ΔAICc: 0.80) included Zn concentration, the infection status and their interaction. The linear predictor of the variables and their confidence intervals showed that i) individuals in Wissous and Cosne-C/L were in better body condition compared to Paris, ii) infected birds were in worse condition compared to non-infected individuals in Wissous, iii) the relationship between body condition and Zn concentration was positive in non-infected birds and negative in *Plasmodium* infected birds.

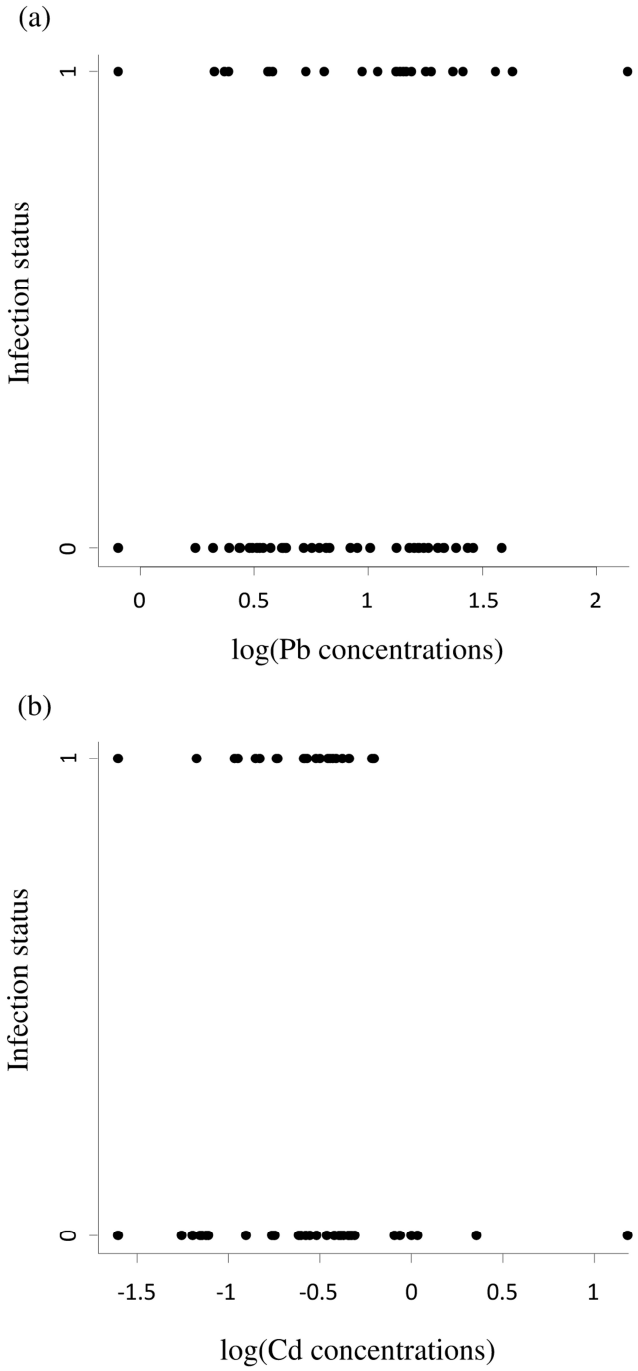


Fig. 3. Association between infection with *Plasmodium* (0 = non-infected, 1 = infected) and Pb and Cd concentration in feathers of house sparrows. Metal concentrations are logged to facilitate the reading of the figure.

## Discussion

It has been established that pollution and especially contamination by TMs can affect human and wildlife health. However, there is little information about the effect of pollutants on the prevalence of infectious diseases. With the current increase in urbanization, it seems relevant to investigate how pollution can be linked with the susceptibility of free-living animals to pathogens. Here, we studied the association between urbanization and TM concentrations in sixteen populations of the house sparrow. In addition, we explored the correlation between TMs and malaria prevalence. We found that i) sparrows living in highly urbanized areas had a higher Pb concentration in their feathers, ii) Cd and Zn concentrations were associated with sites mostly covered by woodlands, iii) Pb concentrations might be associated with higher *Plasmodium* prevalence, while Cd concentrations tended to be negatively correlated with prevalence, iv) body condition was similar or higher in infected than in non-infected birds in all the populations except at Wissous, where sparrows have high Zn concentration in their feathers.

Our finding that Pb concentration follows an urbanization/natural habitat gradient corroborates previous results gathered on a variety of species, including the house sparrow [5-7,16,48], and further shows that, even though Pb is no longer used as a gasoline additive, it persists in urban environments. All urban populations had high values of Pb in the feathers. There is, however, one notable exception to this pattern. A rural population (Réaup-Lisse) had among the highest values of Pb. Although we do not know the local source of this pollution, this shows that other factors in addition to urbanization may determine Pb contamination in birds. Pb feather contamination is generally thought to occur through the incorporation of the TMs ingested with food [49] during feather growth [12]. TM concentrations have also been reported to increase with bird age [50,51], suggesting a time-dependent accumulation. However, samples analyzed here only concerned individuals more than 1-year old, which should reduce the variance due to differential time-dependent exposure.

Few studies have assessed *P. domesticus* or other sparrow species exposure to TMs using feathers and, to our knowledge, investigations on rural populations living in uncontaminated areas have never been reported. Thus, no reference values have been proposed for TM concentrations in sparrow feathers in rural habitats. Data from other species should be generalized to sparrows with care, because of interspecies variations in TM accumulation. Burger [12] determined a median Cd level in feathers of 0.1 µg/g from avian studies worldwide, and 4.5 µg/g of Pb were associated with behavioral abnormalities in birds that can cause lowered survival [52,53]. In our study, 87% of the sparrows had higher Cd residues in feathers than the median values reported above, and 50% were over the Pb concentrations known to have detrimental effects. Considering only those studies performed on sparrows, feather residues in urban populations of *P. domesticus* in Palestine were 0.02 ± 0.00 µg/g for Cd, 8.1 ± 1.3 for Pb, and 54.9 ± 5.3 µg/g for Zn [48]. All the sparrow populations we studied had higher Cd (from 2 to 75-fold) and Zn (from 1.9 to 11.5-fold) concentrations, while 44% of them exhibited higher Pb residues, mainly among those living in the most urbanized environments. Pan et al. (2008) reported TM concentrations in ventral feathers of the tree sparrow (*Passer montanus*) in Beijing [54]. In their sampling site where the traffic was an important source of pollutants, concentrations in male adult tree sparrows reached 0.74 ± 0.41 µg/g and 13.63 ± 2.33 µg/g of Cd and Pb, respectively. These values are in the same range, even if slightly higher, than those we measured in urban house sparrows. The sparrow populations studied here exhibited a wide range of exposure to TMs. Most of the populations living in the most urbanized areas exhibited high exposure level. The population living in the *Jardin des Plantes* (Paris-Jdp), the most urbanized site, is exposed to high levels of both Cd and Pb. Other populations, such as those of Wissous, Gennevilliers, and Réaup-Lisse, suffered high Pb exposure, despite different degrees of urbanization. Finally, populations inhabiting in rural areas (*e.g.* Berck, Thieux, and Arles) generally exhibited low to moderate concentrations of TMs.

The association between Cd and Zn concentrations with sites mainly covered by woodlands is more surprising. Because Cd and Zn median concentrations are of the same order of magnitude for most of the 16 sites and are below 1 and 400 µg $g^{-1}$, respectively, it is possible that they represent mainly a transfer

from geochemical background to sparrow feathers through food. Accordingly, the observed association between Cd and Zn concentrations and woodlands might be due to a higher bioavailability of these two TMs in areas with low pH (which is usually the case in woodlands compared to other habitat types) [55].

Besides identifying the environmental determinants of TM pollution at a large spatial scale, the aim of our study was to investigate the potential link between TM concentrations and malaria prevalence. In agreement with the hypothesis that TMs can disrupt the normal functioning of the immune response and enhance risk of contracting infectious diseases, our results suggest that malaria prevalence could be positively correlated with Pb concentrations. We should however note that, although the overall sample size was quite large, the number of birds sampled for malaria parasites per population ranged from 9 to 16, which might have resulted in an underestimate of the prevalence of the infection.

Pb concentrations were not correlated with parasitemia (nor were the two other TMs). Even though this result might suggest that parasitemia is not affected by environmental pollution, the reduced statistical power and/or the low among-individual variability in parasitemia prevent us to draw a firm conclusion. It should also be noted that birds suffering from high parasitemia might be more difficult to capture because of reduced mobility, possibly biasing the sample towards birds with chronic parasitemia [29].

Given the increasingly rate at which human activity impacts on natural habitats and the well-known effect of pathogens on natural populations of hosts [56], it is surprising to note that the study of the consequences of environmental pollution for the dynamics of infectious diseases has been largely neglected. Among the rare studies where pollutants and infectious diseases have been monitored, Mashima et al. reported that the liver concentration of Cd was higher in avian cholera infected oldsquaws (*Clangula hyemalis*), compared to apparently healthy individuals [57]. However, it should be noted that in this study, avian cholera infected and healthy individuals were collected at different time points (1994 and 1985-87, respectively), which makes difficult to draw a firm conclusion on the link between Cd and susceptibility to avian cholera.

The effects of TMs, notably Cd, Pb and mercury (Hg), on the immune system of birds have been widely demonstrated both *in vitro* and *in vivo*, although the mechanisms involved remain under debate. Metals are generally reported to depress the immune response [23,58-61], resulting in increased susceptibility to infectious diseases and parasites [62]. Other pollutants have also been reported to have similar immunotoxic effects. For example, Camplani et al. (1999) studied the impact of radioactive pollution on barn swallows (*Hirundo rustica*) living in the irradiated site of Chernobyl. They found that lymphocyte and immunoglobulin concentrations were depressed and spleen size was reduced [63].

It should also been noted that subchronic exposure to Pb or Zn may enhance immune functions [64,65], Zn being even considered essential in all aspects of immunity [66,67]. This might explain our finding of a negative correlation between malaria prevalence and Cd concentrations. Alternatively, a negative correlation between pollutants and prevalence might arise if the pollutant exerts its toxic effect on both the host and the parasite. In addition to this, the timing of exposure to pollutants and the infectious agents might affect the sign of the association [62]. Experimental work on lab mice showed that exposure to lead or nickel enhanced mice resistance to a subsequent infection with *Klebsiella pneumoniae*. However, when the pollutant exposure intervened after the infectious challenge, mouse resistance was impaired [68].

To date, no biological function has been identified in birds for both Cd and Pb. The best evidence in support to the idea that TMs can depress the immune response in natural populations of birds comes from Snoeijs et al. [22]. They measured the humoral immune responsiveness of great tits (*Parus major*) (i.e., the proportion of birds that produced detectable antibodies following injection of sheep red blood cells) along a pollution gradient near a metallurgic smelter, and found that birds sampled from the site farthest away from the smelter complex had a significantly higher immune responsiveness than birds from the areas closest to the smelter [22].

Contrary to what could have been expected, body condition was similar or even better in infected birds than in non-infected individuals, except in Wissous (the site with the highest Zn concentration). Previous work has mostly failed to find an association between TMs and

body condition in wild passerines [21], whereas the association between malaria infection and body condition seems to vary depending on environmental conditions. For instance, physiological condition of malaria infected great tits was poorer than for non-infected individuals, even though year and season of sampling had a strong effect on the strength of the association [69]. Finding a statistical support for the association between malaria infection and body condition usually requires either an experimental approach either field studies with very large sample sizes [70,71]. As mentioned above, potential bias in the sampling of heavily infected birds [29,72] further contribute to the difficulty to properly assess the relationship between body condition, TM concentrations, and the infection with *Plasmodium*.

Given the correlative nature of the results reported here, we cannot infer the causality relationship between TM pollution, parasitism and body condition. Although it is tempting to speculate that pollution makes organisms more susceptible to infectious diseases by altering their immune functions, we cannot discard the possibility that sparrows using polluted spots are more exposed to malaria-infected mosquitoes. This would also result in a positive correlation between prevalence of infection and concentration of TMs in the feathers with no role for TM immunotoxicity. Malaria parasites and their vectors can be affected by environmental characteristics, such as temperature, humidity and altitude [73-77].

To conclude, the multiple interactions between pollutants, pathogens and environment are complex and still difficult to disentangle. However, in the current context of emerging infectious diseases and rapid urbanization, understanding the consequences of multi-pollutants and multi-pathogens interactions will be an essential step to predict population persistence and wildlife adaptation to human-made habitats.

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# Manuscrit 7

## Avian malaria, Major Histocompatibility Complex (MHC), and genetic differentiation in house sparrow (*Passer domesticus*)

Coraline Bichet, Stéphane Garnier, Yoshan Moodley, Dustin Penn and Gabriele Sorci

En préparation

# Major Histocompatibility Complex diversity and avian malaria in insular and mainland populations of the house sparrow (*Passer domesticus*)

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**Abstract:** Genes of the major histocompatibility complex (MHC) are highly polymorphic and functionally important. A few nonexclusive hypotheses have been put forward to explain the maintenance of MHC diversity, with a prominent role plaid by parasite-mediated selection. Among these hypotheses, recent work has focused on the idea that spatially and temporally variable selection pressures exerted by parasites can maintain variation within and among populations. Here, we examined microsatellite and MHC class I diversity and differentiation among house sparrows (*Passer domesticus*) originating from 6 mainland and 6 insular populations. We also assessed the diversity of malaria parasites and the prevalence of infection for each population of hosts. The comparison of insular and mainland populations (with different demographic and selective features) provides the opportunity to disentangle the forces (stochastic vs. selective) shaping genetic diversity. We found that insular populations were less variable than mainland ones when looking at microsatellite loci, whereas diversity was similar between types of population based on MHC class I gene. Fst between insular populations was higher than for mainland populations whatever the marker used. In agreement with the idea that selection pressures exerted by parasites shape MHC diversity, we also found that the between-population differentiation based on the MHC was negatively correlated with the similarity of the community of avian malaria parasites (the more similar were the prevalence and diversity of parasite strains between two populations, the less they were genetically differentiated based on the MHC gene). Overall, this study provides some insights on the relative role of selection and neutral process for the maintenance of highly polymorphic MHC genes.

**Keywords:** Avian malaria, *Passer domesticus*, Major Histocompatibility Complex, microsatellites, genetic differentiation, genetic variability, insularity.

## Introduction

The major histocompatibility complex (MHC) is a family of highly polymorphic genes found in all vertebrates (Bernatchez, Landry, 2003). MHC genes code for glycoproteins that bind foreign peptides and present them to T helper cells, initiating an appropriate immune response (Hedrick, 1994). Given this role in the antigen presenting process, MHC genes have been suggested to be under parasite-mediated selection (PMS), and several authors have attempted to understand how PMS could generate and maintain the tremendous variation of MHC genes usually observed (Plachy *et al.*, 1992; Tollenaere *et al.*, 2008; Wegner *et al.*, 2003b). Broadly speaking, three major hypotheses have been put forward: (i) overdominance (also called "heterozygous advantage", (Doherty, Zinkernagel, 1975; Hedrick, 1998; Hughes, Nei, 1992), (ii) negative frequency-dependent selection (also called "rare allele advantage", (Golding, 1992; Takahata, Nei, 1990) and (iii) heterogenous selection in space and time (Hedrick, 2002). In spite of the considerable amount of work that has been devoted in the last decade to identify the form of selection acting on MHC genes and to disentangle the different hypotheses, the effort has been elusive. Whereas several studies have provided evidence in support to each of the three main hypotheses, it has proven difficult to tease apart all of them, especially because they can provide very similar predictions (Apanius *et*

*al.*, 1997; Woelfing *et al.*, 2009). In addition to this, few studies have explicitly assessed the pathogens that might actually exert the selection on MHC genes (but see (Charbonnel, Pemberton, 2005; Loiseau *et al.*, 2009). The most popular approach, up to date, has been to investigate the pattern of genetic differentiation among populations based on MHC genes as to infer the selection acting on it and compare this to the pattern found with neutral (microsatellite) markers (Aguilar, Garza, 2006; Ekblom *et al.*, 2007; Landry, Bernatchez, 2001; Miller *et al.*, 2001; Miller, Withler, 1997; Piertney, 2003; Tollenaere *et al.*, 2008).

