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Dynamics of haemosporidian infections. Causes and consequences of heterogeneity in avian malaria infection

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Dynamics of haemosporidian infections

Causes and consequences of heterogeneity in avian malaria infection

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Causes and consequences of heterogeneity in avian malaria infection

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pour le Doyen
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Prof. Philipp Engel

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Abstract

Host-parasite systems are ubiquitous systems in nature. They are characterized by sustainable interactions between two partners under strong selective processes: they constantly adapt to each other. Heterogeneity in pathogen transmission shapes infectious diseases. Some individuals are highly competent hosts as they are responsible for a disproportionate number of transmission events. This pattern has been observed in a wide range of infectious diseases such as malaria which is the deadliest vector-borne disease. Over the recent years, a certain effort has been devoted to investigating the traits underpinning the development of malaria parasites in the vertebrate host. However, traits that underpin the development of malaria parasites within the vector as well as the strategy evolved to maximize its transmission to the next host remains to be investigated. Using the avian malaria system, I investigated how heterogeneity of infection, both between and within vertebrate hosts, impacts the development of *Plasmodium* within its mosquito vector. I found evidence that variations of parasite intensities shape the resulting mosquito infection rate. Specifically, I have shown that spatial and temporal heterogeneity in the *Plasmodium* distribution within the vertebrate host shapes the infection intensity within the vector. Then, I focused on vector-related traits to investigate to what extent this might drive the *Plasmodium* dynamics. I induced nutritional stress during the mosquito development to lower its immune response and investigate the effect on the parasite development. Interestingly, lowered mosquito immunity was not associated with a reduction in mosquito intensity of infection. I suggested that *Plasmodium* might be able to evolve sophisticated strategies to maximize its fitness both in vertebrate host and the vector. In the current context, where the vector is a promising target for vector-borne disease control programmes there is an urgent need to better understand the relative contribution of each actor in the tripartite interaction in transmission-related traits.

Keywords: host-parasite interaction, malaria, transmission, heterogeneity, vectorial capacity, plastic strategies

Résumé

Les associations hôte-parasite sont omniprésentes dans la nature. Ce sont des interactions durables soumises à des pressions de sélection : les protagonistes s'adaptent constamment l'un à l'autre. Des variations dans la capacité d'un hôte à transmettre des pathogènes peut être déterminant dans le cas des maladies infectieuses. En effet, certains individus sont responsables d'un nombre disproportionné de nouvelles infections. Cela a été observé pour de nombreuses maladies infectieuses et notamment pour la malaria, qui reste la maladie à transmission vectorielle la plus mortelle. Ces dernières années, un certain effort a été consacré à l'étude des facteurs responsables du développement de *Plasmodium* chez l'hôte vertébré. Cependant, les facteurs responsables de son développement au sein du vecteur ainsi que les stratégies que le parasite a mis en place pour maximiser sa transmission à un nouvel hôte restent à étudier. En utilisant le système de la malaria aviaire, j'ai étudié l'impact de l'hétérogénéité d'infection entre et au sein des hôtes vertébrés sur le développement de *Plasmodium* dans le moustique vecteur. J'ai mis en évidence que des variations dans la dynamique intra-hôte de *Plasmodium* façonne la transmission de celui-ci aux moustiques. Plus précisément, j'ai montré l'existence d'une hétérogénéité spatiale et temporelle de la distribution de *Plasmodium* au sein de l'hôte vertébré. J'ai également étudié si des variations dans l'environnement du vecteur pouvaient déterminer la dynamique de *Plasmodium* en son sein. Pour cela, j'ai induit un stress nutritionnel pendant le développement larvaire des moustiques afin de diminuer sa réponse immunitaire et d'étudier l'effet sur le développement du parasite. Il est intéressant de noter que la baisse de l'immunité des moustiques n'était pas associée à une réduction de son intensité d'infection. De plus, *Plasmodium* pourrait être en mesure d'élaborer des stratégies sophistiquées pour maximiser sa fitness à la fois chez l'hôte vertébré et le vecteur. Dans le contexte actuel, où le vecteur est une cible prometteuse pour les programmes de lutte contre les maladies à transmission vectorielle, il est nécessaire d'avoir une meilleure compréhension des contributions relatives de chaque acteur de la tripartite dans la transmission.

Mots-clés : interactions hôte-parasite, malaria, transmission, hétérogénéité, capacité vectorielle, stratégies plastiques

General introduction

Parasitism is a ubiquitous lifestyle in nature (Thompson 1994) and has been reported in all the major taxa (Windsor 1998). Indeed, parasites are widespread among living organisms, and it is estimated that they are represented in up to 40% of all species on earth (Holmes & Price 1980; Windsor 1998; Dobson *et al.* 2008). Host-parasite systems are sustainable interactions under strong selective processes referred as co-evolution or arms race (Combes 1995, 2020). These selective processes assume that parasites have to optimize their phenotype to infect and survive the host and that hosts have to optimize their phenotype to resist and survive parasitism (Combes 1995, 2020). Predicting the emergence, spread and evolution of parasites requires understanding of the transmission dynamics considering the selection pressures exerted by both the parasite, the host and the environments in which they evolve.

Heterogeneities in the ability of a host, or a vector, to transmit pathogens is a fundamental feature of host-parasites systems (Combes 2020). Contrary to what was assumed in early modelling studies of infectious diseases dynamics (Anderson & May 1992; Grenfell *et al.* 1995), all susceptible hosts do not have equal chances to get and/or spread an infection. In many host-parasite systems, individuals host vary widely in their competence, i.e. their ability to cause an infection to another host or vector, and might drive disease spread (Kilpatrick *et al.* 2006; Gervasi *et al.* 2015). Some individuals are highly competent hosts as they are responsible for a disproportionate number of transmission events (Martin *et al.* 2019). The most common form of this extreme competence is super-spreading (Lloyd-Smith *et al.* 2005; Paull *et al.* 2012; Cooper *et al.* 2019; Martin *et al.* 2019). Within a population, most of the hosts induce few secondary infections, while a few individuals are responsible for the majority of them (Woolhouse *et al.* 1997). This transmission pattern is known as the "20/80" rule, whereby 20% of the host population are responsible at least for 80% of new infections (Woolhouse *et al.* 1997; Lloyd-Smith *et al.* 2005; Cooper *et al.* 2019). In addition, parasites are known to be overdispersed or

aggregated in their host populations (Crofton 1971; Poulin 1993, 2013; Shaw & Dobson 1995; Shaw *et al.* 1998; Lord *et al.* 1999). A small fraction of the host population harbors the majority of the parasite population. In addition, superspreaders appears to be the individuals with the highest infection. This pattern has been observed in various diseases, from viruses and fungal parasites of plants (Ruiz *et al.* 2006; Grogan *et al.* 2016) to protozoan and metazoan parasites of humans (Medley *et al.* 1993; Pichon *et al.* 2000; Churcher *et al.* 2005). Investigating the causes and consequences of transmission heterogeneity might improve the understanding and the prediction of diseases spread and outbreaks events (Lloyd-Smith *et al.* 2005; VanderWaal & Ezenwa 2016).

Transmission heterogeneity can have large impact on diseases with public health importance, such as vector-borne diseases (VBDs), which remains a serious threat today. One example of vector-borne disease where heterogeneity in pathogen transmission has been widely reported is malaria. Malaria is one of the deadliest infectious diseases, with an estimated 241 million cases recorded worldwide in 2020, resulting in more than 627 000 deaths (WHO, 2021). Malaria is caused by unicellular haemosporidian parasites of the genus *Plasmodium* and transmitted by hematophagous arthropod vectors, the mosquitoes. In addition to infecting humans, these parasites also infect many other terrestrial vertebrate species (Pereztris *et al.* 2005), including other mammals (Bray & Garnham 1982; Killick-Kendrick 2012), reptiles (Schall 1990) and birds (Garnham 1966; Valkiunas 2004).

The life cycle of *Plasmodium* is similar in all hosts (**Figure 1**), irrespective of their taxa, and comprises two major phases. It first reproduces asexually within a vertebrate host and then sexually within the mosquito. First, the infective stages (called sporozoites) infect the vertebrate hosts through the bite of infected mosquitoes. They are inoculated through the mosquito saliva and start replicating in the reticuloendothelial cells of diverse organs (exoerythrocytic cycle). At the merozoite stage, produced through this cycle, parasites invade red blood cells and are then released into the bloodstream (erythrocytic cycle). While some merozoites end up reinitiating the asexual replication cycle, some of them invade new red blood cells to differentiate into sexual gametocytes, the infective stage for

mosquitoes. The dynamic of infection within the vertebrate includes an acute phase, where the number of parasites in the blood increases until it reaches a peak around 12 days after infection. The number of parasites then progressively decreases to reach a very low density in the blood that can last for years. This second phase is known as the chronic phase. Relapses, characterized by short increases in replication rate during the chronic phase, can occur in response to biotic or abiotic factors. Female and male gametocytes (the sexual stage) are transmitted to the mosquito vector when it takes a blood meal on an infected vertebrate host. They then undergo sexual reproduction inside the mosquito midgut. The zygotes mature into motile ookinetes that penetrate the wall of the midgut and start developing into oocysts. Mature oocysts in turn produce sporozoites that are released into the mosquito hemolymph to invade the salivary glands. Once in the mosquito's salivary glands, the sporozoites can be transmitted to a new vertebrate host.

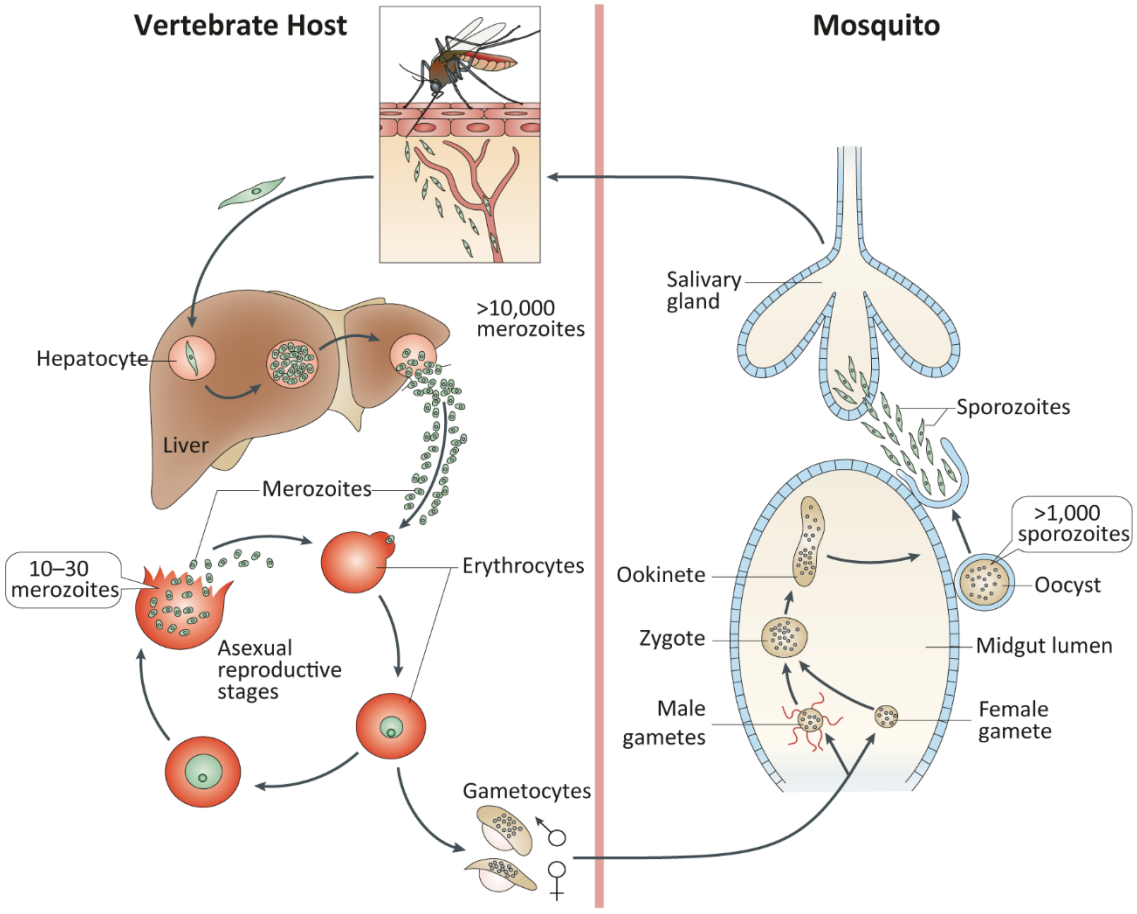

Figure 1: Life cycle of *Plasmodium* spp. Adapted from Ménard et al. (2013).