The rationale behind this approach is that diversifying selection acting on MHC genes should produce a stronger pattern of genetic differentiation among populations than the one observed with microsatellite loci which are only submitted to stochastic factors (ie. drift). In agreement with this view, most of the studies have reported stronger differentiation based on MHC genes than for neutral markers, even though it should be noted that this does not allow teasing apart the negative frequency dependent selection and the heterogeneous selection hypotheses (Aguilar, Garza, 2006; Charbonnel, Pemberton, 2005; Ekblom *et al.*, 2007; Landry, Bernatchez, 2001; Loiseau *et al.*, 2009; Miller *et al.*, 2001; Tollenaere *et al.*, 2008). But, MHC differentiation should also be weaker than neutral differentiation, due to balancing selection (Bernatchez, Landry, 2003; Boyce *et al.*, 1997; Hedrick *et al.*, 2001; Huang, Yu, 2003; Schierup *et al.*, 2000). Under PMS hypothesis, similar parasite pressures between populations could explain this decrease in the differentiation level. If populations differentiation based on MHC mainly reflect demographic factors, we should observe no difference between MHC and microsatellite differentiation patterns. Moreover, an absence of selection, or a weak selection on MHC genes, in our populations could lead to similarities between the differentiation patterns of microsatellites and MHC genes (Aguilar, Garza, 2006; Aguilar *et al.*, 2004; Piertney, 2003).

Although heterogeneous selection is generally considered a widespread phenomenon, little information is usually available on the selection that is locally exerted by parasites and whether this varies among the studied populations. A more powerful approach to study the relative importance of selection vs. drift on the evolution of MHC diversity and to tease apart the frequency-dependent and the heterogeneous selection hypotheses might be to compare populations that differ in terms of both selection regimes and demography. Insular and mainland population should ideally fit these prerequisites. Insular populations usually have smaller population size than mainland populations which makes them more prone to drift (Frankham, 1997; Frankham, 1998). Moreover, in agreement with the insular biogeography theory, organisms living in islands are usually less exposed to the risk of infectious diseases simply because islands harbor less parasite species than mainland (Lenaghan *et al.*, 2006; Maitland *et al.*, 2000; Moro *et al.*, 2003; Nieberding *et al.*, 2006).

In this study, we investigated the genetic variability and differentiation among 12 populations of house sparrow (*Passer domesticus*), six insular and six mainland populations. We also assessed the community of haemosporidian parasites (avian malaria) for each of the 12 populations and the prevalence of infection. Avian malaria is a widespread vector-borne disease of wild birds, extensively studied during the last decades. While haemosporidian parasites that coevolved with birds are usually not lethal to them, recent studies have shown that they could be more detrimental to the host than previously thought, showing direct effects on its fitness (Atkinson, 1999; Atkinson, Van Riper III, 1991; Cellier-Holzem *et al.*, 2010; Knowles *et al.*, 2010; Van Riper, 1986; Williams, 2005).

The comparison of genetic differentiation and parasite diversity between insular and mainland populations allows us to test the following predictions: i) overall, insular population should have a depauperated genetic diversity (whatever the marker used) and an impoverished parasite community compared to mainland populations; ii) population differentiation should be stronger between pairs of populations involving islands compared to mainland for both MHC and microsatellites; iii) if avian parasites play a role as selective agents, the strength of the differentiation at the MHC should be negatively correlated with the similarity in parasite community between two populations.

## Material and methods

*Sampling*

We sample 12 populations of house sparrows located in Brittany, France (Figure 1). 450 adult house sparrows were captured

between 2007 and 2009 using mist-nets (Table 1). Sample sizes vary between the different analyses since not all birds were successfully genotyped for the microsatellites, the MHC, or screened for parasites. Each bird was banded and visually sexed (the house sparrow being a sexually dimorphic species). We collected a small volume of blood samples (ca. 20 µl) by brachial vein puncture. Blood was stored in 500 µl of Queen's Lysis Buffer (QLB, (Seutin *et al.*, 1991). Once in the lab, DNA was extracted using the Wizard® SV 96 Genomic DNA Purification kit (Promega) according to the manufacturer's instructions, for subsequent molecular analyses.

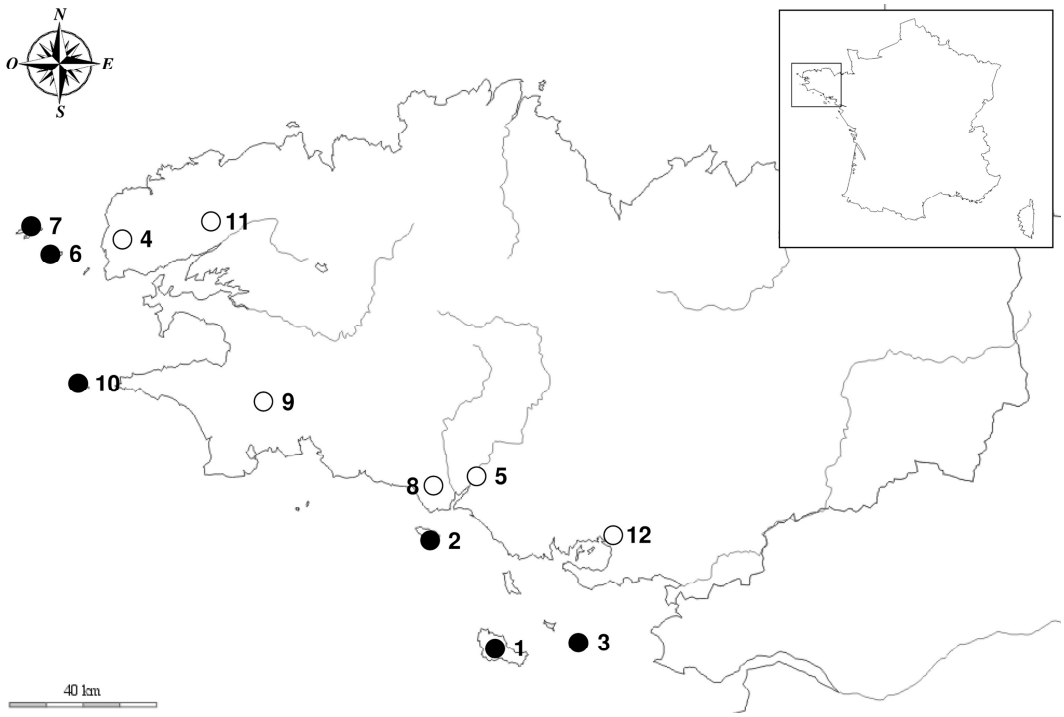


Figure 1. Geographical localization of the 12 house sparrow populations used in this study. 1 - Belle-île, 2 - Groix, 3 - Hoedic, 4 – Kerinou, 5 - Languidic, 6 - Molène, 7 - Ouessant, 8 - Ploemeur, 9 - Quimper, 10 - Sein, 11 - St Elven, 12 - Vannes. Black points localize island populations and white points mainland populations.

*Microsatellite genotyping*

449 individuals were genotyped using twelve microsatellite loci: PdomD09, PdomC11, PdomA08, PdomF09, PdomB01, PdomE09, PdomA04, PdomH05 (Loiseau *et al.*, 2009), Mjg1 (Li *et al.*, 1997), Ase18 (Richardson *et al.*, 2000), Pdo3 and Pdo5 (Griffith *et al.*, 1999). Amplifications were run in a final volume of 10 µl including 10 to 50 ng of DNA, 2 µl of 5X buffer, 1,5 to 2 mM of $MgCl_2$, 400 µl of dNTPs, 1 µM of each primers and 0,2 U of *Taq* DNA polymerase (Promega). The PCR reactions were performed following this program: 94°C 3min, 30 cycles of 94°C 20s, 20s for annealing (48°C to 56°C according to the different loci), and 72°C 40s, followed by a final extension of 72°C 5min. Samples were then run in an ABI3730 automated sequencer. Allele sizes were determinated using GeneMapper 3.0 software (Applied Biosystems 2002).

*MHC class I genotyping*

389 house sparrows were genotyped by amplifying exon 3 of a class I locus, which corresponds to the peptide-binding region (PBR) (Bonneaud *et al.*, 2004b). PCR amplifications were performed using a fluorescent (6'FAM) labelled primer (A23M – GCG CTC CAG CTC CTT CTG CCC ATA) and an unlabelled primer (A21M – GTA CAG CGC CTT GTT GGC TGT GA). Amplifications were run in a final volume of 10 µl, which included 50 to 100 ng of genomic DNA, 0.5 µl of each primer and 5 µl of Multiplex PCR Kit (QIAGEN GmbH) containing hot-start DNA polymerase, buffer and dNTPs. The PCR program began with 5min initial heating at 95°C followed by 35 cycles of 30s denaturation at 94°C, 90s annealing at 56°C and 90s extension at 72°C. A final elongation step was run during 10min

at 72°C. To control for PCR artefacts, we used 2 negative controls (for PCR and for sequencer).

MHC diversity was screened using capillary electrophoresis single conformation polymorphism (CE-SSCP) (Griggio *et al.*, 2011). The fluorescent-labelled PCR samples were prepared for electrophoresis by combining 1 µl PCR product with 8.75 µl Hi-Di formamide and 0.25 µl of in-house prepared ROX size standard (DeWoody *et al.*, 2004). This mix was heated for 5min at 95°C to separate the complementary DNA strands. Analyses were conducted in an automated DNA sequencer (ABI PRISM 3130 xl automated DNA Sequencer, Applied Biosystems). The retention time of allelic variants were indentified relative to the ROX size standard. GeneMapper v4.0 software was used to analyse the SSCP data.

*Parasite screening*

We assessed the presence of malaria parasites (genus *Plasmodium* and *Haemoproteus*) with a nested polymerase chain reaction. This PCR amplified a 524-bp-long fragment of the cytochrome b of the two parasite genus (Waldenstrom *et al.*, 2004). This method is highly repeatable, and the detection limit is one infected blood cell per 100 000. We identified the parasite strain by sequencing PCR products and blasting these sequences in GenBank using the algorithm BLASTN (http://blast.ncbi.nlm.nih.gov/Blast.c gi). We also made alignments between our sequences and sequences listed in the public data base MalAvi (Bensch *et al.*, 2009).

*Analyses*

Genetic variability

Among the 12 populations, 5 populations were sampled in two different years. Since we found no genetic differentiation at microsatellite loci between these two years, we decided to pool the data for the two years.

For each population we tested whether there was any deviation from Hardy-Weinberg equilibrium for each locus and whether there was any linkage disequilibrium between pairs of loci. This was done using exact tests implemented in GenePop 4.0 (Rousset, 2008). Global deviation from Hardy-Weinberg equilibrium was investigated for each population by using Fisher's exact tests. The assumption of neutrality of microsatellite loci was tested using the method implemented by (Beaumont, Nichols, 1996) in LOSITAN (Antao *et al.*, 2008) (see supplementary material).

Within-population genetic diversity of microsatellites was assessed by computing the observed (Ho) and expected (He) heterozygosities using the software Genetix (Belkhir *et al.*, 2004). Microsatellite allelic richness was computed using a rarefaction index for the smallest sample size for one locus in one population (17 individuals in St Elven) using $F_{STAT}$ 2.9.3 (Goudet, 1995).

MHC allelic frequencies were performed using Arlequin 3.5 (Excoffier, Lischer, 2010) and were estimated as the number of individuals carrying a certain allele divided by the total allele count observed in the population. Total allele count is defined as the sum of alleles found per individual in a population (Loiseau *et al.*, 2009). We have to note that this way of determining allele frequencies may underestimate the frequency of common alleles and overestimate the frequency of rare alleles (Ekblom *et al.*, 2007). Allelic richness of MHC class I was estimated using richness cumulative curves with the software ESTIMATES 7.5 (Kavaliers, Colwell, 1995) based on the smallest number of individuals genotyped (11 individuals in Ploemeur).

Estimators of within-population genetic variability (allelic richness and genetic diversity) for the two types of markers were compared between insular and mainland populations with a Mann-Whitney test.

Population differentiation

Genetic differentiation between populations for microsatellite markers was measured by $F_{ST}$ estimators and was tested for each population pair with exact tests implemented in GenePop 4.0 (Rousset, 2008). Genetic differentiation between populations based on MHC class I genes was assessed using the software Arlequin 3.5 (Excoffier, Lischer, 2010).

For each type of markers, isolation by distance was tested by correlating Fst/(1-Fst) to the logarithm of the geographical distance (Rousset, 2008), statistical significance being inferred using a Mantel test in XLSTAT. Similarly, Mantel correlations were used to test whether population differentiation was affected by the particular barrier represented by sea. To this purpose we built a matrix of presence (1) or absence (0) of sea between all population

pairs and correlated it with the matrix of Fst values obtained with microsatellites and MHC.

Parasites analyses

For each sparrow population we assessed the number of different avian malaria strains that were present and the prevalence of infection (proportion of infected birds). The number of strains was compared between insular and mainland populations with Mann-Whitney tests. Differences in prevalence (binomial distribution) between insular and mainland populations were investigated using a general linear mixed model (GLMM) with type of population (island and mainland), year, season, and sex as fixed factors, and population within type as random factor.

To test the idea that genetic differentiation at the MHC depends on the dissimilarity between the parasite communities, we computed the Steinhaus similarity coefficient, S = 2W/(A+B); where W is the sum of the minimal number of infected hosts for each parasite strain between two populations, and A and B are the sum of infected hosts in populations A and B, respectively. In four populations, none of the sampled birds were infected. The similarity index of pairs of populations with zero prevalence was set to 1 because we considered that the absence of the parasites homogenizes the selection pressures exerted by these specific parasites (Table 3). The matrix of parasite similarity was then correlated with the matrices of Fst based on both microsatellites and MHC using Mantel tests.

Unless otherwise attested, statistical tests were performed by using SAS 9.2 (SAS 2002) and JMP 5.0 (SAS 2002).

## Results

*Genetic variability*

For microsatellites, the number of alleles varied from 5 to 26 alleles. Out of 769 exacts tests performed for linkage disequilibrium, 42 were significant at the 0.05 level. However, none of them remained significant after sequential Bonferroni correction. Concerning the deviation from the Hardy-Weinberg equilibrium, 8 of the 144 tests by locus and by population were significant. None of these 8 tests remained significant after sequential Bonferroni correction. None of the 12 Hardy-Weinberg equilibrium global tests were significant. We did not find any evidence showing that the 12 microsatellite loci were under selection (Figure S1, supplementary material).

Allelic richness, expected and observed heterozygosity for each population are given in Table 1. Allelic richness was lower in insular populations than in mainland ones ($Z_1$=2.16, p=0.03).

We found 45 MHC alleles in our populations. Allelic richness and genetic diversity for each population are given in Table 1. There were no differences neither in MHC allelic richness ($Z_1$=1.2, p=0.23).

*Population differentiation*

Fst values based on microsatellites varied between -0.0026 (between Vannes and Ploemeur) and 0.0445 (between Quimper and Molène) (Table 2). 59 out of the 66 Fst values between pairs of populations were statistically significant, and 44 were still significant after sequential Bonferroni correction. All the 15 Fst between island pairs were significant. 75% of the 36 Fst between island and mainland populations, and 6.7% of the 15 Fst between mainland pairs were significant. We did not find any isolation by distance (Mantel test, r=0.091, p=0.47). However, there was a strong correlation between Fst values and the presence/absence of sea between two populations (Mantel test, r=0.426, p<0.0001).

Pairwise Fst values for MHC varied between -0.0109 (Ploemeur and Kerinou) and 0.0399 (Molène and Kerinou) (Table 2). 28 out of the 66 Fst values were statistically significant, 17 of them were still significant after sequential Bonferroni correction (Table 2). 53.3 % of the 15 differentiation tests between island pairs were significant, 25% of the 36 tests between island and mainland populations and 0% of the 15 tests between mainland pairs.

Fst values were not correlated with the geographic distance (Mantel test, r=0.008, p=0.97), but they were strongly correlated with the presence/absence of sea between populations (Mantel test, r=0.379, p=0.0003).

The correlation between $F_{ST}$ for microsatellite loci and $F_{ST}$ for MHC genes was highly significant (Mantel test, r=0.59, p<0.0001, Figure 2). The correlation between microsatellite $F_{ST}$ and MHC $F_{ST}$ was still significant when controlling for the presence/absence of sea (r=0.51, p<0.0001).

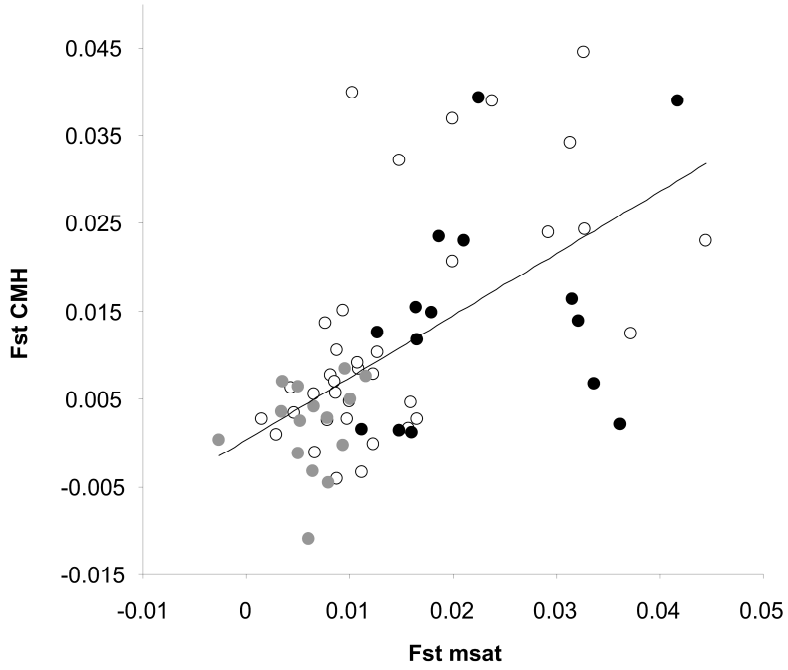


Figure 2. Correlation between microsatellite $F_{ST}$ values and MHC genes $F_{ST}$ values between all populations pairs. White circles represent all population pairs, black circles island population pairs and grey circles mainland population pairs. Black line gives the linear regression between microsatellite $F_{ST}$ values and MHC genes $F_{ST}$ values for all population pairs.

*Avian malaria parasites*

Among the 446 birds screened for *Plasmodium* and *Haemoproteus*, 40 individuals harboured the infection with five different parasite strains. Prevalence in each population is given in Table 1. No parasites were found in 4 of the 12 populations (Groix, Molène, Sein and St Elven). Prevalence did not vary between years ($F_{2,244}$=3.01, p=0.096), or sex ($F_{1,445}$=1.03, p=0.31). Prevalence was higher in winter (12.94%) than in autumn (3.54%) and spring (3.85%) ($F_{2,244}$=10.19, p=0.002), and was higher in mainland (15.46%) than in insular populations (3.92%) ($F_{1,445}$=12.51, p=0.0054).

We computed a Steinhaus similarity coefficient between pairs of populations to putatively infer the similarity of selection pressures exerted by avian malaria on their local hosts (Table 3). This parasite similarity matrix was not correlated with the geographical distance (Mantel test, r=-0.141, p=0.25), nor with the presence/absence of sea between two populations (Mantel test, r=-0.052, p=0.67). There was no correlation between microsatellite Fst and parasite similarity between populations (Mantel test, r=-0.076, p=0.56). However, there was a negative correlation between MHC Fst and parasite similarity (Mantel test, r=-0.246, p=0.039). This correlation remained significant when microsatellite Fst were controlled for (partial Mantel test, r=-0.250, p=0.032).

## Discussion

Allelic richness, measured with microsatellites, was lower is islands than in mainland. Island populations, due to a smaller population size and reduced gene flows, often exhibit a weaker genetic diversity than mainland populations. In a meta-analysis conducted by (Frankham, 1997), 163 of the 202 island populations studies showed a lower genetic variability than mainland populations.

Moreover, island populations seem to be more differentiated than mainland populations. In birds, the differentiation levels are weak, but traditionally observed (*e.g.* (Alcaide *et al.*, 2008; Bouzat, Johnson, 2004; Caizergues *et al.*, 2003; Godoy *et al.*, 2004; Martinez-Cruz *et al.*, 2004), and for the house sparrow see (Loiseau *et al.*, 2009). Our results suggest that neutral differentiation could mainly be explained by the presence or the absence of sea between populations, independently from the geographical distances which separate them. Thus, sea appears to be an important barrier for sparrow migrations, even if the distance to the coast and between two island populations is weak (some dozens of kilometres maximum).

(Altwegg *et al.*, 2000) found that only 10% of the house sparrows were able to disperse between islands, separated from 2 to 20 km. Even in a mainland context, the house sparrow is a sedentary species, with a mean dispersal distance of 1.7 km for juveniles and 1.9 km for adults (Paradis *et al.*, 1998).

This study about neutral genetic variability and differentiation highlights demographical differences between island and mainland house sparrow populations, which could be explained by a smaller population size and gene flows in island populations.

MHC genetic variability, contrary to microsatellite, is similar between island and mainland populations. Usually, this pattern is explained by selective forces strong enough to counteract genetic drift effects (Aguilar *et al.*, 2004; Garrigan, Hedrick, 2001; Gutierrez-Espeleta *et al.*, 2001; Hedrick, 2002). We can notice that the studies previously mentioned concerned only island or threatened populations, without comparison with mainland or with healthy populations of the same species.

MHC genetic differentiation gives similar results to neutral genetic differentiation. Island populations appear to be more differentiated for MHC genes than mainland populations. However, we can notice that MHC differentiation seems to be weaker than neutral differentiation, which could be due to balancing selection(Bernatchez, Landry, 2003; Boyce *et al.*, 1997; Hedrick *et al.*, 2001; Huang, Yu, 2003; Schierup *et al.*, 2000). This decrease in the differentiation level could be due to similar parasite pressures between populations. An absence of selection, or a weak selection on MHC genes, in our populations could also explain the similarities between the differentiation patterns of microsatellites and MHC genes (Aguilar, Garza, 2006; Aguilar *et al.*, 2004; Piertney, 2003). However, it is difficult to draw a conclusion from direct comparison between MHC and microsatellite differentiation levels, since MHC genes and microsatellites differ in their pattern of molecular evolution and in the amount of variability they exhibit. The use of a standardized genetic differentiation measure of Fst (Hedrick, 2005) that controls for marker variability would counteract this problem. But, this measure needs a locus-specific approach, which is not yet the case for MHC genes, in non-model vertebrates.

We found that prevalence of avian malaria infection was lower in islands than in mainland, which could be explained by the insular syndrome (Blondel, 1995). We can suspect that avian malaria parasite selective pressures are weaker is island compared to mainland populations. Parasite-mediated selection is one of the main hypotheses proposed to explain MHC polymorphism in natural populations (Bernatchez, Landry, 2003). More precisely, spatial fluctuations in parasite and pathogen communities are suspected to generate spatial variations in selection, which could contribute to explain MHC observed polymorphism and structure. Several studies have found that MHC allele-frequency spatial variations and MHC differentiation were higher than neutral differentiation (Charbonnel, Pemberton, 2005; Ekblom *et al.*, 2007; Loiseau *et al.*, 2009), and that parasite prevalence varied spatially even at low geographical scale (Loiseau *et al.*, 2011; Wood *et al.*, 2007). However, very few studies had monitored both MHC and parasite variations together (Charbonnel, Pemberton, 2005). In our study, we found that MHC population structure is correlated with malaria parasite community similarity between populations, in agreement with the spatial variation hypothesis. To our knowledge, this is the first study that found direct malaria parasite impact on genetic population structure, at a low geographical scale.

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Table 1. Sample characteristics, sample sizes, genetic and parasite characteristics for each population studied. He, Ho, A are respectively, expected heterozygosity, observed heterozygosity, allelic richness estimated for 11 individuals, obtained for microsatellite loci. D, R are respectively, MHC genetic diversity, MHC allelic richness estimated for 11 individuals. SGS1, GRW11, TURDUS1, BLUTI02 and P43 are the name of the different parasite strains found in our populations.

| | | | | | Microsatellites | | | | MHC class I | | Avian malaria parasites | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| n° | Population | Type | Years | Seasons | N | He | Ho | A | N | R | N | Prevalence (%) | Shannon index | Number of different strains | SGS1 | GRW11 | TURDUS1 | BLUTI02 | P43 |
| 1 | Belle-île | island | 2008 | autumn, winter | 37 | 0.73 | 0.72 | 6.27 | 34 | 14.5 | 37 | 2.7 | 0 | 1 | 1 | 0 | 0 | 0 | 0 |
| 2 | Groix | island | 2008 | autumn, winter | 45 | 0.75 | 0.75 | 6.2 | 41 | 16.1 | 45 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3 | Hoedic | island | 2007,2008 | autumn, winter | 40 | 0.74 | 0.76 | 6.51 | 39 | 18.8 | 40 | 7.5 | 0.64 | 2 | 2 | 1 | 0 | 0 | 0 |
| 4 | Kerinou | mainland | 2008 | spring | 26 | 0.77 | 0.76 | 6.97 | 20 | 14 | 26 | 3.85 | 0 | 1 | 1 | 0 | 0 | 0 | 0 |
| 5 | Languidic | mainland | 2008 | autumn, winter | 55 | 0.73 | 0.74 | 6.49 | 53 | 17.9 | 55 | 23.64 | 0.79 | 4 | 10 | 1 | 1 | 0 | 1 |
| 6 | Molène | island | 2008, 2009 | spring, winter | 30 | 0.7 | 0.74 | 5.9 | 20 | 15 | 30 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7 | Ouessant | island | 2007, 2009 | spring, winter | 71 | 0.71 | 0.7 | 6.41 | 60 | 11.8 | 69 | 8.45 | 0.71 | 2 | 0 | 0 | 2 | 3 | 1 |
| 8 | Ploemeur | mainland | 2008 | winter | 19 | 0.72 | 0.67 | 6.23 | 11 | 14.5 | 19 | 21.05 | 0.56 | 2 | 3 | 0 | 1 | 0 | 0 |
| 9 | Quimper | mainland | 2008, 2009 | spring, winter | 36 | 0.75 | 0.78 | 6.61 | 31 | 15 | 35 | 5.56 | 0 | 1 | 2 | 0 | 0 | 0 | 0 |
| 10 | Sein | island | 2009 | winter | 32 | 0.75 | 0.75 | 6.01 | 30 | 11.2 | 32 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 11 | St Elven | mainland | 2008, 2009 | spring, winter | 17 | 0.77 | 0.78 | 6.88 | 14 | 24.6 | 17 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 12 | Vannes | mainland | 2008 | autumn, winter | 41 | 0.75 | 0.74 | 6.69 | 36 | 14.2 | 41 | 24.39 | 0.67 | 2 | 6 | 4 | 0 | 0 | 0 |

Table 2. Pairwise $F_{ST}$ by population pairs. The half-matrix on the top gives the $F_{ST}$ estimated with microsatellites loci. The half-matrix on the bottom gives the $F_{ST}$ estimated with MHC class I genes. $F_{ST}$ values in bold represent significant differentiation tests. $F_{ST}$ values follow by three stars represent differentiation tests still significant after sequential Bonferonni correction.

| Populations | Belle-île | Groix | Hoedic | Kerinou | Languidic | Molène | Ouessant | Ploemeur | Quimper | Sein | St Elven | Vannes |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Belle-île | - | **0.0127*** ** | **0.0179*** ** | **0.0088*** ** | **0.0082*** ** | **0.0417*** ** | **0.0165*** ** | **0.0066** | **0.0088*** ** | **0.0225*** ** | **0.0109** | **0.0076** |
| Groix | **0.01259*** ** | - | **0.0112*** ** | **0.0087*** ** | **0.0100*** ** | **0.0315*** ** | **0.0160*** ** | **0.0015*** ** | **0.0157*** ** | **0.0186*** ** | **0.0029** | **0.0043** |
| Hoedic | **0.01487*** ** | 0.00146 | - | **0.0127*** ** | **0.0108*** ** | **0.0337*** ** | **0.0148*** ** | **0.0078** | **0.0086*** ** | **0.0164*** ** | **0.0112*** ** | **0.0065*** ** |
| Kerinou | -0.00405 | 0.00573 | **0.01033** | - | **0.0079** | **0.0327*** ** | **0.0123*** ** | 0.0060 | **0.0078*** ** | **0.0103*** ** | 0.0035 | **0.0034** |
| Languidic | **0.00768** | 0.00476 | **0.00916*** ** | -0.00454 | - | **0.0313*** ** | **0.0159*** ** | 0.0064 | **0.0094** | **0.0238*** ** | **0.0116** | **0.0050** |
| Molène | **0.03891*** ** | **0.01637** | 0.00675 | **0.04454*** ** | **0.03416*** ** | - | **0.0322*** ** | **0.0292*** ** | **0.0445*** ** | **0.0362*** ** | **0.0372*** ** | **0.0328*** ** |
| Ouessant | **0.01171*** ** | 0.0012 | 0.00134 | 0.00785 | 0.00466 | **0.0139** | - | 0.0046 | **0.0165*** ** | **0.0211*** ** | **0.0123** | **0.0098*** ** |
| Ploemeur | -0.00114 | 0.00268 | 0.00258 | -0.01093 | -0.00323 | **0.02403** | 0.00342 | - | **0.0101** | **0.0199*** ** | 0.0065 | -0.0026 |
| Quimper | **0.0106** | 0.00168 | 0.00704 | 0.00288 | -0.00031 | **0.02302** | 0.00267 | 0.00494 | - | **0.0199*** ** | **0.0096** | **0.0052** |
| Sein | **0.03925*** ** | **0.0235*** ** | **0.01542*** ** | **0.03994*** ** | **0.03899*** ** | 0.00212 | **0.023*** ** | **0.02063** | **0.03688*** ** | - | **0.0094*** ** | **0.0148*** ** |
| St Elven | 0.00837 | 0.00098 | -0.00336 | 0.00703 | 0.0076 | **0.01247** | -0.0001 | 0.00417 | 0.00843 | **0.01512** | - | 0.0050 |
| Vannes | **0.01369*** ** | **0.00624** | 0.00551 | 0.00354 | -0.00122 | **0.02438*** ** | 0.00272 | 0.00036 | 0.0025 | **0.03215*** ** | 0.00641 | - |

Table 3. Pairwise Steinhaus coefficient between all population pairs. We attributed the coefficient 1 when the two populations had no malaria parasites. Values 1 in bold were excluded in the analyses excluding the comparisons between two populations without malaria parasites.

| Populations | Belle-île | Groix | Hoedic | Kerinou | Languidic | Molène | Ouessant | Ploemeur | Quimper | Sein | St Elven |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Groix | 0 | | | | | | | | | | |
| Hoedic | 0.5 | 0 | | | | | | | | | |
| Kerinou | 1 | 0 | 0.5 | | | | | | | | |
| Languidic | 0.14 | 0 | 0.38 | 0.14 | | | | | | | |
| Molène | 0 | **1** | 0 | 0 | 0 | | | | | | |
| Ouessant | 0 | 0 | 0 | 0 | 0.21 | 0 | | | | | |
| Ploemeur | 0.4 | 0 | 0.57 | 0.4 | 0.47 | 0 | 0.2 | | | | |
| Quimper | 0.67 | 0 | 0.8 | 0.67 | 0.27 | 0 | 0 | 0.67 | | | |
| Sein | 0 | **1** | 0 | 0 | 0 | **1** | 0 | 0 | 0 | | |
| St Elven | 0 | **1** | 0 | 0 | 0 | **1** | 0 | 0 | 0 | **1** | |
| Vannes | 0.18 | 0 | 0.46 | 0.18 | 0.61 | 0 | 0 | 0.43 | 0.33 | 0 | 0 |

# CHAPITRE 3

# Le choix du partenaire : un choix guidé par les pressions environnementales ?

*En populations naturelles, les individus doivent répondre à des pressions de sélection liées à l'environnement, et notamment aux parasites. De plus, le contexte géographique est aussi capable d'avoir une grande influence sur la structure génétique des hôtes, ce qui peut influencer en retour la capacité des individus à se défendre contre les parasites. Toutefois, les caractéristiques de l'environnement ne sont pas fixes. La capacité d'une population à répondre à ces changements implique l'existence d'une variabilité. Cette variabilité peut notamment être générée par la reproduction sexuée. En particulier, choisir son partenaire selon certaines caractéristiques génétiques, en fonction des conditions environnementales, est un moyen pour les individus de produire des descendants adaptés. Dans ce chapitre, nous verrons les causes et les conséquences d'un choix de partenaire non aléatoire, en insistant particulièrement sur le choix de partenaire CMH-dépendant.*

## 1. Le choix du partenaire

Le choix de partenaire peut être défini comme un appariement différentiel de la part des femelles, résultant d'une préférence (Andersson, 1994; Clutton-Brock, 2007; Heisler *et al.*, 1987; Kempenaers, 2007). Il est important de distinguer les termes "préférence" et "choix". En effet, le choix du partenaire est influencé par la préférence des femelles, mais aussi par un grand nombre d'autres facteurs pouvant intervenir dans ce choix. Ces contraintes peuvent être environnementales (risques de prédation, timing lié à la reproduction), ou simplement des contraintes temporelles (la femelle ne dispose pas d'un temps infini pour se reproduire). De ce fait, le choix du partenaire résulte de l'interaction entre les préférences et les contraintes (Wagner, 1998). Dans la plupart des expériences, en laboratoire ou en populations naturelles, les chercheurs ont accès au choix du partenaire et font des inférences sur les préférences des femelles.