Over the recent years, efforts have been made to investigate the traits underpinning the development of malaria parasites in the vertebrate host, and to predict transmission to the vector. Like all organisms, malaria parasites are expected to optimize their resource allocation in growth and survival (i.e. investment in asexual stage) and reproduction (i.e. investment in gametocytes, sexual stages of the parasite) within the vertebrate host (Koella & Antia 1995; Reece *et al.* 2009; Pollitt *et al.* 2011; Mideo & Reece 2012). *Plasmodium* parasites appears to exhibit phenotypic plasticity, i.e. the ability of a genotype to produce distinct phenotypes (Pigliucci 2001) in response to environmental changes they experience throughout their infection within the host (Mideo & Reece 2012). For instance, *Plasmodium* parasites are able to adjust their conversion rate (asexual to sexual ratio; Trager *et al.* 1999; Schneider *et al.* 2018) and the sex ratio of gametocytes (Reece *et al.* 2008) in response to within-host changes (e.g. anemia, EPO levels, host immunes responses; Paul *et al.* 2002; Cameron *et al.* 2013; Carter *et al.* 2014). Even though some responses may result from passive plasticity, some are likely the results of adaptive plasticity. Recently, we have shown that an increase in host stress hormone triggers the replication rate of the avian malaria parasite within the host. This response might be mediated by the stress response of the host, or alternatively, by detection of mosquito cues present in the saliva injected during the blood meal as an adaptive response to mosquito bites (Pigeault et al. 2022, in prep, **Appendix 1**). Indeed, *Plasmodium* is able to detect within-host cues and adjust its transmission traits. For instance, in the presence of others parasite genotypes, the rodent malaria parasite *Plasmodium chabaudii* adjust adaptively, in order to maximize their competitive ability, its investment in sexual stage (Pollitt *et al.* 2011) and its sex ratio (Reece *et al.* 2008) to fine-tune its transmission. More recently, it has been reported that *Plasmodium* is able to respond plastically to mosquito bites by increasing its replication rate within the vertebrate host in order to maximize its transmission to the vector (Cornet *et al.* 2014; Pigeault *et al.* 2018).

While the factors affecting development and resource allocation of *Plasmodium* in the vertebrate host have been widely studied, little attention has been devoted to the traits that underpin the development of *Plasmodium* within the vector and its transmission to the next host (Lefevre *et al.*

2018). To better understand the transmission dynamics of malaria parasites, one must consider the factors related to both the vertebrate host and the vector. In vector-borne diseases, the most common metric of transmission is the vectorial capacity. The individual vectorial capacity (IC) describes the ability of a focal vector to transmit their pathogen (Rivero *et al.* 2010) and is defined as:

$$IC = \frac{a^2 bce^{-gn}}{g}$$

where ***a*** refers to the feeding behaviour of the vector and is defined as the number of bites on a focal host (per vector, per day); ***b*** stands for the probability that a host becomes infected from an infectious bite, defined by a combination of host susceptibility to infection and vector infectiousness; ***c*** is the vector's susceptibility to infection; ***g*** is the mortality rate of the vector; and ***n*** the extrinsic incubation period of the pathogen (i.e. number of days required for the vector to become infectious; EIP)(Rivero *et al.* 2010).

The extent to which one or several components of the vectorial capacity are shaped and trade-off with each other is unclear (Lefevre *et al.* 2018; Ohm *et al.* 2018; Cansado-Utrilla *et al.* 2021). Furthermore, the extent to which malaria parasite have evolved sophisticated strategy to optimize its growth, survival and reproduction within its vector remains to be investigated.

To answer these questions, I used the avian malaria system (Pigeault *et al.* 2015; Rivero & Gandon 2018), which is the only currently available animal experimental system that enables to work with a parasite recently isolated from the wild (*Plasmodium relictum*) and its natural mosquito vector (*Culex pipiens* mosquito; Glaizot *et al.* 2012; Lalubin *et al.* 2013).

The first part of my thesis focused on the traits that underpin parasite dynamics within the mosquito vector. In particular, I studied the effect of both inter- and intra-individual variation of within vertebrate-host parasitaemia on the resulting parasite dynamics within the vector. **In chapter 1**, I investigated the impact of inter-individual variation of vertebrate host parasitaemia on the transmission to mosquitoes, measuring both the resulting parasite burden within the mosquitoes and

the extrinsic incubation period of the parasite. I found that the parasitaemia determine the extrinsic incubation period of the parasite but not the mosquito parasite burden. Mosquitoes that blood fed on highly infected vertebrate hosts were infectious sooner, without displaying higher parasite burden**.** **In chapter 2**, I focused on within-vertebrate host variation in parasite distribution. I attempted to elucidate whether the distribution of *Plasmodium* gametocytes (the stage that is transmissible to mosquitoes) within the vertebrate host blood-stream influences the mosquito infection rate. I showed that gametocytes are non-homogeneously distributed in the blood of both human and bird hosts, which had an impact on the intensity of the mosquito infection. Mosquitoes fed on body parts displaying the highest gametocytes densities had higher parasite burden.

The second part of my thesis focused on the traits related to the vector and the propensity of malaria parasites to set up plastic strategies in order to fine tune their transmission. In **chapter 3**, I investigated the ability of *Plasmodium* parasites to respond plastically to mosquito bites in order to maximize transmission to mosquito. I showed that the overdispersed distribution of malaria parasite within mosquito vectors is partly explained by a temporal heterogeneity in parasite infectivity triggered by the bites of mosquitoes. **In chapter 4**, I induced nutritional stress during the mosquito development to lower its immune response in order to investigate the effect on the parasite development and the extrinsic incubation period of the parasite. I found that the reduction in mosquito immunity was not associated with a reduction in parasite burden. The results of the extrinsic incubation period are still being analyzed and will provide additional insights into the ways parasite and vector traits interact to shape transmission dynamics.

References

Anderson, R.M. & May, R.M. (1992). *Infectious Diseases of Humans: Dynamics and Control*. Oxford University Press. 757pp.

Bray, R.S. & Garnham, P.C.C. (1982). The life-cycle of primate malaria parasites. *British Medical Bulletin*, 38, 117-122.

Cameron, A., Reece, S.E., Drew, D.R., Haydon, D.T. & Yates, A.J. (2013). Plasticity in transmission strategies of the malaria parasite, *Plasmodium chabaudi*: environmental and genetic effects. *Evolutionary Applications*, 6, 365–376.

Cansado-Utrilla, C., Zhao, S.Y., McCall, P.J., Coon, K.L. & Hughes, G.L. (2021). The microbiome and mosquito vectorial capacity: rich potential for discovery and translation. *Microbiome*, 9, 111.

Carter, L.M., Schneider, P. & Reece, S.E. (2014). Information use and plasticity in the reproductive decisions of malaria parasites. *Malaria Journal*, 13, 115.

Churcher, T.S., Ferguson, N.M. & Basáñez, M.-G. (2005). Density dependence and overdispersion in the transmission of helminth parasites. *Parasitology*, 131, 121–132.

Combes, C. (1995). *Interactions durables: écologie et évolution du parasitisme*. Dunod. 524pp.

Combes, C. (2020). *The Art of Being a Parasite*. University of Chicago Press.

Cooper, L., Kang, S.Y., Bisanzio, D., Maxwell, K., Rodriguez-Barraquer, I., Greenhouse, B., *et al.* (2019). Pareto rules for malaria super-spreaders and super-spreading. *Nat Commun*, 10, 3939.

Cornet, S., Nicot, A., Rivero, A. & Gandon, S. (2014). Evolution of Plastic Transmission Strategies in Avian Malaria. *PLoS Pathogens*, 10, e1004308.

Crofton, H.D. (1971). A quantitative approach to parasitism. *Parasitology*, 62, 179–193.

Dobson, A., Lafferty, K.D., Kuris, A.M., Hechinger, R.F. & Jetz, W. (2008). Homage to Linnaeus: How many parasites? How many hosts? *Proceedings of the National Academy of Sciences*, 105, 11482–11489.

Garnham, P.C.C. (1966). *Malaria Parasites and other Haemosporidia.* Blackwell Scientific Publications. 1132pp.

Gervasi, S.S., Civitello, D.J., Kilvitis, H.J. & Martin, L.B. (2015). The context of host competence: a role for plasticity in host–parasite dynamics. *Trends in Parasitology*, 31, 419–425.

Glaizot, O., Fumagalli, L., Iritano, K., Lalubin, F., Rooyen, J.V. & Christe, P. (2012). High Prevalence and Lineage Diversity of Avian Malaria in Wild Populations of Great Tits (*Parus major*) and Mosquitoes (*Culex pipiens*). *PLOS ONE*, 7, e34964.

Grenfell, B.T., Dobson, A.P. & Moffatt, H.K. (1995). *Ecology of Infectious Diseases in Natural Populations*. Cambridge University Press. 521pp.

Grogan, L.F., Phillott, A.D., Scheele, B.C., Berger, L., Cashins, S.D., Bell, S.C., *et al.* (2016). Endemicity of chytridiomycosis features pathogen overdispersion. *Journal of Animal Ecology*, 85, 806–816.

Holmes, J.C. & Price, P.W. (1980). Parasite Communities: The Roles of Phylogeny and Ecology. *Systematic Zoology*, 29, 203–213.

Killick-Kendrick, R. (2012). *Rodent Malaria*. Academic Press.

Kilpatrick M., A., Daszak, P., Jones, M.J., Marra, P.P. & Kramer, L.D. (2006). Host heterogeneity dominates West Nile virus transmission. *Proceedings of the Royal Society B: Biological Sciences*, 273, 2327–2333.

Koella, J.C. & Antia, R. (1995). Optimal Pattern of Replication and Transmission for Parasites with Two Stages in Their Life Cycle. *Theoretical Population Biology*, 47, 277–291.

Lalubin, F., Delédevant, A., Glaizot, O. & Christe, P. (2013). Temporal changes in mosquito abundance (*Culex pipiens*), avian malaria prevalence and lineage composition. *Parasites & Vectors*, 6, 307.

Lefevre, T., Ohm, J., Dabiré, K.R., Cohuet, A., Choisy, M., Thomas, M.B., *et al.* (2018). Transmission traits of malaria parasites within the mosquito: Genetic variation, phenotypic plasticity, and consequences for control. *Evolutionary Applications*, 11, 456–469.

Lloyd-Smith, J.O., Schreiber, S.J., Kopp, P.E. & Getz, W.M. (2005). Superspreading and the effect of individual variation on disease emergence. *Nature*, 438, 355–359.

Lord, C.C., Barnard, B., Day, K., Hargrove, J.W., McNamara, J.J., Paul, R.E.L., *et al.* (1999). Aggregation and distribution of strains in microparasites. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, 354, 799–807.

Martin, L.B., Addison, B., Bean, A.G.D., Buchanan, K.L., Crino, O.L., Eastwood, J.R., *et al.* (2019). Extreme Competence: Keystone Hosts of Infections. *Trends in Ecology & Evolution*, 34, 303–314.

Medley, G.F., Sinden, R.E., Fleck, S., Billingsley, P.F., Tirawanchap, N. & Rodriguez, M.H. (1993). Heterogeneity in patterns of malarial oocyst infections in the mosquito vector. *Parasitology*, 106, 441–449.

Ménard, R., Tavares, J., Cockburn, I., Markus, M., Zavala, F. & Amino, R. (2013). Looking under the skin: the first steps in malarial infection and immunity. Nature Reviews Microbiology, 11, 701—712.

Mideo, N. & Reece, S.E. (2012). Plasticity in parasite phenotypes: evolutionary and ecological implications for disease. *Future Microbiology*, 7, 17–24.

Ohm, J.R., Baldini, F., Barreaux, P., Lefevre, T., Lynch, P.A., Suh, E., *et al.* (2018). Rethinking the extrinsic incubation period of malaria parasites. *Parasites & Vectors*, 11, 178.

Paul, R.E.L., Brey, P.T. & Robert, V. (2002). Plasmodium sex determination and transmission to mosquitoes. *Trends in Parasitology*, 18, 32–38.

Paull, S.H., Song, S., McClure, K.M., Sackett, L.C., Kilpatrick, A.M. & Johnson, P.T. (2012). From superspreaders to disease hotspots: linking transmission across hosts and space. *Frontiers in Ecology and the Environment*, 10, 75–82.

Pereztris, J., Hasselquist, D., Hellgren, O., Krizanauskiene, A., Waldenstrom, J. & Bensch, S. (2005). What are malaria parasites? *Trends in Parasitology*, 21, 209–211.

Pichon, G., Awono-Ambene, H.P. & Robert, V. (2000). High heterogeneity in the number of *Plasmodium falciparum* gametocytes in the bloodmeal of mosquitoes fed on the same host. *Parasitology*, 121, 115–120.

Pigeault, R., Vézilier, J., Cornet, S., Zélé, F., Nicot, A., Perret, P., *et al.* (2015). Avian malaria: a new lease of life for an old experimental model to study the evolutionary ecology of Plasmodium. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 370, 20140300.

Pigeault, R., Caudron, Q., Nicot, A., Rivero, A. & Gandon, S. (2018). Timing malaria transmission with mosquito fluctuations. *Evolution Letters*, 2, 378–389.

Pigliucci, M. (2001). *Phenotypic Plasticity: Beyond Nature and Nurture*. Johns Hopkins University Press. 328pp.

Pollitt, L.C., Mideo, N., Drew, D.R., Schneider, P., Colegrave, N. & Reece, S.E. (2011). Competition and the Evolution of Reproductive Restraint in Malaria Parasites. *The American Naturalist*, 177, 358–367.

Poulin, R. (1993). The disparity between observed and uniform distributions: A new look at parasite aggregation. *International Journal for Parasitology*, 23, 937–944.

Poulin, R. (2013). Explaining variability in parasite aggregation levels among host samples. *Parasitology*, 140, 541–546.

Reece, S.E., Drew, D.R. & Gardner, A. (2008). Sex ratio adjustment and kin discrimination in malaria parasites. *Nature*, 453, 609–614.

Reece, S.E., Ramiro, R.S. & Nussey, D.H. (2009). SYNTHESIS: Plastic parasites: sophisticated strategies for survival and reproduction? *Evolutionary Applications*, 2, 11–23.

Rivero, A. & Gandon, S. (2018). Evolutionary Ecology of Avian Malaria: Past to Present. *Trends in Parasitology*, 34, 712–726.

Rivero, A., Vézilier, J., Weill, M., Read, A.F. & Gandon, S. (2010). Insecticide Control of Vector-Borne Diseases: When Is Insecticide Resistance a Problem? *PLOS Pathogens*, 6, e1001000.

Ruiz, L., Janssen, D., Martín, G., Velasco, L., Segundo, E. & Cuadrado, I.M. (2006). Analysis of the temporal and spatial disease progress of *Bemisia tabaci*-transmitted *Cucurbit yellow stunting disorder virus* and *Cucumber vein yellowing virus* in cucumber. *Plant Pathology*, 55, 264–275.

Schall, J.J. (1990). The ecology of lizard malaria. *Parasitology Today*, 6, 264–269.

Schneider, P., Greischar, M.A., Birget, P.L.G., Repton, C., Mideo, N. & Reece, S.E. (2018). Adaptive plasticity in the gametocyte conversion rate of malaria parasites, *PLOS Pathogens*. 21, e1007371.

Shaw, D.J. & Dobson, A.P. (1995). Patterns of macroparasite abundance and aggregation in wildlife populations: a quantitative review. *Parasitology*, 111, 111-133.

Shaw, D.J., Grenfell, B.T. & Dobson, A.P. (1998). Patterns of macroparasite aggregation in wildlife host populations. *Parasitology*, 117, 597–610.

Thompson, J.N. (1994). *The Coevolutionary Process*. University of Chicago Press. 383pp.

Trager, W., Gill, G.S., Lawrence, C. & Nagel, R.L. (1999). *Plasmodium falciparum*: Enhanced Gametocyte Formationin Vitroin Reticulocyte-Rich Blood. *Experimental Parasitology*, 91, 115–118.

Valkiunas, G. (2004). *Avian Malaria Parasites and other Haemosporidia*. CRC Press. 946pp.

VanderWaal, K.L. & Ezenwa, V.O. (2016). Heterogeneity in pathogen transmission: mechanisms and methodology. *Functional Ecology*, 30, 1606–1622.

Windsor, D.A. (1998). Controversies in parasitology, Most of the species on Earth are parasites. *International Journal for Parasitology*, 28, 1939–1941.

Woolhouse, M.E.J., Dye, C., Etard, J.-F., Smith, T., Charlwood, J.D., Garnett, G.P., *et al.* (1997). Heterogeneities in the transmission of infectious agents: Implications for the design of control programs. *Proceedings of the National Academy of Sciences*, 94, 338–342.

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Chapter one

Impact of within-vertebrate host parasitaemia on the temporal dynamics of *Plasmodium* in mosquitoes

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Authors' contributions. All authors conceived the study and elaborated the experimental design. J.I., M.B. and S.M. performed the experiments. M.B. and J.W. did the molecular analyses. J.I. analyzed the data. J.I. wrote the first draft of the manuscript, and all authors contributed substantially to revision.

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Abstract

Vector-borne parasites have to interact with their environment in dynamic ways to cope with different pressures exerted by both the host and the vector. For *Plasmodium* parasites, most studies focused on the traits underpinning its dynamics within the vertebrate host and their sources of variation. Comparatively, little attention has been given to those underpinning the parasite's dynamics within the vector. Using the avian malaria system, we aimed to investigate whether the intensity of infection in the vertebrate host drives the intensity of infection and the extrinsic incubation period (EIP) of *Plasmodium* within the vector. EIP represents the time it takes for the parasite to develop from its ingestion until it invades the salivary glands. We have shown that mosquitoes that blood-fed on highly infected vertebrate hosts were infectious as soon as four days post blood meal. However, biting on highly infected vertebrate host does not seem to affect the intensity of parasite infection within the mosquito: the number of oocysts did not vary according to the vertebrate host parasitaemia. We hypothesize the density-dependent processes occurring both during the gametocytes-to-ookinetes and the ookinetes-to-oocysts associated with non-competitive strategy in the mosquito resources exploitation might underpins the formation of sporozoites, affecting either their growth rate or their quality. Studying the timing of infection under varying conditions and understanding how it shapes sporozoites development and to what extent this drives malaria transmission potential is needed to reassess the approaches of disease control programs.

Keywords: Avian malaria, Plasmodium, Extrinsic Incubation Period, EIP, Transmission

Introduction

One major goal in ecology is to understand what processes drive the evolution of populations. Of major interest for many population-level processes is density-dependence, whereby the evolution of a population (e.g. growth) is influenced by population density (Fowler 1987; Stiling 1987; Chen *et al.* 2019; Albery *et al.* 2020). Host-parasite systems are especially subject to density-dependent processes, as parasites often have complex life cycles involving bottlenecks upon transmission between hosts, followed by explosive growth (Stiling 1987; Hughes *et al.* 2004; Churcher *et al.* 2005, 2010). Both positive and negative density-dependent processes are common in such associations. While positive density-dependent processes lead to overdispersion (higher variance than the mean) of parasite populations, negative density dependent processes lead to underdispersion (higher mean than the variance) (Anderson & Gordon 1982). Such mechanisms have strong influences on parasite population dynamics and lead to heterogeneity between hosts within population, which ultimately produces different infection outcomes (Anderson & May 1978; Das & Sutradhar 1995; Wetten *et al.* 2007).

Individual heterogeneity in terms of magnitude of infection have been widely described in vector-borne diseases (Medley *et al.* 1993; Woolhouse *et al.* 1997; Cooper *et al.* 2019; Martin *et al.* 2019). For many of these diseases, most studies focused on the traits underpinning parasite dynamics within the vertebrate host and their sources of variation. Comparatively, little attention has been given to those underpinning the parasite's dynamics within the vector. Yet, key traits determining the transmission dynamics of the pathogen (e.g. vector competence, lifespan, the length of the parasite's extrinsic incubation period) depend on the intensity of parasite infection in the vector (Basáñez *et al.* 1994; Dawes *et al.* 2009; Courtenay *et al.* 2017; Gutiérrez-López *et al.* 2019; Childs & Prosper 2020).

Plasmodium parasites, the etiological agents of malaria, remain currently a major global cause of mortality (WHO 2019). These pathogens infect many vertebrate host species including mammals (Bray & Garnham 1982), reptiles (Schall 1990) and birds (Garnham 1966). The life cycle of these parasites is

similar in all hosts, irrespective of their taxa: they reproduce asexually within the vertebrate host and sexually within the dipteran vector. Mosquitoes inoculate sporozoite stage of *Plasmodium* into the blood of vertebrate hosts through a blood meal event. Then, parasites replicate asexually and invade the red blood cells to be released into the bloodstream. Some of the parasite asexual stages will differentiate into male and female sexual gametocytes, the infective stages for mosquitoes. Thus, when a mosquito takes a blood meal on an infected vertebrate host, they ingest both female and male gametocytes, which will then undergo sexual reproduction. The zygotes mature into motile ookinetes that penetrate the wall of the midgut and start developing into oocysts. Mature oocysts produce in turn sporozoites, the infectious stage, that will invade the mosquito's salivary glands to be transmitted to a new vertebrate host ultimately.

Initial infection dose appears to be a key biotic factor for transmission (Leggett *et al.* 2012; Breaux *et al.* 2014; Schmid *et al.* 2017). Regarding malaria parasites, the relationship between gametocyte density and mosquito infection is complex and seems to be specific to *Plasmodium*-mosquito associations. For the human malaria parasite *P. falciparum* and both *An. coluzzii* and *An. gambiae* mosquitoes, the oocyst burden increases with the gametocytes density (Bousema & Drakeley 2011; Stone *et al.* 2013; Da *et al.* 2015; Bradley *et al.* 2018) while the relationship is negative for the rodent malaria species *P.berghei* with *An. stephensi* mosquitoes (Da *et al.* 2015) or unclear for avian malaria species (Churcher *et al.* 2013; Pigeault *et al.* 2015). The majority of the studies trying to elucidate the relationship between the dose and the infection only consider the oocyst stage, which is the simplest and least expensive measure of the mosquito infection. Even though oocyst burden seems to be positively correlated with the likelihood of transmitting sporozoites to a new vertebrate host (Miura *et al.* 2019) the extrinsic incubation period of the parasite (EIP; time for salivary gland invasion) should be considered too (Ohm *et al.* 2018). Indeed, it determines the time the vector will be infectious and able to transmit the parasite to a new host. Predicting when and how the within-vector dynamics vary in terms of intensity but also temporally is relevant because this underpins the vector-to-vertebrate host transmission dynamic.

The aim of this study was to assess the role of the vertebrate host parasitaemia (i.e. number of asexual and sexual stage of the parasite) on the within-vector parasite dynamics. In particular, I investigated whether the parasitaemia in the vertebrate host may determine (i) the intensity of infection of the mosquito, in terms of oocyst burden and (ii) the extrinsic incubation period of *Plasmodium*. Mosquitoes biting highly infected hosts were expected to have higher parasite burden and be infectious sooner (Churcher *et al.* 2017). To test this hypothesis, we used the natural avian malaria system (Pigeault *et al.* 2015), with domestic canaries (*Serinus canaria*), a parasite recently isolated from the wild (*Plasmodium relictum*), and its natural mosquito vector (*Culex pipiens;* Glaizot *et al.* 2012).

Materials and Methods

Biological material

The experiment was carried out using a *Plasmodium relictum* (lineage SGS1) strain isolated from infected house sparrows (*Passer domesticus)* in December 2020 on the campus of the University of Lausanne, Switzerland (46°31‘25.607‘‘N 6°34‘40.714‘‘E). The strain was maintained through regular passages across our stock canaries (*Serinus canaria*) using intraperitoneal (i.p.) injections until the beginning of the experiment (Pigeault *et al.* 2015).

The *Culex pipiens* mosquito population used in this experiment was initiated from wild clutches collected in Lausanne in August 2017, and was maintained in the insectary since. Mosquitoes were reared using standard protocols (Vézilier *et al.* 2010). Females 7–13 days after emergence, which had not had prior access to blood, maintained on glucose solution (10%) since were used and starved (but provided with water to prevent dehydration) 24 h before the experiment.

Experimental design

Infection of vertebrate hosts

Twelves domestic canaries (*Serinus canaria*) were infected with *Plasmodium relictum* (lineage SGS1) by i.p. from our stock canaries. One bird died and was removed from the analyses. The dynamic of infection was monitored for each bird every two days from day 5 to day 20 post-infection by measuring the parasitaemia (*i.e.* number of parasite asexual and sexual stages in the vertebrate host blood) and gametocytaemia (*i.e.* number of mature parasite sexual stages in the vertebrate host blood) by microscopic examination using Giemsa stained blood-smear. The number of infected red blood cells were counted per 3000 erythrocytes in randomly chosen fields on the blood smears (Valkiunas 2004).

Mosquito exposition to *P. relictum*

On day 12 post-infection, corresponding to the peak of infection, birds were exposed individually to 90 uninfected mated female mosquitoes for 3h (6-9 p.m.). At the end of the experiment, blood-fed females were counted and kept individually in plastic tubes under standard laboratory conditions (25°C – 70%RH) with 10% *ad libitum* glucose.

Mosquito dissections

Every two days starting from day 4 to day 20 post-blood meal (pbm), 4 $\pm$ 1 mosquitoes were haphazardly sampled per bird in order to monitor both the dynamic of oocysts formation and sporozoites production. Each mosquito were dissected to (i) count the number of oocysts in their midgut with the aid of a binocular microscope and (ii) quantify the transmissible sporozoites in their head/thorax using real-time quantitative PCR (see molecular analyses below).