Le choix d'une femelle pour un mâle peut se faire en fonction des bénéfices directs que la femelle pourra retirer de cette reproduction, en terme par exemple d'accès à un territoire ou à des ressources. Ce choix pourra également se faire en fonction de bénéfices indirects, aussi appelés "bons gènes", hérités du mâle, et transmis à la descendance (Kempenaers, 2007; Mays *et al.*, 2008; Neff, Pitcher, 2005; Trivers, 1972). Le développement des outils génétiques a permis d'augmenter considérablement les études sur les bénéfices indirects. L'utilisation de marqueurs microsatellites et de marqueurs sélectionnés permet par exemple d'accéder à la

variabilité génétique de chaque individu, de calculer leur proximité génétique et de savoir si le choix de partenaire se fait en fonction de ces caractéristiques.

D'un point de vue théorique, les femelles auraient intérêt à choisir les mâles les plus hétérozygotes et les plus dissemblables, afin d'augmenter la variabilité génétique de leur descendance et éviter la consanguinité (Mays, Hill, 2004; Tregenza, Wedell, 2000). C'est l'hypothèse d'*évitement de la consanguinité* (*inbreeding avoidance*). La plupart des études menées sur ce sujet semblent confirmer cette hypothèse. Pourtant, depuis quelques années, quelques études ont révélé que les femelles pouvaient choisir des mâles génétiquement plus proches qu'attendus avec un appariement aléatoire (*outbreeding avoidance*). Des études théoriques et empiriques ont en effet montré qu'un certain degré de consanguinité pouvait être parfois avantageux et que trop de dissimilarité pouvait avoir des effets délétères (Dolgin *et al.*, 2007; Sherman *et al.*, 2008; Smith, 1979). S'apparier avec des individus trop différents génétiquement peut en particulier conduire à la rupture de complexes de gènes coadaptés (Bateson, 1983; Charlesworth, Charlesworth, 1987; Shields, 1993; Tregenza, Wedell, 2000; Waller, 1993). Une autre hypothèse permettant d'expliquer le choix pour les individus similaires est celle de la sélection de parentèle (*kin selection*). Cette hypothèse, basée sur les théories d'Hamilton (Hamilton, 1964a; Hamilton, 1964b), suggère que des individus génétiquement proches partagent des gènes identiques et que, les femelles s'appariant avec des mâles similaires peuvent augmenter leur fitness inclusive (Bengtsson, 1978; Kokko, Ots, 2006; Lehmann, Perrin, 2003; Oh, 2011; Parker, 2006; Ryder *et al.*, 2010; Wang, Lu, 2011; Waser *et al.*, 1986). L'existence de ce type de choix implique des pressions de sélection particulières, conduisant à une adaptation locale, ainsi que des coûts liés à la consanguinité limités.

Le **Manuscrit 8** présente une étude de choix de partenaire dans une population insulaire de moineaux domestiques. Nous avons mesuré l'homozygotie et la proximité génétique des poussins et des couples à l'aide de 12 loci microsatellites durant 3 années successives. Les résultats présentent un excès significatif d'homozygotes chez les poussins, ce qui suggère que les femelles choisissent plutôt des mâles génétiquement similaires. Ce résultat, bien que surprenant, à été également trouvé chez d'autres populations d'oiseaux, y compris des populations insulaires (Cohen, Dearborn, 2004; Krokene, Lifjeld, 2000; Wang, Lu, 2011).

## 2. Comment choisir ?

Un choix en faveur de certains génotypes implique nécessairement que les femelles sont capables d'évaluer la variabilité et/ou la proximité génétique des mâles. De nombreuses études, comme par exemple chez l'omble chevalier (*Salvelinus alpinus*, Olsen *et al.*, 1998) ou chez le pétrel bleu (*Halobaena caerulea*, Celerier *et al.*, 2011), ont pu mettre en évidence que les individus étaient capables de reconnaître leurs apparentés. Dans les études ayant mis en évidence un choix envers les individus similaires, des mécanismes d'empreintes (*sexual imprinting*, Bateson, 1978), de phénotype référent (*self-referent phenotype matching*, Hauber, Sherman, 2001), et de préférences héritables de micro-habitats (*heritable microhabitat preference*, Petrie *et al.*, 1999) ont été évoqués. L'*imprinting* implique que les jeunes soient élevés suffisamment longtemps par les parents, comme c'est le cas dans de nombreuses espèces d'oiseaux.

Concernant les gènes du CMH, plusieurs études montrent que le génotype CMH d'un individu serait lié à son odeur corporelle (Egid, Brown, 1989; Wedekind *et al.*, 1995; Zavazava, Eggert, 1997). Le processus physiologique est encore mal connu. Une possibilité serait que les gènes du CMH influencent la flore microbienne (Singh *et al.*, 1990), ou la concentration en acides volatiles (Singer *et al.*, 1997), modifiant alors l'odeur de la transpiration et de l'urine (Beauchamp, Yamazaki, 2003; Hurst *et al.*, 2001; Singh *et al.*, 1987; Wedekind, Furi, 1997; Wedekind *et al.*, 1995; Yamazaki *et al.*, 1979). Chez les oiseaux, les preuves de l'existence et de l'utilisation de l'odorat sont relativement récentes. Par exemple, les études menées chez le pétrel bleu (*Halobaena caerulea*) ont montré que les individus étaient capables de détecter les différences d'odeurs entre individus, la similarité d'odeur entre parents et jeunes, et que ces odeurs étaient liées à la glande uropygiale (Celerier *et al.*, 2011; Mardon *et al.*, 2011). Ces odeurs, liées au CMH, ont un rôle dans l'évitement des apparentés chez cette même espèce (Celerier *et al.*, 2011; Mardon, Bonadonna, 2009).

On sait également que les femelles choisissent leurs mâles en fonction de certains traits phénotypiques honnêtes liés à la vigueur des mâles (Hamilton, Zuk, 1982). Ces traits pourraient être liés au génotype CMH (Mays, Hill, 2004; Zahavi, 1975). L'exemple le plus célèbre a été trouvé chez le faisan de Colchide (*Phasianus colchicus*) où les femelles choisissent les mâles ayant les éperons les plus longs. Cette longueur d'éperon est corrélée positivement avec la taille du corps, la viabilité et la survie des jeunes (von Schantz *et al.*, 1989; von Schantz *et al.*, 1996), ainsi qu'avec la variabilité sur les gènes du CMH (von

Schantz *et al.*, 1997; von Schantz *et al.*, 1996). Une autre étude, réalisée chez le cerf de Virginie (*Odocoileus virginianus*), a mis en évidence des associations entre le génotype CMH et le taux de développement des bois, ainsi que la taille du corps (Ditchkoff *et al.*, 2001).

Un autre moyen pour les femelles d'évaluer l'efficacité du génotype CMH des mâles est d'évaluer leur statut parasitaire et d'éviter de se reproduire avec les individus parasités (Kavaliers *et al.*, 2005; Kavaliers, Colwell, 1995).

Dans toutes les études présentées ici, il s'agit d'un choix de partenaire pré-copulatoire. Mais, le choix peut aussi être post-copulatoire et cryptique. Certaines études montrent en effet que les femelles détruisent sélectivement le sperme ou les jeunes de certains mâles incompatibles (Birkhead, 1998; Eberhard, Cordero, 1995; Jennions, 1997). Des avortements spontanés en fonction du niveau de similarité du génotype CMH du mâle ont également été décrits (Apanius *et al.*, 1997; Ober, 1992). Dans l'étude décrite dans le **Manuscrit 8**, la formation des couples est compatible avec des appariements aléatoires, tandis que l'excès d'homozygotes chez les poussins penche plutôt en faveur d'un choix préférentiel pour les individus similaires. Ce résultat semble indiquer que le choix des femelles ne se reflète pas au niveau du choix du mâle, mais au niveau de sa descendance, ce qui est plutôt en faveur d'un choix post-copulatoire.

## 3. Le choix de partenaire lié au CMH

Si le choix du partenaire peut être évalué avec des marqueurs neutres, il est également très intéressant d'étudier ce choix en examinant des gènes soumis à sélection. Dans ce cadre, les gènes du CMH apparaissent être des marqueurs de choix, de part leur polymorphisme important et leur fonction connue dans le système immunitaire. Leur utilisation permet d'étudier le choix de partenaire pour la variabilité de ces gènes, mais également pour la présence ou l'absence de certains allèles (approche allèle spécifique). La sélection sexuelle est le deuxième mécanisme sélectif, après la sélection exercée par les parasites (PMS, chapitre 2), évoqué pour expliquer le maintien du polymorphisme sur ces gènes (Brown, Eklund, 1994; Jordan, Bruford, 1998; Milinski, 2006; Penn, Potts, 1999; Piertney, Oliver, 2006).

De nombreuses hypothèses adaptatives ont été suggérées pour expliquer comment le CMH peut influencer le comportement de reproduction. Parmis elles, les plus populaires sont : les fécondations CMH-dépendantes (Rulicke *et al.*, 1998; Wedekind *et al.*, 1996), les

avortements CMH-dépendants (Alberts, Ober, 1993) et les appariements CMH-dépendants (Penn, Potts, 1999).

Deux possibilités sont généralement avancées pour expliquer un choix de partenaire CMH-dépendant. Ce type de choix permettrait d'augmenter la résistance aux parasites de la descendance, ou bien d'éviter les appariements consanguins. En effet, de part leur polymorphisme important, deux individus possédant des allèles CMH communs ont de grande chance d'être apparentés. Il apparaît que l'évitement de la consanguinité serait plus bénéfique que la seule production d'hétérozygotes sur les gènes du CMH (Potts *et al.*, 1994).

Les toutes premières études, réalisées en laboratoire, chez la souris, ont montré que les femelles choisissaient préférentiellement des mâles possédant un CMH différent du leur (Egid, Brown, 1989; Yamazaki *et al.*, 1976). Des études menées chez l'homme ont obtenu des résultats similaires (Ober *et al.*, 1997; Wedekind, Furi, 1997; Wedekind *et al.*, 1995). Ces études sont aujourd'hui discutées. Les études chez l'homme sont surtout critiquées sur la méthodologie, tandis que les études sur les souris ont été remises en cause car elles utilisaient des lignées congéniques (Manning *et al.*, 1992). Les résultats obtenus sont alors difficilement extrapolables aux populations naturelles. Chez la souris, d'autres études menées en laboratoire n'ont d'ailleurs pas pu mettre en évidence un choix de partenaire CMH-dépendant (Beauchamp *et al.*, 1988; Eklund *et al.*, 1991). Toutefois, en condition semi-naturelle, Potts *et al.* (1991) ont mesuré, toujours chez la souris, des déficits en homozygotes pour les gènes du CMH, indiquant un choix préférentiel pour des individus ayant un génotype CMH différent. Dans les mêmes conditions, Manning *et al.* (1992) n'ont pas décelé de choix de partenaire CMH-dépendant. Pour des organismes non-modèles, quelques études en laboratoire ont aussi été réalisées. Par exemple, une étude menée sur le lézard des souches (*Lacerta agilis*) a montré un choix pour des mâles dissimilaires au niveau du CMH (Olsson *et al.*, 2003).

Par ailleurs, les études menées en populations naturelles restent encore limitées et très contrastées. Des études chez le bruant des prés (*Passerculus sandwichansis*, Freeman-Gallant *et al.*, 2003), la rousserole des Seychelles (*Acrocephalus seychellensis*, Richardson *et al.*, 2005), ou encore du saumon Atlantique (*Salmo salar*, Landry *et al.*, 2001) ont mis en évidence un choix CMH-dépendant en faveur des individus dissimilaires. Par contre, d'autres études sur le mouton de l'île de Saint Kilda (*Ovis aries*, Paterson, Pemberton, 1997), la rousserole turdoïde (*Acrocephalus arundinaceus*, Westerdahl, 2004), le macaque rhésus (*Macaca mulatta*, Sauermann *et al.*, 2001), et sur la bécassine double (*Gallinago media*, Ekblom *et al.*, 2004) n'ont pas révélé de choix de partenaire CMH-dépendant. Finalement,

une étude menée chez le moineau domestique a montré que les femelles choisissaient des mâles, ni trop similaires, ni trop différents, avec donc un nombre d'allèles en commun intermediaire (Bonneaud *et al.*, 2006a).

Un moyen de savoir si le choix du partenaire est lié aux gènes du CMH, ou si ces gènes sont plutôt un moyen d'évaluer la proximité génétique à l'échelle du génome du partenaire, est d'étudier le choix de partenaire à l'aide de marqueurs neutres. Nous avons réalisé cette étude dans le **Manuscrit 8** et nous n'avons pas mis en évidence de choix de partenaire CMH-dépendant. Les résultats vont dans le même sens que ceux obtenus avec les loci microsatellites, ce qui est plutôt en faveur d'un choix pour un génome entier et non pour ces gènes en particulier.

## 4. Un choix de partenaire contexte-dépendant ?

Les différentes études consacrées au choix de partenaire peuvent donner des résultats très contrastés, surtout en populations naturelles. Il existe des facteurs intrinsèques à l'espèce étudiée, ou des facteurs environnementaux, capables de moduler considérablement l'existence et la direction de ce choix.

Si les gènes du CMH sont à la base de la réponse immunitaire, le contexte parasitaire dans lequel se trouve la population étudiée semble être une caractéristique de première importance, pouvant influencer le choix du partenaire. En effet, des études menées chez la souris ont montré que les femelles produisaient plus de jeunes hétérozygotes sur les gènes du CMH lors d'un pic épidémiologique, ou quand elles étaient elles-mêmes infectées (Rulicke *et al.*, 1998; Wedekind *et al.*, 1996). Ainsi, dans la région où notre étude chez le moineau domestique a été réalisée (**Manuscrit 8**), la prévalence en parasites sanguins est faible (entre 7.5 et 11.1 % à Hoëdic et entre 0 et 24% en Bretagne, **Manuscrits 5 et 7**), comme c'est souvent le cas dans les populations insulaires. Ces pressions de sélection relâchées sur les gènes du CMH pourraient expliquer le fait qu'un choix de partenaire CMH-dépendant n'a pas été détecté dans cette population.