Molecular analyses

Real-time quantitative PCR was used to measure the prevalence of *Plasmodium* sporozoites in the mosquito head/thorax. Beads were added to each head/thorax samples and flash frozen in liquid

nitrogen in order to grind the samples. DNA was then extracted using the Qiagen DNeasy 96 blood and tissue kit following the manufacturer's instructions with one modification: samples were incubated overnight. For each individual (mosquitoes), two qPCRs were carried out: one targeting the mtDNA cytb gene of *Plasmodium* (Primers L4050Plasmo 5'-GCTTTATGTATTGTATTTATAC-3', H4121Plasmo 5'-GACTTAAAAGATTTGGATAG-3', Probe TexasRed-CYTB-BHQ2 5'-CCTTTAGGGTATGATACAGC-3') and the other targeting the CQ11 gene of *Culex pipiens* mosquitoes (Primers 1725-F 5'- GCGGCCAAATATTGAGACTT-3', 1726-R 5'- CGTCCTCAAACATCCAGACA-3', Probe FAM-CQ11-BHQ1 5'- GGAACATGTTGAGCTTCGGK-3'). All samples were run in triplicate (QuantStudio 6 and 7 Pro Real-Time PCR Systems) and samples with a threshold Ct value higher than 35 for the parasite were considered uninfected. Relative quantification values (RQ) were calculated to assess the parasite prevalence and can be interpreted as the fold-amount of target gene (*Plasmodium* CYTB) with respect to the amount of the reference gene (*Cx. pipiens* CQ11) and are calculated as $2^{-(Ct\text{CYTB } Plasmodium - Ct\text{CQ11 } Cx.\ pipiens)}$.

Statistical analyses

Analyses were carried out using the R statistical software (v. 4.2.1). Oocyst burden and sporozoite prevalence were analyzed fitting bird as a random factor into the models using *lmer* or *glmer* (package: lme4; Bates *et al.* 2015) according to whether the errors were normally (oocyst burden) or binomially distributed (prevalence of sporozoite infection). Parasitaemia at day 12 post infection, used as a proxy of quantity of parasites ingested by the mosquitoes, was used as fixed factor. Extrinsic incubation period (EIP) was analyzed using a Spearman's rank correlation.

The different statistical models (maximal and minimal models) built to analyze the data are described in the supplementary material (**Table S1**). Maximal models, including all higher-order interactions, were simplified by sequentially eliminating non-significant terms and interactions to establish a minimal model (Crawley 2012). The significance of the explanatory variables was established using a likelihood ratio test (Bolker 2008). The significant Chi-square given in the text are for the minimal

model, whereas non-significant values correspond to those obtained before the deletion of the variable from the model.

Results

This experiment was carried out to investigate whether parasitaemia in the vertebrate host the day of the infectious blood meal underpinned the parasite development within the mosquitoes. Eleven birds were infected with *P. relictum* (SGS1) to generate eleven different parasitaemia ranging from 0.29 to 17.29 % of infected red blood cells. On day 12 post-infection, mosquitoes were allowed to blood-feed on infected birds and then dissected every two days to monitor the dynamic of parasite infection within the vector.

Effect of vertebrate host parasitaemia on the oocyst production dynamics

The temporal dynamic of oocyst production within the mosquitoes was consistent across the parasitaemia (or birds, **Figure 1A**). All mosquitoes already had oocysts in their midgut when we started the dissections (day 4 post blood meal, pbm), thereby we were not able to investigate the relationship between parasitaemia and the first appearance of oocysts. On average, the oocyst burden peaked at day 6 pbm before gradually decreasing due to oocyst bursting and sporozoite release. To investigate whether parasitaemia underpinned the oocyst intensity of infection, we restricted our analyses at the day of the oocyst peak of infection, day 6 pbm, which is the gold standard for microscopic enumeration of oocysts in malaria transmission studies. The oocyst burden within the mosquito midgut did not significantly vary according to the parasitaemia on which they fed (model 1, X^2=1.29, p = 0.25). Mosquitoes feeding on high infected birds had similar oocyst burden than mosquitoes feeding on low infected birds (**Figure 1B**). Differences in parasitaemia in the birds did not drive the development dynamics of the oocyst stage within the mosquitoes.

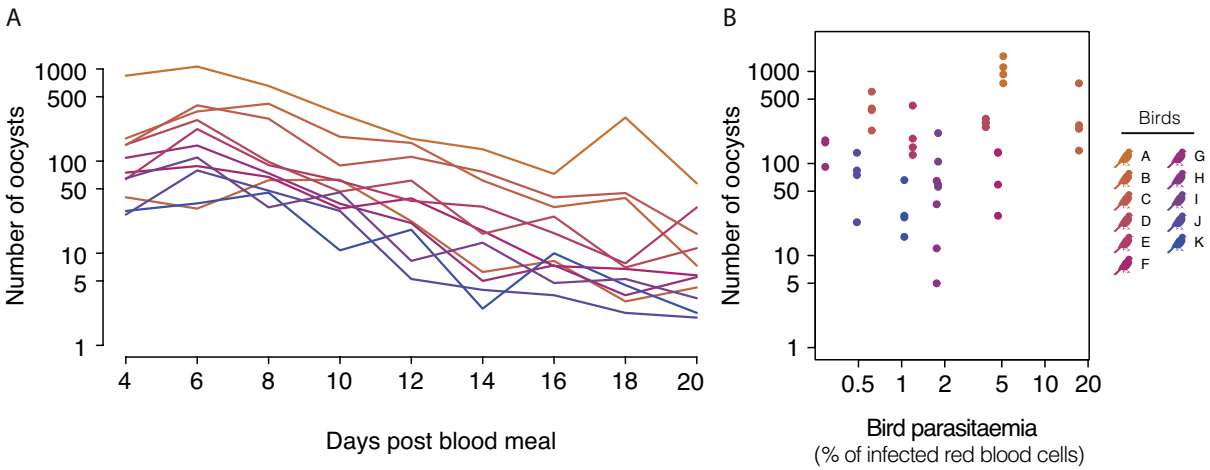

Figure 1: **Relationship between bird parasitaemia and mosquito oocyst burden.** (A) Mean oocyst burden in mosquitoes fed on different birds, i.e. different parasitaemia, over the course of infection. (B) Effect of bird parasitaemia on the mosquito oocyst burden at the peak of oocyst infection, day 6 pbm. Each point corresponds to one mosquito. Note the logarithmic scale of the y and x axes.

Effect of parasitaemia on the EIP

Variations in the prevalence of sporozoite infection (i.e. proportion of mosquitoes that had sporozoites) were observed trough the overall mosquito population (model 2, X^2=252.73, $p < 0.001$, **Figure 2**). This allow us to measure the time of salivary gland invasion by the sporozoites, known as the extrinsic incubation period (EIP). Ten per cent of mosquitoes already had sporozoites in their thorax at day 6 (EIP10: 6 days pbm) while it took up to 2 more days for ninety per cent of the population to show sporozoites (EIP90: 7.8 days pbm).

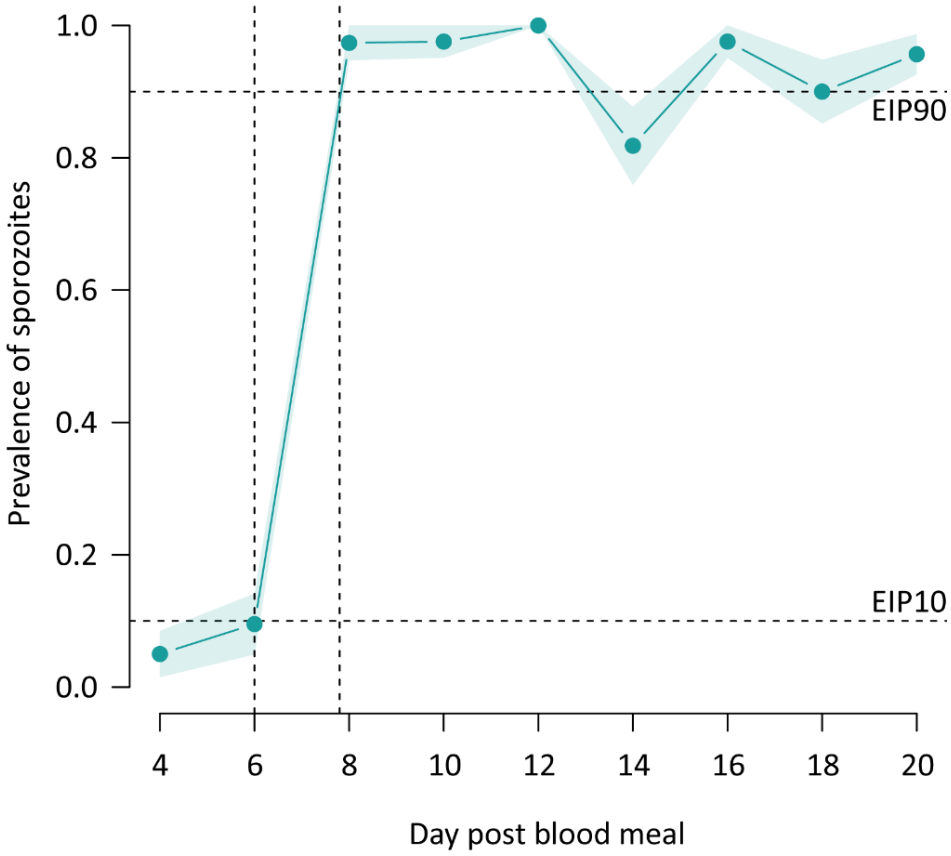

Figure 2: **Prevalence of sporozoite infection**. Proportion of mosquitoes that had sporozoites in their head/thorax for each day of dissection across the overall mosquito population. Shaded area represents the 95% confidence interval. Dashed line highlights the time at which ten and ninety per cent of the mosquito population had sporozoites respectively.

We then investigated whether parasitaemia in the birds might determine the time of salivary glands invasion in the mosquito population that fed on. The EIP, defined for this analysis as the first appearance of sporozoites in mosquito head/thorax, varied significantly according to the parasitaemia of the birds (correlation test, rho = -0.88, $p < 0.05$, **Figure 3**). The higher was the intensity of infection within the bird, the earlier the sporozoites invaded mosquito salivary glands. Females that bit on the most infected birds carried their first sporozoites as early as day 4 pbm which is up to 4 days earlier than females biting the least infected birds.

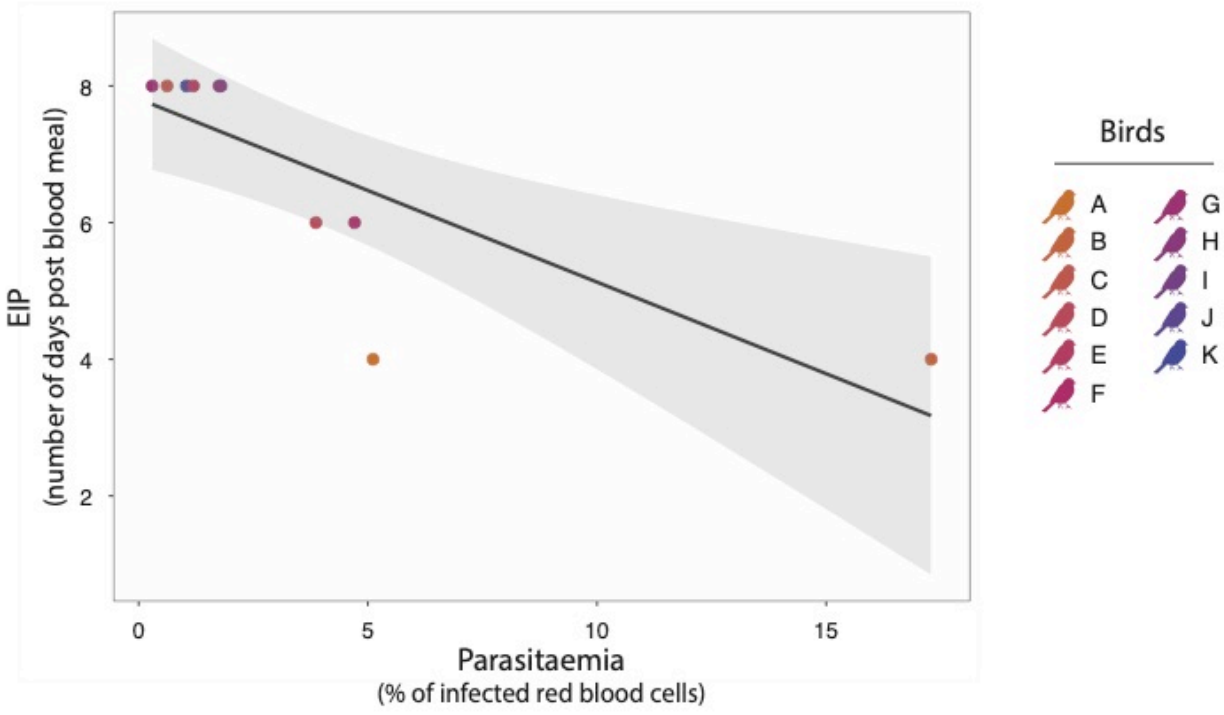

Figure 3: **Relationship between bird parasitaemia and EIP.** Correlation between the time at which the first mosquito had sporozoites in its head/thorax (EIP) and the parasitaemia of the bird it fed on. Spearman correlation test, rho=-0.88, $p < 0.05$. Each point corresponds to the first mosquito having sporozoites for each bird.

Discussion

Our study suggests that the parasitaemia within vertebrate hosts determine the time after biting required for the first vectors to become infectious. It is one of the first times that EIP was reported for the natural association *Cx pipiens* - *P. relictum*. Mosquitoes that blood-fed on highly infected vertebrate hosts were found with sporozoites as soon as four days post blood meal. However, biting on highly infected vertebrate hosts does not seem to affect the intensity of parasite infection within the mosquito: the number of oocysts did not vary according to the vertebrate host parasitaemia. In this study, we highlight that the time of parasite development might be more important, or at least as important as the intensity of infection in terms of transmission.

How does this accelerated development of sporozoites occur?

Density-dependent processes are at play in the regulation of the parasite journey within the vector (Sinden *et al.* 2007; Dawes *et al.* 2009; Churcher *et al.* 2010). *Plasmodium* experiences successive life-stage transitions (gametocytes, ookinetes, oocyst, sporozoites) and go through intense variations in terms of parasite density within the mosquito, especially during the early sporogony. Major bottlenecks occur during both the transition from gametocytes to ookinetes and from ookinetes to oocysts (Sinden *et al.* 2007; Vaughan 2007; Poudel *et al.* 2008). Density-dependent processes occurring during one of these transitions might shapes the formation of sporozoites, affecting either their growth rate or their quality.

Gametocytes-to-ookinetes transition. Gametocyte sex ratio is often female-biased: one male gametocyte produces up to 8 gametes while one female gametocyte produces 1 gamete (Schall 1989; Paul *et al.* 2002; but see Tadesse *et al.* 2019). *Plasmodium* is known to plastically alter its sex ratio in response to within-host environment changes as a form of fertility insurance (Paul *et al.* 2002; West *et al.* 2002; Reece *et al.* 2008; Mitri *et al.* 2009) Vertebrate host infections with high parasitaemia are associated with strong immune responses, decreasing the probability of *Plasmodium* fertilization within the blood meal and successful transmission (Paul *et al.* 2002; West *et al.* 2002). Indeed, even though vertebrate host antibodies target gametes of both sexes, they especially agglutinate male gametes and alter their motility and their ability to find a female gamete and mate (Ramiro *et al.* 2011). One likely hypothesis is that *Plasmodium* male-biases its own sex ratio to ensure fertilization (Schall 2000). Natural selection should favor parasites that adjust their sex ratio, based on cues provided by changes within their vertebrate host (e.g. EPO, lysed RBC levels; Paul *et al.* 2002; Cameron *et al.* 2013; Carter *et al.* 2014) to ensure that a female gamete will encounter a male gamete within the mosquito blood meal. In this context, a simple hypothesis might be that a high number of male gametes competing for female gametes within a highly infected blood meal speeds up fertilization time and consequently the development of the parasites within the mosquito.

Ookinetes-to-oocyst transition. There is growing evidence that the timing of salivary gland invasion might be determined by the parasite burden within the vector (Childs & Prosper 2020; Shaw *et al.* 2020; Stopard *et al.* 2020) and especially, by the density of ookinetes (Childs & Prosper 2020). Churcher et al. (2010) showed that both negative and positive density-dependent processes operate during the ookinetes-to-oocysts transitions. Formation of oocysts appears to be limited at both low and high ookinete densities. The ookinete development into the oocyst stage relies on non-competitive relationship with mosquito resource exploitation: parasites exploit the surplus of nutritional resources that was not invested in oogenesis (Costa *et al.* 2018; Werling *et al.* 2019). In case of ookinetes over-crowding, when mosquito resources become limited, malaria parasites might respond adaptively using two complementary strategies. To avoid intra-specific competition for resources and damage for the vector, *Plasmodium* is expected to reduce its own ookinete population using apoptosis (Al-Olayan *et al.* 2002; Ramiro *et al.* 2011). Along with that, a recent study has experimentally shown that *Plasmodium* respond to metabolic changes due to resource limitation by increasing the speed of its growth rate and becoming infectious sooner (Werling *et al.* 2019). This is consistent with studies trying to model human malaria sporogony, where the fastest growth rates were associated to intermediate levels of ookinetes (Childs & Prosper 2020). A combination of these two strategies might explain why mosquitoes taking highly-parasitized blood meals were infectious earlier with no significant increase in parasite burden. The time of salivary gland invasion may partly be due to density-dependent processes occurring on early sporogony.

To date, density-dependent factors driving the parasite dynamics within the vector are still complex to identify. The adaptive hypothesis seems likely: in case of over-crowding during the gametocytes – to – oocysts transition, *Plasmodium* might respond adaptively by speeding up parasite development in order to ensure its transmission while sparing of the vector. Even though we cannot directly test these hypotheses with our data, they support the possible existence of selective pressure for parasite to modulate growth and reproduction within the vector too (Pollitt *et al.* 2013; Lefevre *et al.* 2018).

What would be the benefit of such strategy?

Vector-to-vertebrate host transmission depends, among other things, on the time at which the vector will be able to take a new blood meal. Mosquitoes blood-feed to get the essential nutrients for the gonotrophic cycle, which is the period of egg development between the blood meal and the oviposition. The duration of the gonotrophic cycle, along with the duration of parasite EIP, delineates the number of opportunities for transmission to a new host. Females *Culex pipiens sp.* mosquitoes are able to oviposit as soon as four days after a blood meal (Vézilier *et al.* 2012; Pigeault & Villa 2018). In our study, mosquitoes fed on highly infected vertebrate hosts already had sporozoites four days following the blood meal while it took eight days to the ones fed on slightly infected hosts. The possibility that *Plasmodium* shifts its EIP as a form of fertility insurance is a likely hypothesis. Under over-crowding context, when vector's resources become limited, shortening EIP, i.e. having sporozoites sooner, matching the end of the first gonotrophic cycle results in (i) one more transmission opportunity and (ii) a refill of nutrients to continue and improve its development within the mosquito (Pigeault & Villa 2018; Shaw *et al.* 2020). Besides the potential to infect more hosts overall, the ability to adjust the speed of its own development might be adaptive when there is a risk that the vector might not live long enough to be able to transmit the parasite.

Why shift in EIP is of epidemiological interest?

EIP is a key determinant transmission trait of vectorial capacity, which is the standard measure of malaria transmission potential (Dye 1992; Rivero *et al.* 2010; Lefevre *et al.* 2018; Ohm *et al.* 2018). Even small changes in EIP might drive the transmission outcomes as it delineates the number of hosts a vector might infect over its lifespan. In this study, we suggest that density-dependent processes occurring during early sporogony play a crucial role in the determination of the parasite extrinsic incubation period. Furthermore, recent studies on human and rodent malaria parasites highlight that multiple factors induce changes in EIP (e.g. temperature, blood meals, nutritive environment of the vector; Carrington *et al.* 2013; Shapiro *et al.* 2016; Ohm *et al.* 2018; Shaw *et al.* 2020). Despite its

epidemiological importance, experimental data are still scarce. Studying the timing of infection under varying conditions and understanding how it shapes sporogony and to what extent this drives malaria transmission potential are needed to reassess accordingly the approaches of disease control programs (Stopard *et al.* 2020).

Caveats of the study?

Some limitations in our study deserve considerations. We are aware that our experimental design based on destructive sampling methods to monitor infection limited our sample sizes. Development of non-invasive methods to follow the course of infection within mosquitoes are ongoing with encouraging results (Fontaine *et al.* 2016; Brugman *et al.* 2018; Gutiérrez-López *et al.* 2019; Ramírez *et al.* 2019; Guissou *et al.* 2021). This will create the opportunity to get a better picture of the *Plasmodium* development within individual mosquito throughout the infection.

References

Albery, G.F., Newman, C., Ross, J.B., MacDonald, D.W., Bansal, S. & Buesching, C. (2020). Negative density-dependent parasitism in a group-living carnivore. *Proceedings of the Royal Society B: Biological Sciences*, 287, 20202655.

Al-Olayan, E.M., Williams, G.T. & Hurd, H. (2002). Apoptosis in the malaria protozoan, *Plasmodium berghei*: a possible mechanism for limiting intensity of infection in the mosquito. *International Journal for Parasitology*, 32, 1133–1143.

Anderson, R.M. & Gordon, D.M. (1982). Processes influencing the distribution of parasite numbers within host populations with special emphasis on parasite-induced host mortalities. *Parasitology*, 85, 373–398.

Anderson, R.M. & May, R.M. (1978). Regulation and Stability of Host-Parasite Population Interactions: I. Regulatory Processes. *The Journal of Animal Ecology*, 47, 219.

Basáñez, M.G., Boussinesq, M., Prod'hon, J., Frontado, H., Villamizar, N.J., Medley, G.F., *et al.* (1994). Density-dependent processes in the transmission of human onchocerciasis: intensity of microfilariae in the skin and their uptake by the simuliid host. *Parasitology*, 108, 115–127.

Bates, D., Mächler, M., Bolker, B. & Walker, S. (2015). Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software*, 67, 1–48.

Bolker, B.M. (2008). *Ecological Models and Data in R*. Princeton University Press. 408pp.

Bousema, T. & Drakeley, C. (2011). Epidemiology and Infectivity of *Plasmodium falciparum* and *Plasmodium vivax* Gametocytes in Relation to Malaria Control and Elimination. *Clinical Microbiology Reviews*, 24, 377–410.

Bradley, J., Stone, W., Da, D.F., Morlais, I., Dicko, A., Cohuet, A., *et al.* (2018). Predicting the likelihood and intensity of mosquito infection from sex specific *Plasmodium falciparum* gametocyte density. *eLife*, 7, e34463.

Bray, R.S. & Garnham, P.C.C. (1982). The life-cycle of primate malaria parasites. *British Medical Bulletin*, 38, 117-122.

Breaux, J.A., Schumacher, M.K. & Juliano, S.A. (2014). What does not kill them makes them stronger: larval environment and infectious dose alter mosquito potential to transmit filarial worms. *Proceedings of the Royal Society B: Biological Sciences*, 281, 20140459.

Brugman, V.A., Kristan, M., Gibbins, M.P., Angrisano, F., Sala, K.A., Dessens, J.T., *et al.* (2018). Detection of malaria sporozoites expelled during mosquito sugar feeding. *Scientific Reports*, 8, 1–9.

Cameron, A., Reece, S.E., Drew, D.R., Haydon, D.T. & Yates, A.J. (2013). Plasticity in transmission strategies of the malaria parasite, *Plasmodium chabaudi*: environmental and genetic effects. *Evolutionary Applications*, 6, 365–376.

Carrington, L.B., Armijos, M.V., Lambrechts, L. & Scott, T.W. (2013). Fluctuations at a Low Mean Temperature Accelerate Dengue Virus Transmission by *Aedes aegypti*. *PLOS Neglected Tropical Diseases*, 7, e2190.

Carter, L.M., Schneider, P. & Reece, S.E. (2014). Information use and plasticity in the reproductive decisions of malaria parasites. *Malaria Journal*, 13, 115.

Chen, L., Swenson, N.G., Ji, N., Mi, X., Ren, H., Guo, L., *et al.* (2019). Differential soil fungus accumulation and density dependence of trees in a subtropical forest. *Science*, 366, 124–128.

Childs, L.M. & Prosper, O.F. (2020). The impact of within-vector parasite development on the extrinsic incubation period. *Royal Society Open Science.*, 7, 192173.

Churcher, T.S., Bousema, T., Walker, M., Drakeley, C., Schneider, P., Ouédraogo, A.L., *et al.* (2013). Predicting mosquito infection from *Plasmodium falciparum* gametocyte density and estimating the reservoir of infection. *eLife*, 2, e00626.

Churcher, T.S., Dawes, E.J., Sinden, R.E., Christophides, G.K., Koella, J.C. & Basáñez, M.-G. (2010). Population biology of malaria within the mosquito: density-dependent processes and potential implications for transmission-blocking interventions. *Malaria Journal*, 9, 311.

Churcher, T.S., Ferguson, N.M. & Basáñez, M.-G. (2005). Density dependence and overdispersion in the transmission of helminth parasites. *Parasitology*, 131, 121–132.