Les caractéristiques génétiques de la femelle peuvent également avoir une influence sur le choix du mâle. Chez l'épinoche, les femelles possédant beaucoup d'allèles CMH différents préfèreront les mâles en ayant peu, tandis que les femelles portant peu d'allèles choisiront des mâles en possédant un nombre élevé (Aeschlimann *et al.*, 2003; Reusch *et al.*, 2001). De même, chez le moineau domestique, les femelles avec un faible nombre d'allèles CMH

s'apparieront avec les mâles en possédant un grand nombre (Griggio *et al.*, 2011). Dans une autre population de moineau, les femelles semblent choisir des partenaires intermédiaires, ni trop proches, ni trop éloignés génétiquement (Bonneaud *et al.*, 2006a). Ces phénomènes de compensation et d'ajustement du nombre d'allèles (*count alleles*) tendent à montrer qu'une diversité maximale n'est pas toujours optimale. Chez les épinoches, un nombre intermédiaire d'allèles CMH conduit à une meilleure résistance aux parasites (Wegner *et al.*, 2003a). Chez le moineau domestique, les femelles possédant un nombre intermédiaire d'allèles CMH ont des tailles de ponte plus élevées (Bonneaud *et al.*, 2004a). La compensation peut également avoir lieu après le premier appariement, en réalisant des copulations avec d'autres mâles que le mâle social, qui va élever les jeunes (dans le cas des espèces où les soins parentaux sont partagés). Ce phénomène de compensation a été décrit chez des populations d'oiseaux, dont certaines donnent des résultats très similaires à l'étude présentée dans le **Manuscrit 8** (Ferretti *et al.*, 2011; Kleven *et al.*, 2005; Wang, Lu, 2011). Dans notre étude, le taux de paternité hors couple est très élevé (45% des poussins sont issus de copulations hors-couple). De plus, le mâle qui est alors choisi par la femelle pour réaliser ces copulations hors-couple est génétiquement plus proche de la femelle que ne l'était le mâle social, au niveau des loci microsatellites mais également au niveau des gènes du CMH.

Les connaissances acquises jusqu'à aujourd'hui sur le choix de partenaire tendent à montrer que, plutôt que de se focaliser sur un choix en faveur des individus les plus variables génétiquement, une approche allèle-spécifique pourrait se révéler beaucoup plus informative (Mays, Hill, 2004; Tregenza, Wedell, 2000). Des études semblent en effet montrer que certaines combinaisons alléliques déterminées par la mère et le père confèrent une meilleure immunocompétence (Johnsen *et al.*, 2000; von Schantz *et al.*, 1997), et donc que certains génotypes sont plus compatibles que d'autres. Si les études portant sur l'hétérozygotie donnent des résultats significatifs, c'est peut-être en partie parce que les femelles qui choisissent des mâles génétiquement dissemblables ont plus de chances de s'apparier, par "hasard", avec un mâle qui possédera le bon "allèle" (Milinski, 2006).

En effet, le plus important n'est peut-être pas de posséder beaucoup d'allèles, mais de posséder les bons, ceux qui confèreront la meilleure adaptation à l'environnement local. Ainsi, le contexte génétique de la population étudiée est très important à prendre en compte. En particulier, le risque de consanguinité est un paramètre capable d'influencer complètement la direction du choix de partenaire (Milinski, 2006; Penn, Potts, 1999). Quand le risque de consanguinité est grand, comme par exemple dans des petites populations isolées, les femelles

auront intérêt à mettre en place des mécanismes pour éviter les appariements avec les mâles apparentés. Au contraire, quand le risque de consanguinité est très faible, les appariements pourront être aléatoires ou en fonction de caractéristiques, autres que génétiques. Dans une population où l'adaptation locale est importante et les coûts liés à la consanguinité faibles, les études prédisent que la sélection de parentèle conduira à un choix de partenaire pour les individus génétiquement proches.

Dans notre étude chez le moineau domestique (**Manuscrit 8**), nous avons mis en évidence des signes de choix de partenaire pour les individus similaires. Plusieurs hypothèses peuvent être alors avancées. Bien que nous nous trouvions dans une population insulaire, supposée petite et isolée, les niveaux de variabilité génétique ne sont pas forcément plus faibles que ceux observés dans des populations continentales proches, chez la même espèce (**Manuscrit 7**). Le risque d'appariements consanguins est peut-être beaucoup plus faible que ce que nous aurions pu supposer *a priori*. Aussi, cette population insulaire est soumise à des conditions environnementales bien particulières (isolement, espace limité, conditions climatiques plus rigoureuses). Des phénomènes d'adaptation locale peuvent guider le choix du partenaire vers les apparentés, et augmenter ainsi l'aptitude phénotypique de la descendance. Pour finir, une forte structuration spatiale des individus de la population peut expliquer nos résultats. Les individus voisins sont plus facilement accessibles et disponibles pour la reproduction, mais ce sont aussi les plus apparentés (Cohen, Dearborn, 2004).

Le paradigme du choix de partenaire pour la dissimilarité et vers l'augmentation de la variabilité génétique de la descendance semble peu à peu être remis en question. Les études réalisées en populations naturelles mettent en évidence que le choix du partenaire peut passer d'un extrême à l'autre, en fonction des caractéristiques environnementales de la population, et en particulier du contexte parasitaire et des risques de consanguinité. Les gènes du CMH sont soumis à un choix de partenaire permettant d'augmenter la résistance aux parasites de la descendance, mais ils peuvent aussi être des indices, accessibles aux femelles, permettant de connaître la variabilité/similarité/compatibilité du génome des potentiels partenaires (Figure 16).

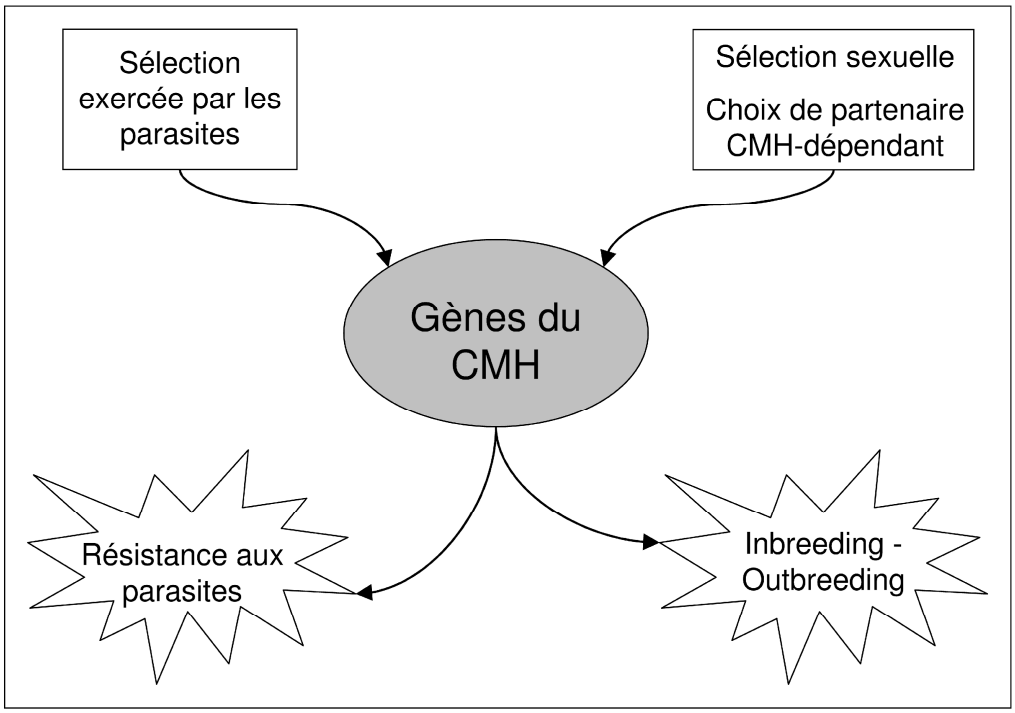


*Figure 16 : Les mécanismes de sélection liés au CMH. La sélection exercée par les parasites et la sélection sexuelle, à travers un choix de partenaire CMH-dépendant, sont deux mécanismes non-exclusifs permettant d'expliquer le polymorphisme de ces gènes. Un choix de partenaire CMH-dépendant pourra permettre d'augmenter la résistance aux parasites de la descendance et/ou d'évaluer la compatibilité/diversité du génome.*

*Pour résumer :*

| Manuscrit | Etude | Hypothèses | Résultats | Conclusion |
|---|---|---|---|---|
| 8 | Etude du choix de partenaire dans une population insulaire de moineaux domestiques. | Dans une population insulaire, petite et isolée, le risque d'appariements consanguins peut être élevé. Les femelles devraient choisir des mâles génétiquement différents. | Les poussins sont plus homozygotes qu'attendu pour un appariement aléatoire. Les couples se forment de façon aléatoire. Indices en faveur d'un choix post-copulatoire pour des individus similaires. | Le choix du partenaire dans cette population de moineaux domestiques tend plutôt en faveur d'un évitement de l'outbreeding ou d'une sélection de parentèle. Dans cette population insulaire, des pressions de sélection particulières peuvent conduire à une forte adaptation locale. |
| | | Le choix de partenaire pour les gènes du CMH devrait permettre d'augmenter la résistance aux parasites de la descendance et/ou d'éviter la consanguinité. | Pas de choix CMH-dépendant. Les résultats sont similaires à ceux obtenus avec les microsatellites. | |
| | | Les paternités hors couples devraient être relativement faibles dans cette population insulaire. | Plus de 45% des poussins sont issus de copulations hors couple. Les mâles choisis pour réaliser ces copulations sont encore plus similaires à la femelle que le mâle social. | Les femelles sont capables de compenser le fait de s'être appariées avec des individus trop différents en réalisant des copulations hors-couples avec des individus génétiquement plus proches. |

# Manuscrit 8

## Preference for genetically similar mates in an island population of house sparrows

Coraline Bichet, Dustin Penn, Yoshan Moodley, Luc Dunoyer, Elise Cellier-Holzem, Stéphane Garnier and Gabriele Sorci

En préparation

# Preference for genetically similar mates in an island population of house sparrows

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**Abstract:** It is often suggested that females should select mates that are genetically dissimilar to maximize offspring genetic diversity and avoid inbreeding. Yet, several recent studies find no such mating preferences, and in some instances, females seem to prefer to mate with genetically similar males. A preference for genetically similar mates may evolve if the fitness consequences of outbreeding are greater than those from inbreeding, or if females gain inclusive fitness benefits by mating with close kin. Here, we investigated mating patterns and offspring genetic diversity in an insular population of house sparrow (*Passer domesticus*), over a three-year period, using 12 microsatellite markers and one major histocompability complex (MHC) class I gene. We expected that females would show mating preferences for genetically dissimilar mates, especially given the small population size, the distance from the mainland and the presumed reduced gene flow. Contrary to our expectation, however, we found that offspring were less genetically diverse (multi-locus heterozygosity) than expected under a random mating, suggesting that females in this population show preferences for genetically similar males. We found high levels of extra-pair offspring, and offspring sired by extra-pair males had a better fledging success than those sired by the social male. Again, unexpectedly, females were more closely related to the extra-pair male than with the social mate. Our results did not depend on the type of marker used, since microsatellites and MHC genes provided similar results and we found little evidence for MHC-dependent mating patterns.

**Keywords**
Mate choice, *Passer domesticus*, Major Histocompatibility Complex, microsatellites, extra-pair paternity.

## Introduction

Mate choice remains an important research topic in behavioural ecology, as there appear to be several possible indirect, genetic benefits (Andersson, 1994; Clutton-Brock, 2007; Kempenaers, 2007). For example, it is often suggested that females should prefer heterozygous or genetically dissimilar males to produce more genetically diverse progeny or avoid inbreeding (Brown, 1997; Charlesworth and Willis, 2009; Ferretti et al., 2011; Mays and Hill, 2004; Penn, 2002; Richardson et al., 2005; Tregenza and Wedell, 2000). The rapid development of genetic tools during the last decade has considerably promoted the study of the genetic benefits of mate choice and, in certain cases, allowed disentangling the different mechanisms underlying it. Previous studies have both used heterozygosity at neutral loci (microsatellites) and diversity of genes under selection to infer genome-wide diversity and relatedness (Andersson, 1994; Clutton-Brock, 2007; Kempenaers, 2007; Mays et al., 2008; Neff and Pitcher, 2005). Highly polymorphic genes, such as those of the major histocompatibility complex (MHC), have been suggested as ideal candidates as relatedness-markers (Piertney and Oliver, 2006). MHC genes also control immune self/non-self and heterozygosity can sometimes enhance disease resistance (Penn et al., 2002). Therefore, MHC-dependent mating preferences could also confer the benefit of increased parasite protection for offspring (Apanius et al., 1997; Landry et al., 2001; Milinski, 2006; Penn and Potts, 1999; Piertney and Oliver, 2006; Reusch et al., 2001).

Yet, several recent studies have surprisingly reported evidence for mating preferences for genetically related reproductive partners (Cohen and Dearborn, 2004; Krokene and Lifjeld, 2000; Wang and Lu, 2011). One possible function of mating with kin is reducing outbreeding depression (Frankham et al., 2011). If local environmental conditions select for co-adapted ensembles of genes, mating with genetically distant partners could disrupt these assemblages and result in a loss of fitness (Shields, 1993; Waller, 1993). Pioneering work conducted by Bateson (1978, 1983) suggested that maximal reproductive success can be achieved by pairs with intermediate level of genetic relatedness though there is mixed evidence for this 'optimal outbreeding' hypothesis (Dolgin et al., 2007; Ryder et al., 2010; Sherman et al., 2008). Preference for mates with intermediate or even high relatedness could arise as a consequence of kin selection (Hamilton, 1964a; Hamilton, 1964b) since relatives share genes that are identical by descent, thereby allowing females to increase their own inclusive fitness (Kokko and Ots, 2006; Oh, 2011; Waser et al., 1986), assuming no inbreeding depression (Bateson, 1978; Lehmann and Perrin, 2003; Parker, 2006).

We studied mating patterns by measuring offspring genetic diversity in house sparrows (*Passer domesticus*), using microsatellite loci and MHC class I genes over three consecutive years. This indirect assessment only allowed us to infer realized mate choice, whereas assessing female preference would have required letting females choose a partner in the absence of constraints (Wagner, 1998). Given the small population size and the isolated nature of the studied population, we expected that females should preferentially mate with diverse and genetically dissimilar males as to i) reduce the risk of inbreeding; ii) enhance genetic diversity of the progeny. Also, to obtain insights into female mating preferences, we also examined offspring from extra-pair mates. Although the vast majority of bird species are socially monogamous, a substantial fraction of females also engage in extra-pair copulations with the result that broods are usually composed of chicks sired by different fathers (Cockburn, 2006; Griffith et al., 2002; Lack, 1968; Moller and Ninni, 1998). Extra-pair mating presents the opportunity of examining the processes governing mate choice in the absence of any potential direct benefit since the extra-pair male does not contribute to parental care. According to the inbreeding avoidance hypothesis, females mated with closely related males should engage in extra-pair copulations with genetically dissimilar mates (Kempenaers, 2007), whereas the outbreeding avoidance and the kin selection hypotheses predict that females should engage in extra-pair copulations with genetically similar males (Ferretti et al., 2011; Kleven et al., 2005).

## Material and methods

*The study population*

The house sparrow population studied here is located in Hoëdic, a small (2.08 km²) island off the French coast of Brittany (47°20'24.40"N-2°52'43.09"W). Adult house sparrows were captured using mist nets and banded with a metal ring and a unique combination of colour rings which allowed individual recognition. At the first capture, we took a small amount of blood (20 µl) by brachial vein puncture and stored it in 500 µl of Queen's Lysis Buffer (QLB) (Seutin et al., 1991). In 2009, 2010 and 2011 we monitored pairs breeding in nest boxes that were set up in the village. For logistic reasons, we were only able to monitor the first two broods during each year, even though house sparrows can lay up to 3-4 clutches per breeding season. Between late April and the end of June, we visited nest boxes at least twice per week and recorded clutch size and number of hatched and fledged chicks. When chicks were 8 days old, they were banded with a metal ring and a drop of blood was collected and stored as for adults. Parental identity was assessed during focal observations when they were brooding or feeding the chicks. Sample sizes are summarized in the Table 1.