Churcher, T.S., Sinden, R.E., Edwards, N.J., Poulton, I.D., Rampling, T.W., Brock, P.M., *et al.* (2017). Probability of Transmission of Malaria from Mosquito to Human Is Regulated by Mosquito Parasite Density in Naïve and Vaccinated Hosts. *PLOS Pathogens*, 13, e1006108.

Cooper, L., Kang, S.Y., Bisanzio, D., Maxwell, K., Rodriguez-Barraquer, I., Greenhouse, B., *et al.* (2019). Pareto rules for malaria super-spreaders and super-spreading. *Nature Communications*, 10, 3939.

Costa, G., Gildenhard, M., Eldering, M., Lindquist, R.L., Hauser, A.E., Sauerwein, R., *et al.* (2018). Non-competitive resource exploitation within mosquito shapes within-host malaria infectivity and virulence. *Nature Communications*, 9.

Courtenay, O., Peters, N.C., Rogers, M.E. & Bern, C. (2017). Combining epidemiology with basic biology of sand flies, parasites, and hosts to inform leishmaniasis transmission dynamics and control. *PLOS Pathogens*, 13, e1006571.

Crawley, M.J. (2012). *The R Book*. John Wiley & Sons.

Da, D.F., Churcher, T.S., Yerbanga, R.S., Yaméogo, B., Sangaré, I., Ouedraogo, J.B., *et al.* (2015). Experimental study of the relationship between *Plasmodium* gametocyte density and infection success in mosquitoes; implications for the evaluation of malaria transmission-reducing interventions. *Experimental Parasitology*, 149, 74–83.

Das, K. & Sutradhar, B.C. (1995). Score Tests for Fixed Effects and Overdispersion Components in Nonlinear Models for Repeated Measures. *Journal of Multivariate Analysis*, 54, 126–146.

Dawes, E.J., Churcher, T.S., Zhuang, S., Sinden, R.E. & Basáñez, M.-G. (2009). *Anopheles* mortality is both age- and *Plasmodium*-density dependent: implications for malaria transmission. *Malaria Journal*, 8, 228.

Dye, C. (1992). The Analysis Of Parasite Transmission By Bloodsucking Insects. *Annual review of entomology*, 37, 1–19.

Fontaine, A., Jiolle, D., Moltini-Conclois, I., Lequime, S. & Lambrechts, L. (2016). Excretion of dengue virus RNA by *Aedes aegypti* allows non-destructive monitoring of viral dissemination in individual mosquitoes. *Scientific Reports*, 6, 24885.

Fowler, C.W. (1987). A Review of Density Dependence in Populations of Large Mammals. In: *Current Mammalogy* (ed. Genoways, H.H.). Springer US, Boston, MA, pp. 401–441.

Garnham. (1966). *Malaria Parasites and Other Haemosporidia*. Blackwell Scientific Publications. 1132pp.

Glaizot, O., Fumagalli, L., Iritano, K., Lalubin, F., Rooyen, J.V. & Christe, P. (2012). High Prevalence and Lineage Diversity of Avian Malaria in Wild Populations of Great Tits (*Parus major*) and Mosquitoes (*Culex pipiens*). *PLOS ONE*, 7, e34964.

Guissou, E., Waite, J.L., Jones, M., Bell, A.S., Suh, E., Yameogo, K.B., *et al.* (2021). A non-destructive sugar-feeding assay for parasite detection and estimating the extrinsic incubation period of *Plasmodium falciparum* in individual mosquito vectors. *Scientific Reports*, 11, 9344.

Gutiérrez-López, R., Martínez-de la Puente, J., Gangoso, L., Yan, J., Soriguer, R. & Figuerola, J. (2019). Experimental reduction of host *Plasmodium* infection load affects mosquito survival. *Scientific Reports*, 9, 8782.

Hughes, W.O., Petersen, K.S., Ugelvig, L.V., Pedersen, D., Thomsen, L., Poulsen, M., *et al.* (2004). Density-dependence and within-host competition in a semelparous parasite of leaf-cutting ants. *BMC Evolutionary Biology*, 4, 45.

Lefevre, T., Ohm, J., Dabiré, K.R., Cohuet, A., Choisy, M., Thomas, M.B., *et al.* (2018). Transmission traits of malaria parasites within the mosquito: Genetic variation, phenotypic plasticity, and consequences for control. *Evolutionary Applications*, 11, 456–469.

Leggett, H.C., Cornwallis, C.K. & West, S.A. (2012). Mechanisms of Pathogenesis, Infective Dose and Virulence in Human Parasites. *PLOS Pathogens*, 8, e1002512.

Martin, L.B., Addison, B., Bean, A.G.D., Buchanan, K.L., Crino, O.L., Eastwood, J.R., *et al.* (2019). Extreme Competence: Keystone Hosts of Infections. *Trends in Ecology & Evolution*, 34, 303–314.

Medley, G.F., Sinden, R.E., Fleck, S., Billingsley, P.F., Tirawanchap, N. & Rodriguez, M.H. (1993). Heterogeneity in patterns of malarial oocyst infections in the mosquito vector. *Parasitology*, 106, 441–449.

Mitri, C., Thiery, I., Bourgouin, C. & Paul, R.E.L. (2009). Density-dependent impact of the human malaria parasite *Plasmodium falciparum* gametocyte sex ratio on mosquito infection rates. *Proceedings of the Royal Society B: Biological Sciences*, 276, 3721–3726.

Miura, K., Swihart, B.J., Deng, B., Zhou, L., Pham, T.P., Diouf, A., *et al.* (2019). Strong concordance between percent inhibition in oocyst and sporozoite intensities in a *Plasmodium falciparum* standard membrane-feeding assay. *Parasites & Vectors*, 12, 206.

Ohm, J.R., Baldini, F., Barreaux, P., Lefevre, T., Lynch, P.A., Suh, E., *et al.* (2018). Rethinking the extrinsic incubation period of malaria parasites. *Parasites & Vectors*, 11.

Paul, R.E.L., Brey, P.T. & Robert, V. (2002). *Plasmodium* sex determination and transmission to mosquitoes. *Trends in Parasitology*, 18, 32–38.

Pigeault, R., Vézilier, J., Cornet, S., Zélé, F., Nicot, A., Perret, P., *et al.* (2015). Avian malaria: a new lease of life for an old experimental model to study the evolutionary ecology of *Plasmodium*. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 370, 20140300.

Pigeault, R. & Villa, M. (2018). Long-term pathogenic response to *Plasmodium relictum* infection in *Culex pipiens* mosquito. *PLOS ONE*, 13, e0192315.

Pollitt, Laura.C., Churcher, T.S., Dawes, E.J., Khan, S.M., Sajid, M., Basáñez, M.-G., *et al.* (2013). Costs of crowding for the transmission of malaria parasites. *Evolutionary Applications*, 6, 617–629.

Poudel, S.S., Newman, R.A. & Vaughan, J.A. (2008). Rodent *Plasmodium* Population Dynamics of Early Sporogony within *Anopheles stephensi* Mosquitoes. *Journal of Parasitology*, 94, 999–1008.

Ramírez, A.L., van den Hurk, A.F., Mackay, I.M., Yang, A.S.P., Hewitson, G.R., McMahon, J.L., *et al.* (2019). Malaria surveillance from both ends: concurrent detection of *Plasmodium falciparum* in saliva and excreta harvested from *Anopheles* mosquitoes. *Parasites & Vectors*, 12, 355.

Ramiro, R.S., Alpedrinha, J., Carter, L., Gardner, A. & Reece, S.E. (2011). Sex and Death: The Effects of Innate Immune Factors on the Sexual Reproduction of Malaria Parasites. *PLOS Pathogens*, 7, e1001309.

Reece, S.E., Drew, D.R. & Gardner, A. (2008). Sex ratio adjustment and kin discrimination in malaria parasites. *Nature*, 453, 609–614.

Rivero, A., Vézilier, J., Weill, M., Read, A.F. & Gandon, S. (2010). Insecticide Control of Vector-Borne Diseases: When Is Insecticide Resistance a Problem? *PLOS Pathogens*, 6, e1001000.

Schall, J.J. (1989). The sex ratio of Plasmodium gametocytes. *Parasitology*, 98, 343–350.

Schall, J.J. (1990). The ecology of lizard malaria. *Parasitology Today*, 6, 264–269.

Schall, J.J. (2000). Transmission success of the malaria parasite *Plasmodium mexicanum* into its vector: role of gametocyte density and sex ratio. *Parasitology*, 121.

Schmid, M.A., Kauffman, E., Payne, A., Harris, E. & Kramer, L.D. (2017). Preparation of Mosquito Salivary Gland Extract and Intradermal Inoculation of Mice. *Bio-Protocol*, 7.

Shapiro, L.L.M., Murdock, C.C., Jacobs, G.R., Thomas, R.J. & Thomas, M.B. (2016). Larval food quantity affects the capacity of adult mosquitoes to transmit human malaria. *Proceedings of the Royal Society B: Biological Sciences*, 283, 20160298.

Shaw, W.R., Holmdahl, I.E., Itoe, M.A., Werling, K., Marquette, M., Paton, D.G., *et al.* (2020). Multiple blood feeding in mosquitoes shortens the *Plasmodium falciparum* incubation period and increases malaria transmission potential. *PLOS Pathogens*, 16, e1009131.

Sinden, R.E., Dawes, E.J., Alavi, Y., Waldock, J., Finney, O., Mendoza, J., *et al.* (2007). Progression of *Plasmodium berghei* through *Anopheles stephensi* Is Density-Dependent. *PLoS Pathogens*, 3, e195.

Stiling, P.D. (1987). The Frequency of Density Dependence in Insect Host-Parasitoid Systems. *Ecology*, 68, 844–856.

Stone, W.J.R., Eldering, M., Gemert, G.-J. van, Lanke, K.H.W., Grignard, L., Vegte-Bolmer, M.G. van de, *et al.* (2013). The relevance and applicability of oocyst prevalence as a read-out for mosquito feeding assays. *Scientific Reports*, 3, 1–8.

Stopard, I.J., Churcher, T.S. & Lambert, B. (2020). Estimating the extrinsic incubation period of malaria using a mechanistic model of sporogony (preprint). *Microbiology*.

Tadesse, F.G., Meerstein-Kessel, L., Gonçalves, B.P., Drakeley, C., Ranford-Cartwright, L. & Bousema, T. (2019). Gametocyte Sex Ratio: The Key to Understanding *Plasmodium falciparum* Transmission? *Trends in Parasitology*, 35, 226–238.

Takken, W. & van den Berg, H. (2019). *Manual on prevention of establishment and control of mosquitoes of public health importance in the WHO European Region (with special reference to invasive mosquitoes)*. World Health Organization. Regional Office for Europe, Copenhagen.

Valkiunas, G. (2004). *Avian Malaria Parasites and other Haemosporidia*. CRC Press. 946pp.

Vaughan, J.A. (2007). Population dynamics of *Plasmodium* sporogony. *Trends in Parasitology*, 23, 63–70.

Vézilier, J., Nicot, A., Gandon, S. & Rivero, A. (2010). Insecticide resistance and malaria transmission: infection rate and oocyst burden in *Culex pipiens* mosquitoes infected with *Plasmodium relictum*. *Malaria Journal*, 9, 379.

Vézilier, J., Nicot, A., Gandon, S. & Rivero, A. (2012). *Plasmodium* infection decreases fecundity and increases survival of mosquitoes. *Proceedings of the Royal Society B: Biological Sciences*, 279, 4033–4041.

Werling, K., Shaw, W.R., Itoe, M.A., Westervelt, K.A., Marcenac, P., Paton, D.G., *et al.* (2019). Steroid Hormone Function Controls Non-competitive *Plasmodium* Development in *Anopheles*. *Cell*, 177, 315-325.e14.

West, S.A., Smith, T.G., Nee, S. & Read, A.F. (2002). Fertility insurance and the sex ratios of malaria and related hemospororin blood parasites. *Journal of Parasitology*, 88, 258–263.

Wetten, S., Collins, R.C., Vieira, J.C., Marshall, C., Shelley, A.J. & Basáñez, M.-G. (2007). Vector competence for *Onchocerca volvulus* in the Simulium (Notolepria) exiguum complex: Cytoforms or density-dependence? *Acta Tropica*, 103, 58–68.

Woolhouse, M.E.J., Dye, C., Etard, J.-F., Smith, T., Charlwood, J.D., Garnett, G.P., *et al.* (1997). Heterogeneities in the transmission of infectious agents: Implications for the design of control programs. *Proceedings of the National Academy of Sciences*, 94, 338–342.

World malaria report (2019). *Geneva: World Health Organization*; 2019. Licence:CC BY-NC-SA 3.0 IGO.