*Microsatellite genotyping*

DNA was extracted using the Wizard® SV 96 Genomic DNA Purification kit (Promega) according to the manufacturer's instructions. All individuals were genotyped using the following twelve microsatellite loci: PdomD09, PdomA08, PdomB01 (Garnier et al., 2009), Mjg1 (Li et al., 1997), Ase18 (Richardson et al., 2000), Pdo3, Pdo5 (Griffith et al., 1999), Pdo1 (Neumann and Wetton, 1996), Pdo10 (Griffith et al., 2007), Pdo16 and Pdo27 (Dawson et al., 2010). Polymerase chain reactions (PCRs) were performed in a

final volume of 10 µl including 10 to 50 ng of DNA, 2 µl of 5X buffer, 1.5 to 2 mM of $MgCl_2$, 400 µl of dNTPs, 1 µM of each primers and 0,2 U of *Taq* DNA polymerase (Promega). The PCR program comprised: 94°C 3 to 4min, 30 to 35 cycles of 94°C 20s, 20s for annealing (48°C to 62°C according to the different loci), and 72°C 30 to 40s, followed by a final extension of 72°C 5 to 7min. Samples were then run in an ABI3730 automated sequencer. Allele sizes were determinate using GeneMapper v4.0.

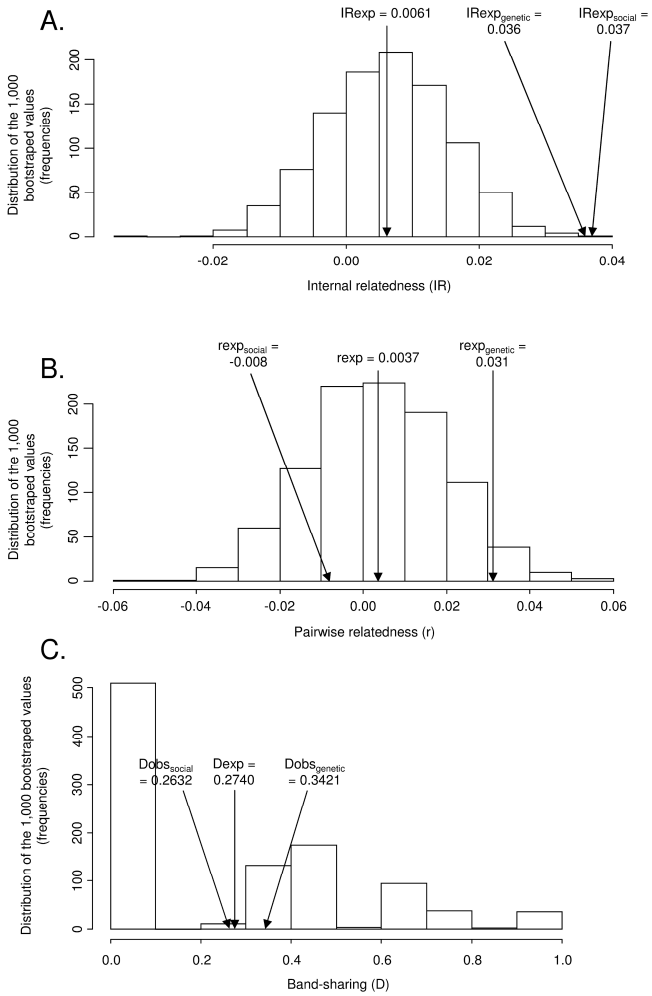


Figure 1. Distribution of the 1,000 bootstraped IR (A.), r (B.) and MHC allele-sharing (C.) values for pairs breeding in the nest boxes. IRobs$_{social}$, robs$_{social}$, Dobs$_{social}$ correspond to the mean of the observed IR, r and allele-sharing between the female and its social mate, respectively. IRobs$_{genetic}$, robs$_{genetic}$, Dobs$_{genetic}$ corresponds to the mean of the observed IR, r and allele-sharing between the female and the extra-pair mate, respectively. IRexp, rexp and Dexp correspond respectively to the mean of the expected IR, r and allele-sharing.

*MHC class I genotyping*

We amplified the MHC class I exon 3, which corresponds to the peptide-binding region (PBR) (Bonneaud et al., 2004). PCR amplifications were performed using a fluorescent (6'FAM) labelled primer (A23M – GCG CTC CAG CTC CTT CTG CCC ATA) and an unlabeled primer (A21M – GTA CAG CGC CTT GTT GGC TGT GA). PCRs were performed in a final volume of 10 µl, including 50 to 100 ng of genomic DNA, 0.6 µM of each primer and 5 µl of Multiplex PCR reagent (QIAGEN GmbH) containing hot-start DNA polymerase, buffer and dNTPs. The PCR program began with 5min initial denaturation at 95°C, followed by 35 cycles of 30s denaturation at 94°C, 90s annealing at 56°C and 90s extension at 72°C. A final elongation step was run for 10min at 72°C. To control for PCR artefacts, we used 2 negative controls, for PCR and for sequencer, by adding purified water instead of DNA or PCR products. MHC diversity was screened using capillary electrophoresis single conformation polymorphism (CE-SSCP) (Griggio et al., 2011). PCR samples were prepared for

electrophoresis by combining 1 µl PCR product with 8.75 µl Hi-Di formamide and 0.25 µl of in-house prepared ROX size standard (DeWoody et al., 2004). This mix was heated for 5min at 95°C to separate the complementary DNA strands. Electrophoresis was conducted in an automated DNA sequencer (ABI PRISM 3130 xl automated DNA Sequencer, Applied Biosystems). The retention time of allelic variants was assessed relative to the ROX size standard. GeneMapper v4.0 software was used to analyse the SSCP data.

Table 1. Total number of individuals, breeding pairs, and individuals genotyped.

| Year | Number of individuals | Number of breeding pairs with known identity | Number of microsatellite genotyped individuals (number of chicks) | Number of MHC genotyped individuals (number of chicks) |
|---|---|---|---|---|
| 2009 | 225 | 15 | 222 (51) | 190 (51) |
| 2010 | 341 | 40 | 335 (82) | 294 (82) |
| 2011 | 316 | 49 | 311 (89) | 275 (89) |
| Total* | 574 | 96 | 565 (222) | 494 (222) |

* Total of unique individuals

*Statistical analyses*

*Mate choice*

Genome-wide inbreeding was assessed with individual multi-locus heterozygosity (He, (Chapman et al., 2009) and Internal Relatedness (IR, (Amos et al., 2001). Multi-locus heterozygosity corresponds to the number of heterozygous loci divided by the number of genotyped loci, while IR corresponds to the number of homozygous loci, divided by the number of genotyped loci, weighed by the allele frequencies. To measure the genetic proximity between paired males and females, we also computed unbiased pairwise relatedness (r, (Li et al., 1993), where each locus is weighed using the method described in (Lynch and Ritland, 1999) and (Van de Casteele et al., 2001).

Observed values of IR and r were compared to expected distributions under the assumption of random mate-choice. Expected values were generated using the software STORM (Frasier, 2008) by randomly sampling (1000 iterations) reproductive males and females in order to generate the same number of chicks and mating pairs to the observed ones. Observed values were then compared to the distribution of the simulated values.

Allele-sharing was calculated to estimate MHC similarity between males and females forming a pair-bond. Allele-sharing is twice the number of shared alleles divided by the number of different alleles of each individual [$D=2F_{ab}/(F_a+F_b)$] (Bonneaud et al., 2006; Wetton et al., 1987). The observed mean allele-sharing (D) was compared with the expected allele-sharing obtained by randomly generating mating pairs (1000 bootstraps) in R version 2.15.0 (R Development Core Team 2011). We used two different sets of individuals to generate the distribution of expected allele-sharing values. The first set involved the individuals known to have bred in the nest boxes, the second set involved all the adults sampled in the population (irrespective of whether they bred in the nest boxes or not).

*Paternity*

In each nest, parents were identified during the brooding and chick feeding period. To assess extra-pair paternity, we used the likelihood-based approach implemented in the software CERVUS 3.0. (Kalinowski et al., 2007). The software allows excluding and assigning putative fathers based on their multi locus genotypes. The probability of exclusion and assignment was fixed to 95%. We also tested if there was any mis-match between the maternal identity based on the field observations and the one based on the microsatellite. Maternal mis-matches would indicate that brood parasitism had occurred. Over the entire study period, in only two instances we found a mis-match between the maternal identity based on the field observations and the genetic markers. However, these mis-matches involved complete clutches which likely reflect errors in the reading of the color bands rather than brood parasitism. Accordingly these two records were excluded from the statistical analyses.

*Hypothesis testing*

We used General Linear Mixed Models (GLMMs) to test the idea that females mated with more closely related males would also engage in extra-pair fertilization. Brood type was modeled as a binary response variable

(with or without extra-pair chicks). The explanatory (fixed) variables were male He, male IR, male relatedness to the female (r), band-sharing within a male-female pair, and year. Given that He and IR were highly correlated ($rs_{60}$=-0.96, p<0.0001), they were entered in different sets of models to avoid co-linearity. Female identity nested within year was declared as a random factor, because some females laid several clutches during the three-year period covered by the study.

Table 2. A. GLMM exploring the effects of He, IR, r, and D of the social male on the female likelihood to engage in extra-pair fertilizations. B. Relative variable importance given by Akaike weights (ΣAIC$\omega$). He and IR were not included in the same model because they were highly correlated (see text). K = number of parameters.

A.

| Variables | Model | K | AICc | ΔAICc | $\omega$ |
|---|---|---|---|---|---|
| Brood type | | | | | |
| (broods with extra-pair chicks vs. broods with no extra-pair chicks) | Null | 1 | 84.0 | 0.00 | 0.296 |
| ($n$ = 70) | D | 2 | 84.8 | 0.80 | 0.197 |
| | r | 2 | 86.1 | 2.10 | 0.105 |
| | He | 2 | 86.2 | 2.20 | 0.101 |
| | D + r | 3 | 86.8 | 2.80 | 0.075 |
| | He + D | 3 | 87.1 | 3.10 | 0.062 |
| | year | 2 | 88.1 | 4.10 | 0.039 |
| | He + r | 3 | 88.3 | 4.30 | 0.035 |
| | He + D + r | 4 | 89.1 | 5.10 | 0.023 |
| | D + year | 3 | 89.2 | 5.20 | 0.022 |
| | r + year | 3 | 90.2 | 6.20 | 0.013 |
| | He + year | 3 | 90.4 | 6.40 | 0.012 |
| | D + r + year | 4 | 91.3 | 7.30 | 0.008 |
| | He + D + year | 4 | 91.7 | 7.70 | 0.006 |
| | He + r + year | 4 | 92.6 | 8.60 | 0.004 |
| | He + D + r + year | 5 | 93.9 | 9.90 | 0.002 |
| Brood type | | | | | |
| (broods with extra-pair chicks vs. broods with no extra-pair chicks) | Null | 1 | 84.0 | 0.00 | 0.297 |
| ($n$ = 70) | D | 2 | 84.8 | 0.80 | 0.197 |
| | r | 2 | 86.1 | 2.10 | 0.106 |
| | IR | 2 | 86.2 | 2.20 | 0.099 |
| | D + r | 3 | 86.8 | 2.80 | 0.075 |
| | IR + D | 3 | 87.1 | 3.10 | 0.063 |
| | year | 2 | 88.1 | 4.10 | 0.039 |
| | IR + r | 3 | 88.3 | 4.30 | 0.034 |
| | IR + D + r | 4 | 89.1 | 5.10 | 0.023 |
| | D + year | 3 | 89.2 | 5.20 | 0.022 |
| | r + year | 3 | 90.2 | 6.20 | 0.013 |
| | IR + year | 3 | 90.4 | 6.40 | 0.012 |
| | D +Sr + year | 4 | 91.3 | 7.30 | 0.008 |
| | IR + D + year | 4 | 91.7 | 7.70 | 0.006 |
| | IR + r + year | 4 | 92.7 | 8.70 | 0.004 |
| | IR + D + r + year | 5 | 93.9 | 9.90 | 0.002 |

B.

| Variables | Source of variation | ΣAIC$\omega$ |
|---|---|---|
| Brood type | | |
| (broods with extra-pair chicks vs. broods with no extra-pair chicks) | D | 0.39 |
| | r | 0.27 |
| | He | 0.25 |
| | year | 0.11 |
| Brood type | | |
| (broods with extra-pair chicks vs. broods with no extra-pair chicks) | D | 0.4 |
| | r | 0.26 |
| | IR | 0.24 |
| | year | 0.11 |

We also looked at the fledging success and hatching success of chicks and eggs sired by the social male compared to extra-pair males. Here, fledging success was entered as a binomial response variable, year was also included as a fixed factor and female identity was nested within year as a random factor.

Genetic diversity (He) of chicks produced by social and extra-pair males was fitted with a binomial distribution and compared to field data using a GLMM. He, year, chick type (sired by the social or the extra-pair male), and their interactions were added as fixed factors. Female identity was nested within year and entered as a random factor.

Table 3. A. GLMM exploring the effects of brood type (broods with extra-pair chicks vs. broods with no extra-pair chicks) on hatching and fledging success. B. Relative variable importance given by Akaike weights (ΣAIC$\omega$). K = number of parameters.

A.

| Variables | Model | K | AICc | ΔAICc | $\omega$ |
|---|---|---|---|---|---|
| Hatching success ($n$ = 61) | Null | 1 | 69.6 | 0.00 | 0.440 |
| | brood type | 2 | 69.9 | 0.30 | 0.379 |
| | year | 2 | 72.8 | 3.20 | 0.087 |
| | year + brood type | 3 | 73.1 | 3.50 | 0.075 |
| | year + brood type + year*brood type | 4 | 75.8 | 6.20 | 0.019 |
| Fledging success ($n$ = 53) | year | 2 | 92.8 | 0.00 | 0.565 |
| | year + brood type | 3 | 94.0 | 1.20 | 0.317 |
| | Null | 1 | 97.3 | 4.50 | 0.060 |
| | brood type | 2 | 98.5 | 5.70 | 0.034 |
| | year + brood type + year*brood type | 4 | 99.2 | 6.40 | 0.023 |

B.

| Variables | Source of variation | ΣAIC$\omega$ |
|---|---|---|
| Hatching success | brood type | 0.47 |
| | year | 0.18 |
| | year*brood type | 0.02 |
| Fledging success | year | 0.91 |
| | brood type | 0.37 |
| | year*brood type | 0.02 |

We also compared the genetic dissimilarity between mates for the restricted sample of females that engaged in extra-pair copulations. We constructed a GLMM where male status (social or extra-pair) was entered as a binomial response variable. Again, due to co-linearity, male multi-locus heterozygosity (He) and male internal-relatedness (IR) were entered in different models. Male He (or male IR), D, r and year were included as fixed factors. Breeding event nested within female and within year was also declared as a random factor. When a female mated with several extra-pair mates during a single reproductive event, we computed the mean IR, He, r and D and used these values in the statistical models.

We used a similar GLMM to test if chicks sired by the social or the extra-pair male(s) differed in their fledging success. Fledging success corresponds to the number of fledged chicks sired by a given male, divided by the total number of eggs laid and this was modeled as a binomial response variable. Male type (social or extra-pair), year and their interaction were added as fixed factors. Breeding event, nested within female identity and year was added as a random factor.