Table S1: Description of statistical models used in the study. "N" gives the sample size. "Maximal Model" includes the complete set of explanatory variables. "Minimal model" gives the model containing only the significant variables and their interactions. Square brackets indicate variables fitted as random factors. Curly brackets indicate the error structure used (n: normal errors, b: binomial errors). The response variable was not transformed unless otherwise stated.

Variable of interest	Resp. variable	Model Nb.	N	Maximal model	Minimal model	Rsubroutine [err struct.]
Oocyst burden - Day 6 pbm	log(Oocyst+1)	1	40	Para_J12 + (1|Ring)	1 + (1|Ring)	lmer{n}
Sporozoites Prevalence	cbind(Inf, Uninf)	2	348	Day + (1|Ring)	Day + (1|Ring)	glmer{b}

Chapter Two

Different distribution of malaria parasite in left and right extremities of vertebrate hosts translates into differences in parasite transmission

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Authors' contributions.

Contributed to experiment design and conception: R.P., J.I., T.L., P.C.; contributed materials/samples: R.P., J.I., T.L., S.R.Y., A.C., J.B.O., K.R.D.; analysed data: R.P.; contributed to paper writing: R.P., J.I., T.L., S.R.Y., A.C., P.C. All authors reviewed the manuscript.

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Different distribution of malaria parasite in left and right extremities of vertebrate hosts translates into differences in parasite transmission

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Malaria, a vector-borne disease caused by *Plasmodium spp.*, remains a major global cause of mortality. Optimization of disease control strategies requires a thorough understanding of the processes underlying parasite transmission. While the number of transmissible stages (gametocytes) of *Plasmodium* in blood is frequently used as an indicator of host-to-mosquito transmission potential, this relationship is not always clear. Significant effort has been made in developing molecular tools that improve gametocyte density estimation and therefore prediction of mosquito infection rates. However a significant level of uncertainty around estimates remains. The weakness in the relationship between gametocyte burden, measured from a blood sample, and the mosquito infection rate could be explained by a non-homogeneous distribution of gametocytes in the bloodstream. The estimated gametocyte density would then only be a single snapshot that does not reflect the host infectivity. This aspect of *Plasmodium* infection, however, remains largely neglected. In both humans and birds, we found here that the gametocyte densities differed depending on which side of the body the sample was taken, suggesting that gametocytes are not homogeneously distributed within the vertebrate host. We observed a fluctuating asymmetry, in other words, the extremity of the body with the highest density of parasites is not always the same from one individual to another. An estimation of gametocyte density from only one blood sample, as is commonly measured, could, therefore, over- or underestimated the infectivity of gametocyte carriers. This might have important consequences on the epidemiology of the disease since we show that this variation influences host-to-mosquito transmission. Vectors fed on the least infected body part had a lower parasite burden than those fed on the most infected part. The heterogeneous distribution of gametocytes in bloodstream should be considered to improve diagnosis and test new malaria control strategies.

The World Health Organisation estimates that in 2018, there were 228 million cases of malaria worldwide, resulting in more than 405 000 deaths[1]. African countries are disproportionally affected, and while the region experienced a 50% decline in malaria-related mortality between 2000 and 2015, the 2017 and 2018 estimates indicate a recent increase in the number of malaria cases in several countries.

In Africa and elsewhere, control strategies have focused on reducing malaria transmission through early diagnosis and treatment as well as vector control[2]. The efficacy of these interventions is however continually challenged and threatened by the evolution of insecticide[3,4] and drug resistances[5,6]. To overcome resistance issues, the re-emergence of the concept of malaria transmission-blocking strategies[7–10] has boosted the research efforts to find vaccines[11,12], molecules[13,14] or microorganisms[15–17] able to inhibit the transmission of parasites or to disturb the life cycle of *Plasmodium* in the mosquito vector. This vector-borne parasite is also found infecting many other terrestrial vertebrate species, including other mammals, reptiles and birds and may have negative impact on vertebrate host populations[18–20]. A thorough understanding of the fundamental processes underlying the transmission

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of the parasite from the vertebrate host to the mosquito vector is essential to develop transmission-blocking strategies, but also to understand the dynamic of infection in natural populations.

Mosquito blood meal volumes range from 1.5 to 4µl[21,22] and must contain at least one gametocyte (sexual stage) of each sex to result in successful transmission to the mosquito vector. The likelihood of mosquito infection appears to be mainly dictated by gametocyte density[23,24], estimates of which are often used as an indicator of vertebrate host-to-mosquito transmission potential[25–27]. Robust estimation of gametocyte density and its relationship with the likelihood of mosquito infection are, therefore, essential to the identification of the host infection reservoir. While in human and rodent malaria systems the development of sensitive molecular techniques has significantly improved the detection, quantification, and possible sex determination of gametocytes[23,25,28,29], the temporal and spatial dynamics of gametocyte distributions in the vertebrate host, and effects on successful transmission remain relatively neglected. These two important aspects of the dynamics of *Plasmodium* infection may partly explain why the relationship between gametocyte densities measured from blood samples and the infection rates of mosquitoes is often tenuous (Fig. S1, Table S1).

Gametocyte densities are usually estimated from a single blood sample taken from a single location in the body (e.g., finger prick or antecubital venous blood for human, wing vein and tail for bird and rodent respectively, references in Table S1) at a single point in time. Such spatiotemporal snapshots inevitably fail to capture the complex temporal and spatial dynamics of infection within the vertebrate host[30,31]. For example, a recent study found that rodent malaria *P. chabaudi* gametocytes are twice as infective at night despite being less numerous in the blood[32]. Similarly, a periodic, late afternoon increase in parasitemia, coinciding with a peak in biting activity by the mosquito vector, is observed in the avian malaria system[33]. It has also been shown that when infected birds were exposed to mosquito bites during a short period of time (3 hours), parasite transmission from host-to mosquitoes increased gradually with the biting order of vectors[34].

Regarding spatial distribution in the vertebrate host, while mature gametocytes have long been considered to be passively displaced by the blood flow[35–37], suggesting a random or even homogeneous distribution in the peripheral circulatory compartment[38–41], a report of their overdispersion in mosquitoes which fed on three naturally-infected volunteers suggests an aggregated, and not homogeneous, distribution[42,43]. Furthermore, by reviewing the literature, we show that the proportion of studies showing a positive relationship between gametocyte density and transmission of the parasite to the mosquito increases significantly when mosquitoes are fed by an artificial membrane compared to mosquitoes fed directly on the skin of an infected host (Fig. S1, Table S1), a result possibly explained by a lack of heterogeneity in gametocyte spatial distribution in artificial feeding systems. Lastly, gametocyte densities may vary among different body compartments. A handful of studies have reported a higher density of gametocytes in capillary than in venous blood[44–48] but see[49–51]. A single study estimating gametocyte densities from the same blood compartment (veins or capillaries) but from different parts of the body, found a 3-fold higher prevalence of gametocytes in arm capillary blood compared to capillary blood collected from a finger-prick[52].

To date, no study has empirically examined the distribution of *Plasmodium* gametocytes in the peripheral blood compartment of the vertebrate host and the impact on parasite transmission. Our specific aims in this study were to answer two questions. First, are *Plasmodium* gametocyte densities similar (homogenous) between two blood samples taken at the same time but from different body parts of the vertebrate host? If not, we hypothesize that a heterogeneous distribution of gametocyte densities could be detected through random deviations from a symmetric distribution between different body parts (i.e. fluctuating asymmetry[53]). Second, does vertebrate host-to-mosquito transmission of *Plasmodium* vary according to the location of mosquito bites? If gametocyte density is heterogeneous in the bloodstream, we predicted a higher transmission rate in higher infected body part. This work uses both human (*P. falciparum/Anopheles gambiae s.s*) and avian malaria (*P. relictum/Culex pipiens*) systems to measure gametocyte density at two different body locations: the left and right hand in humans and the left and right leg in birds. The presence of directional and/or fluctuating asymmetry was then investigated while taking into account the measurement variations. Due to ethical reasons, the effect of mosquito bite location on parasite transmission was carried out only with the avian malaria system. Avian malaria is the oldest experimental model for investigating the life cycle of *Plasmodium* parasites and is an ideal surrogate for understanding the biology of human malaria parasites[54,55].

Results

Spatial heterogeneity of Plasmodium infection in the vertebrate host. *Human malaria.* Gametocyte densities, from the right and left hands of each volunteer, were determined by two independent microscopists. The repeatability of measurements between observers was highly significant ($R = 0.914$, $CI = 0.866, 0.945$, $p < 0.0001$) and the variation in gametocyte densities within each extremity of each individual represented only 0.01% of the variance. On the other hand, heterogeneity between infected individuals explained a large part of the variance (89%, Fig. 1A). Regarding variation in gametocyte densities between human extremities, while the analysis showed absence of directional asymmetry (i.e. it was not always the same hand, right or left, that had the highest gametocyte density, $F = 0.52$, $P = 0.474$), a fluctuating asymmetry was observed ($\chi^2_1 = 4.50$, $P = 0.033$, Fig. 1A, Fig. S2A). Fluctuating asymmetry explained 2.4% of the total variance in gametocyte density. Although the asymmetry analysis considers the measurement variations, this result may be largely influenced by the individuals showing extremely low intensities of infection. However, when individuals with one or less than one gametocyte on average per 1000 leucocytes per hand, and then individuals with 5 or less than 5 gametocytes, were removed from the dataset, fluctuating symmetry was still detected ($\chi^2_1 = 5.88$, $P = 0.015$, $\chi^2_1 = 4.23$, $P = 0.039$, respectively). The individual asymmetry index corrected for size-dependence averaged 0.67 ± 0.11 for the complete dataset, 0.36 ± 0.05 for the dataset containing only individuals with more than one detected gametocytes and 0.25 ± 0.05 for the dataset containing only individuals with more than 5 detected gametocytes.

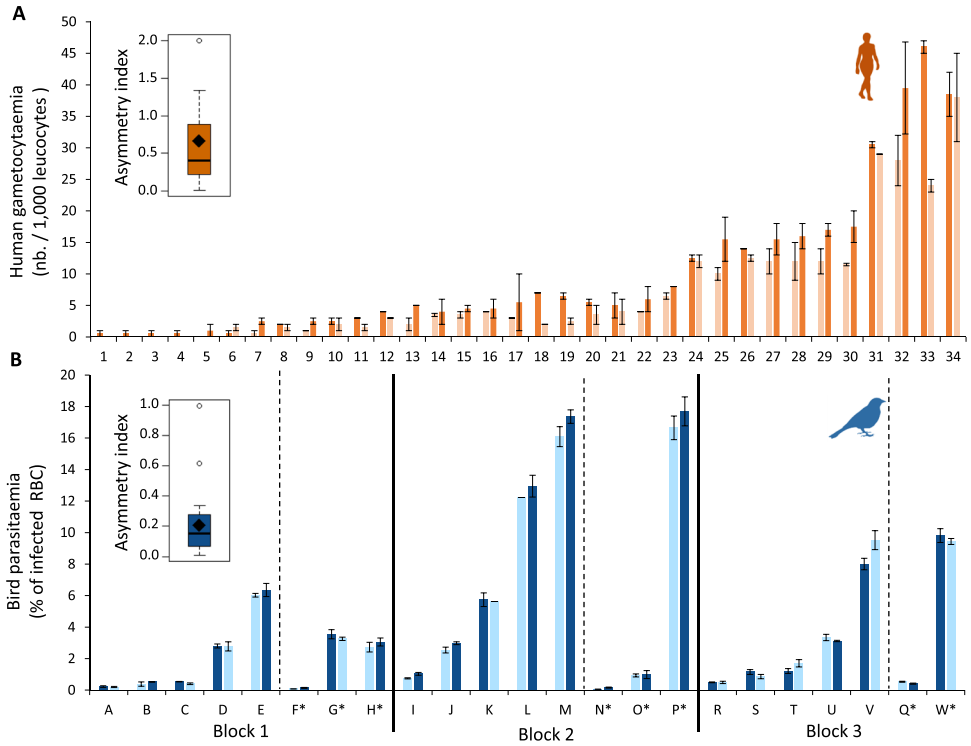

Figure 1. Variation in infection density between two body parts. (**A**) Variation in human gametocyte density (nb. of gametocytes gametocytes per 1000 leucocytes) between the left hand (left bar) and the right hand (right bar). (**B**) Variation in bird parasitaemia (% of infected red blood cells) between the left leg (left bar) and the right leg (right bar). The black vertical lines on panel B separates the three experimental blocks (see materials & methods). Each number (humans) or letter (birds) corresponds to one individual. The * for individuals to the left of the vertical dotted line in each block corresponds birds unexposed to mosquito bites. Light colored bars correspond to the body part with the lower gametocyte density (human) or parasite density (bird), and dark colored bars correspond to the body part with higher densities. Error bars represent standard error around the mean. Boxplots represent the individual asymmetry index corrected for size-dependence. Boxes above and below the medians (horizontal lines) show the first and third quartiles, respectively. Black diamond represent the means.