We used the package lme4 (Bates et al., 2011), implemented in R 2.15.0 to run all GLMMs. Rather than traditional null hypothesis testing, we used the information-theoretic (IT) approach as it has recently been suggested to be more appropriate for observational studies (Burnham and Anderson, 2002). We used the Akaike Information Criterion (AIC) and ΔAIC to infer support for models in the candidate set (Bolker et al., 2009). We calculated the Akaike weights ($\omega$) which is the probability that a model is selected as the best in a model set (Burnham and Anderson, 2002). Using the package MuMIn (Barton, 2012), we also calculated the summed AIC weight (ΣAIC$\omega$) for each variable. This corresponds to the sum of the weights of the models in which the variable is present and can be interpreted as the probability that a given variable is retained in the selected model. We deemed that there was essentially no evidence in support to a model when its ΔAIC value was greater than 10 (Burnham and Anderson, 2002). It is also worth noting that when a fitted parameter was added to a model, a penalty of 2 is added to the model's AIC value (Burnham and Anderson, 2002). Thus, we considered that a variable

improved the fit of the model only when the ΔAIC was lower than 2.

## Results

*Mate choice*

Individuals had a mean of 14.67 (±0.97 SE) microsatellite alleles and a mean of 2.31 (±0.05 SE) MHC class I alleles. The individual MHC allele number varied between 1 and 6 and a total of 37 MHC alleles where found in the entire population.

The observed IR mean was 0.037 (±0.0096 SE) and was significantly higher than expected under a random mate choice ($p < 0.001$) (Figure 1A). Pairwise relatedness (r) mean was -0.008 (±0.016 SE) and did not differ from expected values under a random mate choice (Figure 1B). Pair allele-sharing (D) mean was 0.263 (±0.024 SE) and did not differ from expected values under a random mate choice (Figure 1C), whatever the sample used to compute them (pairs breeding in the nest boxes or the whole population).

Table 4. GLMM exploring the effects of chick type (sired by the social or extra-pair mate) on chick multi-locus heterozygosity. B. Relative variable importance given by Akaike weights (ΣAIC$\omega$). K = number of parameters.

A.

| Variables | Model | K | AICc | ΔAICc | $\omega$ |
|---|---|---|---|---|---|
| He | Null | 1 | 236.5 | 0.00 | 0.394 |
| ($n$ = 222) | year | 2 | 237.7 | 1.20 | 0.222 |
| | chick type | 2 | 238.4 | 1.90 | 0.153 |
| | year + chick type + year*chick type | 4 | 238.5 | 2.00 | 0.143 |
| | year + chick type | 3 | 239.5 | 3.00 | 0.089 |

B.

| Variables | Source of variation | ΣAIC$\omega$ |
|---|---|---|
| He | year | 0.45 |
| | chick type | 0.38 |
| | year*chick type | 0.14 |

*Paternity*

In 2009, 2010 and 2011, respectively 41.2, 43.9 and 50.6 % of chicks were extra-pair fathered. Similarly, there were 71.4, 57.7 and 72.4 % of broods with at least one extra-pair offspring, respectively. Given the relatively high proportion of extra-pair chicks, we compared the observed IR, r and D based on actual paternity (social or extra-pair) to the expected values under random choice. We found that the observed IR mean was 0.036 (±0.0095 SE) and was significantly higher than expected under a random mate choice ($p = 0.003$) (figure 1A). Pairwise relatedness (r) mean was 0.031 (±0.019 SE) and did not differ from expected values under a random mate choice (figure 1B). Pair allele-sharing (D) mean was 0.342 (±0.032 SE) and did not differ from expected values under a random mate choice (figure 1C), whatever the sample used.

None of the variables describing genome-wide inbreeding of the social mate affected the female likelihood to engage in extra-pair fertilizations, since the model with the highest AIC value was the null model (Table 2). Similarly, none of the variables improved the fit of the model for hatching rate (the null model had the highest AIC (69.6) and $\omega$ (0.44), Table 3). However, fledging rate did vary among years (0.89 in 2009, 0.75 in 2010, and 0.59 in 2011) and the model including year had the highest AIC (92.8) and $\omega$ (0.565) values (Table 3). The null model was the one that better accounted for chick heterozygosity (AIC = 36.5, $\omega$ = 0.394, Table 4).

We then focused on broods that contained both within and extra-pair chicks. This allowed us to compare the genetic similarity between social and extra-pair mates. When male He was included as fixed factor, the null model was the best model (AIC = 104, $\omega$ = 0.176) (Table 5). When IR was included as fixed factor, the best model was the model included r (AIC = 100.9, $\omega$ = 0.198) (Table 5). The ΔAICs and ΣAICω of the other competitive models suggest a possible role for MHC band sharing and pairwise relatedness (Table 5). The mean pairwise relatedness was 0.068 (±0.030 SE) between females and extra-pair mates whereas it was 0.003 (±0.029 SE) between females and social mates. For the genetic pairs, MHC allele-sharing was 0.34 (±0.050 SE) between females and extra-pair mates, and 0.25 (±0.046 SE) between females and social mates.

Fledging success of chicks sired by extra-pair males (0.43 ±0.038 SE) was higher than for chicks sired by the social mate (0.23 ±0.030 SE). The model including sire type (social vs. extra-pair) had the highest AIC

(AIC = 112.9, $\omega$ = 0.799, Table 6), and sire type had a very high ΣAICω (0.99).

## Discussion

Contrary to our expectations, we found evidence suggesting a preference for genetically similar mates in an insular population of house sparrows. Over the three years covered by the study, we found that offspring were less genetically diverse than expected under random mate choice (based on 12 microsatellite loci), whereas there was little support for an involvement of MHC class I genes in the process of pair formation. This pattern was consistent even when taking into account the relatively high proportion of extra-pair fertilizations. When focussing on broods containing both within and extra-pair chicks, we also found that extra-pair mates were genetically more similar to the females than social mates both for microsatellite markers and MHC genes. Interestingly, fledging success of chicks sired by extra-pair males was higher than for chicks sired by social mates. This finding suggests that offspring from extra-pair males had greater fitness than those sired from the social mate. Thus, our results do not provide support for the "inbreeding avoidance hypothesis", despite the particular demographic and ecological characteristics of the studied population should have promoted the preference for dissimilar mates as to avoid the depletion of genetic diversity and inbreeding (Charlesworth and Willis, 2009; Cohen and Dearborn, 2004; Ferretti et al., 2011; Frankham, 1997; Krokene and Lifjeld, 2000; Mays and Hill, 2004; Richardson et al., 2005; Tregenza and Wedell, 2000; Wang and Lu, 2011).

The main assumption that guided our prediction for a preference of dissimilar mates is that the studied population has a depleted genetic variance and is potentially vulnerable to inbreeding. As a part of a larger study on the genetic structuring of house sparrow populations, we found that the within-population diversity of both microsatellite and MHC was similar between Hoëdic and six mainland populations located within a radius of 200 km (Bichet et al., in prep). Gene flow is therefore higher than previously assumed, which decreases the risk of inbreeding and weakens the strength of selection against mating with close relatives.

Our results are more in agreement with the outbreeding avoidance hypothesis, since females seem to prefer genetically similar males both as social and extra-pair mates. This may be because spatially variable environmental conditions (such as variable risks to contract infectious diseases) have promoted the evolution of co-adapted genes that confer benefits under conditions that are locally predominant on Hoëdic. On the other hand, preference for genetically similar mates might also evolve through kin selection where females seek to increase their own inclusive fitness by breeding with genetically related males (Kokko and Ots, 2006; Waser et al., 1986). Because we only indirectly assessed realized mate choice and not female preference, our results could also arise as the consequences of environmental constraints (Wagner, 1998). For example, related individuals might tend to cluster in the same flock and to occupy nearby nest boxes, enhancing the chance of mating with genetically similar mates. An analogous phenomenon has been reported for lekking peacocks (*Pavo cristatus*), where genetically related males tend to cluster in the same lek (Petri et al. 1999).

Another argument supporting the view that ecological constraints can limit a free expression of female preference comes from the finding of high rate of extra-pair paternity. The proportion of extra-pair chicks reported here is higher than those previously reported for other house sparrow populations (Cordero et al., 1999; Griffith et al., 1999; Stewart et al., 2006; Veiga and Boto, 2000; Whitekiller et al., 2000). We found that 45.2 % of chicks were sired by extra-pair males and 67.2 % of broods contained at least one extra-pair chick. These extra-pair matings tended to favour genetically similar mates. One possible bias in the estimates of extra-pair matings could come from errors in the reading of colour bands. However, we believe that this only marginally affected the reported values since in only 2.9% (2/69) of broods we observed a mis-match of the maternal identity likely to reflect reading mistakes (and in both cases the entire brood was assigned to a single mother). For five broods, none of the chicks was assigned to the social father, possibly indicating errors in the reading of the color bandings; however, in all of these five broods, chicks were assigned to at least two different males. This therefore strongly suggests that the high proportion of extra-pair chicks was not the result of mistakes in the identification of the social father. Even

though it is generally thought that the occurrence of extra-pair matings is low in insular populations (Griffith, 2000; Griffith et al., 1999; Krokene and Lifjeld, 2000), some exceptions to this rule have been reported (Charmantier and Blondel, 2003; Conrad et al., 2001; Fridolfsson et al., 1997). A few tentative explanations have been provided for these results, even though none appears to be conclusive.

Table 5. A. GLMM exploring the effects of He, IR, r, and D of the male on the likelihood of being a social or an extra-pair male. B. Relative variable importance given by Akaike weights (ΣAIC$\omega$). He and IR were not included in the same model because they were highly correlated (see text). K = number of parameters.

A.

| Variables | Model | K | AICc | ΔAICc | $\omega$ |
|---|---|---|---|---|---|
| Male type (social vs. extra-pair) | Null | 1 | 104.0 | 0.00 | 0.176 |
| ($n$ = 72) | D | 2 | 104.2 | 0.20 | 0.161 |
| | r | 2 | 104.3 | 0.30 | 0.152 |
| | MHe | 2 | 104.7 | 0.70 | 0.122 |
| | D + r | 3 | 105.2 | 1.20 | 0.096 |
| | MHe + D | 3 | 105.6 | 1.60 | 0.079 |
| | MHe + r | 3 | 105.6 | 1.60 | 0.078 |
| | MHe + D + r | 4 | 107.0 | 3.00 | 0.040 |
| | year | 2 | 108.4 | 4.40 | 0.019 |
| | D + year | 3 | 108.6 | 4.60 | 0.018 |
| | r + year | 3 | 108.8 | 4.80 | 0.016 |
| | MHe | 3 | 109.2 | 5.20 | 0.013 |
| | D + r + year | 4 | 109.7 | 5.70 | 0.010 |
| | MHe + D + year | 4 | 110.0 | 6.00 | 0.009 |
| | MHe + r + year | 4 | 110.1 | 6.10 | 0.008 |
| | MHe + D + r + year | 5 | 111.5 | 7.50 | 0.004 |
| Male type (social vs. extra-pair) | r | 2 | 100.9 | 0.00 | 0.198 |
| ($n$ = 70) | Null | 1 | 101.2 | 0.30 | 0.170 |
| | D | 2 | 101.3 | 0.40 | 0.160 |
| | D + r | 3 | 102.0 | 1.10 | 0.118 |
| | MIR | 2 | 102.7 | 1.80 | 0.081 |
| | MIR + r | 3 | 102.8 | 1.90 | 0.075 |
| | MIR + D | 3 | 103.2 | 2.30 | 0.062 |
| | MIR + D + r | 4 | 104.1 | 3.20 | 0.040 |
| | r + year | 3 | 105.4 | 4.50 | 0.021 |
| | year | 2 | 105.7 | 4.80 | 0.018 |
| | D + year | 3 | 105.8 | 4.90 | 0.017 |
| | D + r + year | 4 | 106.4 | 5.50 | 0.013 |
| | MIR + year | 3 | 107.3 | 6.40 | 0.008 |
| | MIR + r + year | 4 | 107.5 | 6.60 | 0.007 |
| | MIR + D + year | 4 | 107.8 | 6.90 | 0.006 |
| | MIR + D + r + year | 5 | 108.7 | 7.80 | 0.004 |

B.

| Variables | Source of variation | ΣAIC$\omega$ |
|---|---|---|
| Male type (social vs. extra-pair) | D | 0.42 |
| | r | 0.40 |
| | He | 0.35 |
| | year | 0.10 |
| Male type (social vs. extra-pair) | r | 0.48 |
| | D | 0.42 |
| | IR | 0.28 |
| | year | 0.10 |

Our finding that females engaging in extra-pair matings showed a preference for more genetically similar males is in agreement with the results of two recent studies in two other birds species, the ground tit (*Parus humilis*) (Wang and Lu, 2011) and the barn swallow (*Hirundo rustica*) (Kleven et al., 2005). Wand and Lu (2011) investigated the pattern of extra-pair paternity in the ground tit. Even though the propensity of females to engage in extra-pair matings did not depend on the relatedness with the social mates, they seeked extra-pair copulations with males with whom they were more related than to their social mates. Since there was no cost due to mating with relatives, Wang and Lu (2011) suggested that these

results support the kin selection hypothesis where females may gain inclusive fitness by mating with related males.

There have been extensive reports on the role plaid by the MHC on the process of mate-choice (Egid and Brown, 1989; Ekblom et al., 2004; Freeman-Gallant et al., 2003; Olsson et al., 2003; Potts et al., 1991; Reusch et al., 2001; Richardson et al., 2005; von Schantz et al., 1997; Westerdahl, 2004). Because MHC molecules are involved in the process of antigen presentation, MHC genes have been often assumed as being the good genes females might be looking for when choosing a mate. Alternatively, MHC could be a general marker of relatedness and MHC-based mate choice might rather reflect a way to discriminate between closely related individuals. We found no support for a potential role of MHC genes in the process of mate choice other than the general assessment of relatedness. Of course, insular populations might be less exposed to parasites and pathogens (Lenaghan et al., 2006; Maitland et al., 2000; Moro et al., 2003; Nieberding et al., 2006) which could weaken the selection for specific MHC alleles and diversity. Further work should be devoted to the role of MHC-based mate choice in populations differing in their exposure to infectious diseases such as mainland and insular populations.

Table 6. A. GLMM exploring the effects of sire type (social vs. extra-pair) on fledging success. Fledging success corresponds to the number of chicks sired by a male, divided by the total number of eggs laid in the clucth. B. Relative variable importance given by Akaike weights (ΣAICω). K = number of parameters.

A.

| Variables | Model | K | AICc | ΔAICc | $\omega$ |
|---|---|---|---|---|---|
| Fledging success ($n$ = 74) | male type | 2 | 112.9 | 0.00 | 0.799 |
| | male type + year | 3 | 116.2 | 3.30 | 0.152 |
| | male type + year + year*sire type | 4 | 118.9 | 6.00 | 0.039 |
| | Null | 1 | 122.0 | 9.10 | 0.009 |
| | year | 2 | 125.2 | 12.30 | 0.002 |

B.

| Variables | Source of variation | Σ AIC$\omega$ |
|---|---|---|
| Fledging success | male type | 0.99 |
| | year | 0.19 |
| | year*male type | 0.04 |


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# CONCLUSIONS ET PERSPECTIVES

*Au cours de cette thèse, nous avons essayé d'avoir une approche intégrative en étudiant l'interaction hôte-parasite de la malaria aviaire à plusieurs niveaux. Les interactions hôtes-parasites sont ainsi nommées car hôtes et parasites exercent l'un sur l'autre des pressions de sélection importantes.*

Dans un premier temps, une approche expérimentale en laboratoire a permis de mettre en évidence les effets de la réponse immunitaire et d'autres caractéristiques de l'hôte (statut social, statut nutritionnel) sur l'interaction oiseau-malaria (**Manuscrits 1, 2 et 3**). Si la réponse immunitaire permet aux individus de se défendre contre la malaria, nous avons démontré qu'elle peut aussi engendrer des coûts, ou immunopathologie. L'existence de tels coûts permet en partie d'expliquer l'importante variabilité de la réponse immunitaire que l'on peut observer dans les populations hôtes.

En populations naturelles, malgré tous les facteurs confondants liés à ce type d'études, nous avons pu mettre également en évidence que des caractéristiques des hôtes, telles que l'âge ou le sexe, pouvaient avoir un effet sur l'interaction hôte-parasite, et plus précisément sur la parasitémie, mesure facilement associable à la virulence (**Manuscrit 4**).

Dans un deuxième temps, nous nous sommes intéressés, en populations naturelles, à un troisième acteur, l'environnement, qui peut intervenir et bouleverser complètement les résultats qui auraient pu être obtenus en laboratoire. L'environnement et ses caractéristiques ont un effet sur l'hôte, sur le parasite et dans notre modèle d'étude également sur le vecteur. Il est par exemple assez intuitif de supposer que la température peut affecter drastiquement le développement du vecteur ectotherme, ainsi que la survie ou la rapidité de multiplication du parasite. Une étude nous a permis de montrer que les variations journalières de température pouvaient expliquer en grande partie les prévalences observées en France (**Manuscrit 5**). Par contre, les parasitémies n'ont pu être corrélées avec aucune des variables bioclimatiques étudiées. Dans un contexte de changements climatiques et d'anthropisation de l'environnement, ces études se révèlent d'une importance capitale pour prédire l'émergence de maladies infectieuses et pour étudier l'apparition et l'évolution de ces parasites dans des populations naïves. L'anthropisation de l'environnement est une menace de plus en plus globale et brutale pour les populations naturelles. Il est donc fort probable que ces changements rapides influencent les interactions hôtes-parasites.

Nous avons également montré, en population naturelle, que la pollution au plomb était corrélée à la prévalence dans les populations (**Manuscrit 6**). Les individus infectés par la

malaria aviaire étaient aussi les plus contaminés au plomb. Cette étude a permis d'illustrer la complexité des facteurs intervenant dans les interactions hôtes-parasites, puisque l'effet des autres métaux lourds étudiés semble varier selon la population hôte étudiée.

Nos études, expérimentales en laboratoire ou en populations naturelles, ont pu donc mettre en évidence un aspect majeur concernant la malaria aviaire : le statut infectieux d'un individu semble en grande partie déterminé par les caractéristiques de l'environnement (température, pollution), tandis que la parasitémie est fortement dépendante des caractéristiques de l'hôte (âge, sexe, capacités immunitaires, statuts nutritionnel et social).

Dans un troisième temps, nous nous sommes focalisés sur l'isolement des populations hôtes qui a un effet bien connu sur la variabilité génétique, et donc sur la capacité de survie à long terme de ces populations. Les petites populations isolées ont une variabilité génétique faible par rapport aux grandes populations interconnectées. Cependant, la variabilité génétique sélectionnée peut être maintenue sous l'action d'agents de sélection, et parmi eux les parasites sanguins. Les parasites peuvent exercer des pressions de sélection capables de maintenir l'important polymorphisme des gènes du CMH dans les populations naturelles. Notre étude a permis de montrer que les populations de moineaux domestiques étaient affectées par les caractéristiques géographiques (îles ou continent) en termes de variabilité et de différenciation génétique (**Manuscrit 7**). Au niveau des gènes du CMH, il apparaît que les populations les plus différenciées entre elles au niveau de ces gènes sont aussi les populations les plus dissimilaires au niveau de leurs communautés de parasites sanguins. Les parasites sanguins ont donc une influence sur la structure génétique du CMH dans des populations de moineaux domestiques, même à une échelle géographique restreinte. De plus, les résultats montrent que les niveaux de différentiations entre populations, mesurés avec le CMH, sont plus faibles que ceux obtenus avec les microsatellites, ce qui laisse sous-entrevoir l'action de la sélection balancée sur ces gènes. Cette étude est particulièrement intéressante car elle nous a permis d'intégrer à la fois les caractéristiques de l'hôte (caractéristiques génétiques), le parasite (communautés des parasites sanguins et prévalence) et l'environnement (géographie, insularité) dans une seule et même étude, ce qui reste encore relativement rare à l'heure actuelle.

Ces résultats pourraient être renforcés par la mise au point de marqueurs microsatellites spécifiques des parasites de la malaria. Ces marqueurs permettraient de conduire les mêmes analyses de génétique des populations (variabilité et structure génétiques) que celles réalisées dans les populations hôtes de moineaux. Nous serions alors en mesure de montrer de façon

encore plus efficace si la structure génétique des populations est liée à la structure génétique des populations de parasites. Il serait alors possible de conduire également des analyses de phylogénie et d'éventuellement mettre en évidence une coévolution entre hôtes et parasites. La mise au point de ce type de marqueurs reste un défit technique, de nombreuses tentatives de ce type ayant déjà échouées. Cependant, Vardo et Schall ont réussis à mettre au point sept marqueurs microsatellites spécifiques de *Plasmodium mexicanum*, un parasite de la malaria chez les lézards (Schall, Vardo, 2007; Vardo-Zalik *et al.*, 2009; Vardo, Schall, 2007).

La prévalence en malaria aviaire restant relativement faible en Bretagne, nous pourrions également envisager d'étudier d'autres parasites ou agents pathogènes qui pourraient avoir une influence sur la structure génétique, en particulier au niveau des gènes du CMH. Des ectoparasites comme les tiques pourraient être récoltés. Les communautés bactériennes intestinales pourraient également être cultivées et identifiées. Ce type de méthodologie est déjà développé chez d'autres espèces d'oiseaux, comme la mouette tridactyle (*Rissa tridactyla*) dans un contexte de suivi épidémiologique de maladies sexuellement transmissibles (White *et al.*, 2010).

Pour finir, nous avons étudié le choix du partenaire en prenant en compte les pressions de sélection exercées par les parasites et l'environnement sur les caractéristiques génétiques des populations hôtes (**Manuscrit 8**). Le choix du partenaire est un moyen pour les individus de transmettre leurs gènes dans un environnement donné et de produire des descendants adaptés. Au niveau des gènes du CMH, un choix de partenaire CMH-dépendant a été montré dans de nombreuses études et permettrait notamment d'augmenter la résistance aux parasites de la descendance et/ou d'éviter les appariements consanguins. Nous avons étudié le choix du partenaire dans une population insulaire de moineau domestique avec comme prédiction que cette population isolée pouvait être soumise à des risques de consanguinité non négligeables. Pourtant, nous avons observé une tendance pour la femelle à choisir un mâle plus proche génétiquement, aussi bien au niveau des marqueurs neutres qu'au niveau des gènes du CMH. Nous n'avons donc pas observé de choix de partenaire spécifiquement lié aux gènes du CMH. Ce résultat surprenant semblerait illustrer un certain bénéfice à l'homozygotie pour la descendance. Dans cette population insulaire, l'adaptation locale est sans doute plus forte que le risque de consanguinité. Par exemple, les pressions de sélection parasitaires sont telles que seuls les individus porteurs de certains allèles sont capables de survivre ou sont avantagés. Le meilleur moyen pour la femelle de transmettre ces allèles à sa descendance est de s'apparier avec un individu génétiquement proche, qui les possédera également. Dans l'hypothèse de la

sélection de parentèle, s'apparier avec un individu similaire permettrait également à la femelle d'augmenter sa fitness inclusive. Les résultats de cette étude remettent en cause le dogme du choix de partenaire pour des individus génétiquement différents, ce qui maximiserait la variabilité génétique de la descendance, et/ou la résistance aux parasites, en ce qui concernent les gènes du CMH. Il apparaît donc aujourd'hui dans plus en plus d'études, dont la nôtre, que le choix du partenaire serait fortement dépendant des caractéristiques de la population (communautés de parasites présents, risques de consanguinité, pressions de sélection locales). Cette étude illustre également comment les caractéristiques locales et environnementales peuvent influencer les caractéristiques génétiques d'une population, via le choix du partenaire, qui pourront influencer en retour les capacités immunitaires de ces populations et donc les communautés de parasites.

Dans ce contexte, des études de choix de partenaire en populations expérimentales permettraient de savoir si certaines caractéristiques de la population sont primordiales dans le choix du partenaire. Il serait alors possible de faire varier certaines caractéristiques de l'environnement comme la pression parasitaire (population infectée versus population saine) ou la quantité de nourriture (population avec restriction alimentaire versus population avec nourriture *ad libitum*). La reproduction dans ces populations contrastées pourrait être suivie plusieurs années. Il serait même possible de changer drastiquement ces caractéristiques environnementales au sein de la même population (infecter la population avec le parasite alors qu'elle était saine jusqu'à présent). Cela permettrait de savoir à quelle vitesse les individus sont capables de modifier leur comportement de choix de partenaire en fonction des pressions environnementales. De la même manière, le niveau de consanguinité pourrait être contrôlé artificiellement et le choix de partenaire pourrait être comparé dans deux populations contrastées (population consanguine versus population en outbreeding).

En conclusion, nous avons tenté d'explorer tous les volets des interactions hôtes-parasites, en y incluant l'environnement (Figure 1). Nous proposons dans la Figure 17 de résumer nos différents résultats, ainsi que les liens de cause à effet que nous avons pu mettre en évidence. Nous espérons avoir, au cours de cette thèse, apporter notre contribution au décorticage complexe que représente l'étude des interactions hôtes-parasites, en proposant une approche multi-niveaux. Bien sûr, nous sommes conscients de n'avoir étudié qu'un seul type de parasite sur deux espèces hôtes, dans deux conditions (expérimentales et naturelles) différentes. L'espèce hôte étudiée peut potentiellement modifier l'interaction avec le parasite. De plus, nous nous sommes focalisés seulement sur l'hôte intermédiaire. La partie de

l'interaction concernant le vecteur a toute son importance et doit être prise en compte. De nombreuses équipes de recherche se penchent à l'heure actuelle sur ces questions. La mise en commun de nos études et de nos résultats pourrait représenter une perspective intéressante permettant d'étudier l'écologie évolutive de la malaria aviaire dans la totalité de son cycle.

L'immuno-écologie fait face aujourd'hui à un défit de taille. D'un côté, les études des interactions hôtes-parasites doivent se diriger de plus en plus vers l'échelle des communautés, en considérant à la fois les communautés d'hôtes et les communautés de parasites, notamment les infections multiples et les interactions des différents parasites au sein de l'hôte. D'un autre côté, les études à des échelles très fines, comme les caractéristiques génétiques et les mécanismes impliqués dans l'immunité, doivent être poursuivies et surtout replacées dans un contexte environnemental et écologique. Dans ce but, les parasites sanguins et plus particulièrement les parasites de la malaria aviaire apparaissent comme de très bons modèles pour ce type d'études.

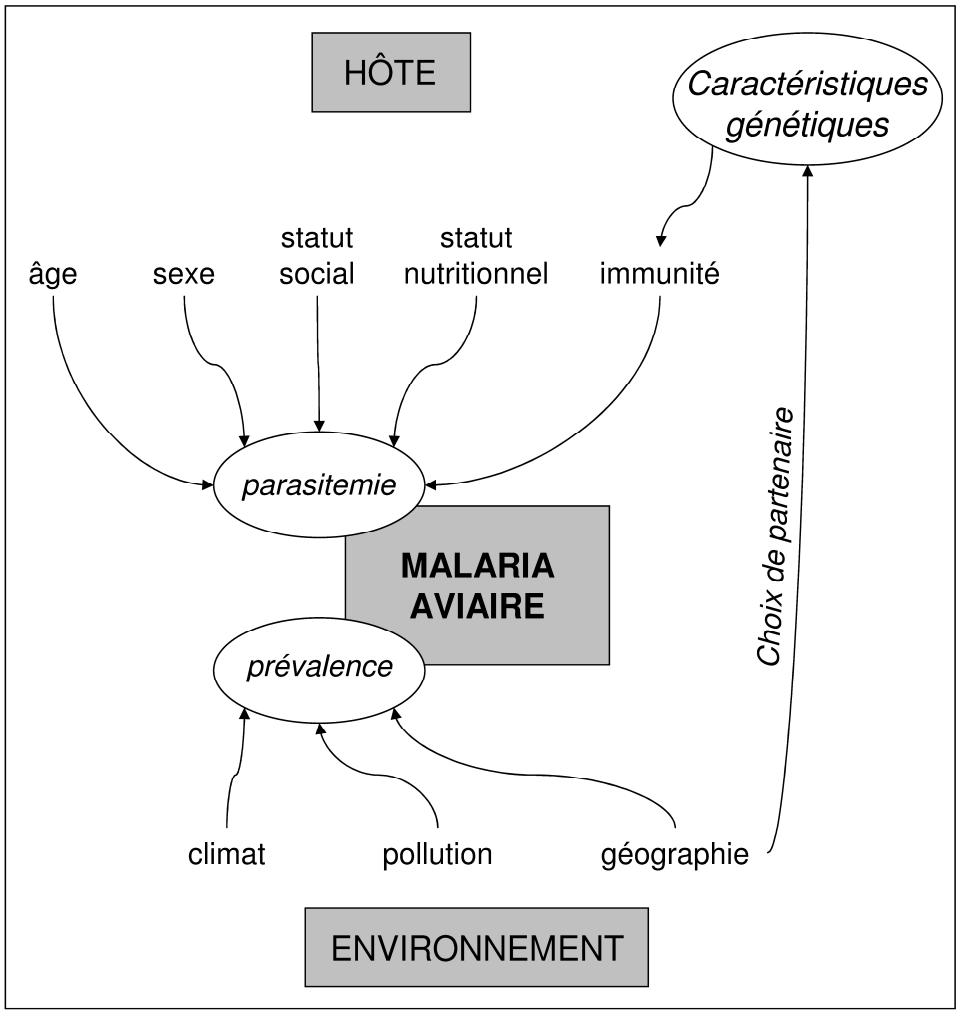


*Figure 17 : L'interaction hôte-parasite-environnement pour la malaria aviaire étudiée au cours de cette thèse. Les effets de certaines caractéristiques de l'hôte et de l'environnement ont pu être mis en évidence.*

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# Résumé

L'étude des interactions hôtes-parasites est devenue un thème de recherche incontournable pour les sciences de l'évolution. Cette coévolution complexe dépend de nombreux compromis évolutifs et peut être grandement influencée par les facteurs environnementaux. Nous nous proposons ici d'étudier les interactions hôtes-parasites à plusieurs échelles, à travers des approches expérimentales et des études en populations naturelles, en étudiant les parasites de la malaria aviaire. Dans un premier temps, nous nous sommes intéressés à l'influence des caractéristiques de l'hôte et notamment au système immunitaire. Le système immunitaire est bénéfique pour l'hôte dans sa lutte contre le parasite, mais peut également engendrer des coûts immunopathologiques. Des traits d'histoire de vie, comme l'âge ou le statut social peuvent modifier la parasitémie au sein des hôtes, sans toutefois avoir d'effet sur la prévalence. Dans un second temps, l'effet de certains facteurs environnementaux a été évalué au sein des interactions hôtes-parasites. La température et la contamination en métaux lourds ont un effet sur la prévalence dans les populations, mais n'affectent pas la parasitémie. Au cours de cette thèse, nous avons également montré l'influence directe des parasites sanguins sur la structure génétique des populations hôtes, notamment au niveau des gènes du CMH.

**Mots-clés :** malaria aviaire, canari domestique, moineau domestique, complexe majeur d'histocompatibilité, système immunitaire, traits d'histoire de vie, environnement, choix de partenaire.

# Abstract

Host-parasite interactions are one of the main topics in evolutionary sciences. This complex coevolution depends on several trade-offs and can be influenced by environmental factors. Here, we propose to study host-parasite interactions with a multi-level approach, using experimental and natural population studies, focusing on avian malaria parasites. First, we studied the effect of host characteristics, and more precisely the immune system. The immune system confers benefits in terms of protection against the parasite, but can also generated immunopathological costs. Life history traits, like age or social status, appear to modify parasitemia but not prevalence. In a second part, we evaluated the effect of environmental factors on host-parasite interactions. We found that temperature and heavy metal contamination had an effect on population prevalence, but not on host parasitemia. We also showed the direct parasite influence on host population genetic structure, and more precisely on MHC genes.

**Keywords :** avian malaria, domestic canaries, house sparrow, major histocompatibility complex, immune system, life history traits, environment, mate choice.