Avian malaria. For each leg of each bird, three independent measurements of parasitaemia were made from three different drops of blood. The measurements repeatability between the three blood smears was highly significant ($R = 0.987$ $CI = 0.980, 0.992$, $p < 0.0001$). Variation in parasite densities within each leg of each bird represents only 0.03% of the variance. As for human malaria, heterogeneity between birds explains a large part of the variance (97%, Fig. 1B). While we showed absence of directional asymmetry ($F = 2.45$, $P = 0.135$), a fluctuating asymmetry of parasitaemia between the two bird sides was observed (1.7% of the variance, $\chi^2_1 = 10.81$, $P = 0.001$, Fig. 1B, Fig. S2B). When individuals with a parasitaemia of less than 0.1% where removed from the dataset, the fluctuating asymmetry of parasite density was still detected ($\chi^2_1 = 9.052$, $P = 0.002$). However, when individuals with a parasitaemia of less than 0.5% where removed from the dataset, the fluctuating asymmetry was no longer detected ($\chi^2_1 = 1.014$, $P = 0.314$). The individual asymmetry index corrected for size-dependence averaged 0.21 ± 0.04 for the full dataset, 0.17 ± 0.03 for the dataset containing only birds with a parasitaemia of more than 0.1% and 0.13 ± 0.02 for the dataset containing only birds with a parasitaemia of more than 0.5%.

Parasite transmission to mosquito vectors. To investigate the effect of the location of mosquito bites on *Plasmodium* transmission, the left and right legs of infected birds (*Serinus canaria*) were independently and simultaneously exposed to mosquitoes (*Culex pipiens*) for 3 hours. Immediately following exposure to mosquitoes, blood from both legs was collected to measure parasite densities (see above), and each leg was classified as either lower infected leg (LIL) or higher infected leg (HIL). Blood-fed mosquitoes were dissected one week post blood meal to count the number of parasites in their midgut (oocyst stage).

We found no significant differences among mosquitoes fed on the lower (LIL) or on the higher infected leg (HIL) in either the proportion of females taking a blood meal (mean ± s.e., $HIL = 0.44 \pm 0.05$, $LIL = 0.36 \pm 0.04$; $\chi^2_1 = 3.03$ $P = 0.082$), blood meal size ($HIL = 15.01 \pm 0.67$, $LIL = 14.57 \pm 0.63$; $\chi^2_1 = 0.795$ $P = 0.373$), or infection prevalence (proportion of mosquitoes containing at least 1 oocyst, $HIL = 0.40 \pm 0.04$, $LIL = 0.41 \pm 0.04$; $\chi^2_1 = 0.005$ $P = 0.994$). The analysis of oocyst burden included only mosquitoes having at least one oocyst in the midgut. We found that infected females who fed on the HIL had a significantly higher oocyst burden than females fed on the LIL ($HIL = 12.70 \pm 2.18$ vs. $LIL = 7.79 \pm 1.93$; $\chi^2_1 = 5.24$ $P = 0.022$, Fig. 2).

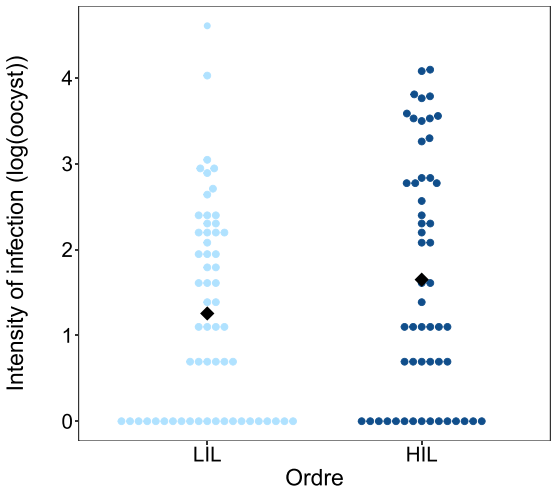

Figure 2. Oocyst burden in mosquitoes fed on either the lower infected leg (LIL) or on the higher infected leg (HIL). Black diamond represent the means.

Discussion

Reducing *Plasmodium* transmission from the vertebrate host to the insect vector is a critical component of global efforts to control malaria[56]. Understanding the processes underlying the relationship between *Plasmodium* gametocyte densities and mosquito infection is, therefore, crucial to assess the effectiveness of control programs and their effects on transmission. An essential first step is to obtain accurate estimations of gametocyte densities in an infected host. To this end, new molecular tools have been developed to detect and estimate more precisely gametocyte densities, including densities of males and females. It is noteworthy that these methods have significantly improved the prediction of mosquito infection rates[23,51]. Nevertheless, a significant level of uncertainty in predicting mosquito infection rates remains.

Another factor potentially affecting the development of better predictors of parasite transmission to the mosquito host is variation in the spatial distribution of mature gametocytes within the vertebrate host[42,43]. Although largely ignored, a non-homogeneous distribution of the parasite in the blood could bias the estimation of the vertebrate host infectivity. In the vast majority of studies, gametocyte densities are exclusively estimated from a blood sample taken from a single body location[24,55,57–61]. The objective of our study was to assess variation in parasite densities among different locations of a similar organ (hand or leg) at a given timestamp (i.e., sampling time), rather than to specifically measure differences between the right and left extremities of individuals. Here, we showed that the gametocyte densities differed depending on which side of the body the sample was taken, suggesting that gametocytes are not homogeneously distributed within the vertebrate host. In both humans and birds, we observed a fluctuating asymmetry, in other words it was not always the same body extremities, right or left, that had the highest parasite density.

The differences in parasite load observed between the extremities of vertebrate hosts may impact the transmission of *Plasmodium* to mosquitoes. Indeed, in the birds we observed that mosquitoes fed on the least infected body part had a lower parasite burden than those fed on the most infected part. In turn, variation in oocyst density could have epidemiological consequences as recent work suggested that increased parasite burden in mosquitoes can result in increased probability of transmission to vertebrate hosts[62,63]. Consequently, using a single measure of gametocyte density from a single blood sample taken from only one side of the body, may not provide an accurate estimate of a host's infectivity. Our results suggest that the most efficient way to obtain a more accurate estimate of gametocyte density, and, therefore, a better predictor of infectiousness, would be to combine several independent density measurements from different body parts.

The mechanisms leading to the establishment of a non-homogeneous distribution of *Plasmodium* in the blood of the vertebrate host are unknown. Gametocytes are not motile and cannot actively migrate to accumulate in the capillaries. Passive accumulation of gametocytes in some sub-dermal capillaries could induce a non-homogeneous distribution of *Plasmodium* in the vertebrate host. For instance, the elongated asymmetric curvature of *P. falciparum* gametocytes may facilitate their blockage in the dermal capillaries[64]. Mature gametocyte aggregation might also partly explain the spatial heterogeneity in the distribution of gametocytes[42,65]. Active aggregation mechanisms potentially involving binding interactions between infected red blood cells containing late developmental stages of gametocytes have however not been observed in either human or avian malaria parasites ("rosetting-like" adhesion[51,66]). Nevertheless, a study described a very unusual clustering behavior between *Leucocytozoon toddi* gametocytes (another haemosporidian parasite). Authors observed significant proportions of male and female gametocytes in aggregations involving substantial contact[67].

When the gametocyte density is low, an adaptive strategy allowing the aggregation of several sexual stages of *Plasmodium* within a blood capillary or even within a same red blood cell[68] may increase the probability that a mosquito will be infected[42,43,69]. In this case, while the majority of blood-fed mosquitoes did not ingest any parasite, those biting an area containing aggregated gametocytes will be undoubtedly infected by malaria. However, the benefits of such a strategy are reduced once gametocyte density increases to a level ensuring the successful

transmission regardless of the mosquito biting site. In this case, homogeneous distribution should maximize transmission[43]. Thus, a plasticity in the level of aggregation in response to changes in gametocyte density would allow for optimization of transmission to the mosquito vector throughout the infection.

Although our data did not allow us to test this prediction we observed that the asymmetry index does indeed seem to be lower in hosts with higher parasite densities. In addition, in the avian malaria system, when individuals with low gametocytaemia (<0.5%) were removed from the analysis, the fluctuating asymmetry was no longer detected. The distribution of parasites within highly infected birds therefore appeared to be more homogeneous than that observed in the lowest infected hosts. Nevertheless, another explanation could be that the non-homogeneous distribution of *Plasmodium* gametocytes detected in weakly infected birds is an artifact which could be due to a stochastic effect (*i.e.* the chance to detect very few parasites regardless of the body location). Although in human subjects, fluctuating asymmetry was always detected even after removing individuals with low gametocyte loads, it should be noted that only one blood sample was taken at each hand (due to ethical reasons). Gametocyte density was estimated by two independent microscopists and our analyses consider measurement variations but it cannot be ruled out that by increasing the number of blood samples taken from each extremity of humans, the difference between the two locations will diminish. Nevertheless, whether the difference in parasite density observed here is due to heterogeneity in the distribution of gametocytes between extremities or to stochastic effects during sampling, in both cases it would affect the mosquitoes when they take blood meals and thus increase the heterogeneity of vector exposure.

Given that malaria infection is temporally dynamic[30,33], the single measurement used in this study to compare the number of parasites between different sides of the body does not allow us to determine whether the apparent non-homogeneous distribution changes over time and is simply a single snapshot of an underlying process that is more complex and dynamic. A logical next step is to monitor gametocyte densities at different body parts with repeated measurements over the course of the infection. Furthermore, of particular relevance would be to compare gametocyte densities among different body locations in regard to variation in mosquito attraction to these sites. For instance, it is known that the major vectors of *P. falciparum* (*An. gambiae s.s.*, *An. arabiensis*, *An. funestus*) all have a strong preference for feeding close to the ground which is associated to increased biting rate on legs, ankles, and feet[70,71]. However, a recent study of 8 infected donors found no significant increase in gametocyte density estimated from leg skin biopsies compared to that from arm skin as would be predicted[51].

Improving the detection and estimation of gametocyte density in infected hosts is fundamental to improve the diagnosis of gametocyte carriers and therefore identify infectious reservoirs but also to develop and test new malaria control strategies. In this study, we found that the gametocyte burden varies between different body parts. We argue that it is essential to collect several blood samples from different body parts to depict accurately gametocyte density and infectiousness. We further propose that such sampling, combined with a better understanding of mosquito biting preferences, may better our understanding of within-host malaria infection dynamics and, ultimately, the fundamental processes underlying parasite transmission from human-to-mosquito.

Materials and Methods

Human malaria. The study was conducted at the Institut de Recherche en Sciences de la Santé in Bobo Dioulasso, South-Western Burkina Faso. The intensity of malaria transmission is high and perennial in this area, with a peak from August to November. Blood slides were collected from December 2018 to July 2019 from 42 asymptomatic children aged 5–12 years attending the elementary schools of Dandé, Soumousso, Klesso, Samandeni - four villages located in the surroundings of Bobo Dioulasso. *P. falciparum* is the predominant parasite species in these villages, accounting for more than 95% of malaria cases[72].

Separate finger-prick blood samples from the right and left hand of each volunteer were collected, Giemsa-stained and screened for asexual parasites and gametocytes. Gametocyte densities were determined from each slide as the number of gametocytes per 1000 leucocytes. Each slide was read twice by two independent qualified microscopists[72]. Slides were declared negative after a minimum reading of 100 fields. We discovered 8 gametocyte-free individuals leaving 34 individuals for analysis.

Avian malaria. *Parasite strain.* *Plasmodium relictum* is the most prevalent form of avian malaria in Europe[73]. The lineage used in these experiments (lineage SGS1) was isolated from infected great tits (*Parus major*) caught in the region of Lausanne (Switzerland) in 2015. The strain has since been maintained by regular passage between infected and naïve canaries (*Serinus canaria*) via intraperitoneal injection. Twenty three uninfected canaries were split into three experimental blocks (Block 1: 8, Block 2: 8, Block 3: 7) and inoculated by means of an intraperitoneal injection of 150–200 μL of a blood mixture collected from five chronically infected canaries. Birds in the same experimental block were infected with a blood mixture from an independent group of infected birds. For each block infected birds were then either "exposed" (block 1, 2, 3 = 5) or "unexposed" (block 1, 2 = 3, block 3 = 2) to mosquito bites (Fig. 1B).

Mosquito rearing. *Culex pipiens* mosquitos used in the experiment were from a population collected from the field (Lausanne, 46°31′25.607″N 6°34′40.714″E, altitude: 380 m) in August 2017 and since maintained under laboratory conditions. Mosquitoes were reared as described by Vézilier *et al.*[74] in an insectary at 25 °C ± 1 °C, 70 ± 5% RH and with 12 L:12D photoperiod. On the day prior to mosquito exposure, 500 7–10 day old female mosquitoes were haphazardly chosen from different emergence cages and placed inside new cages (100 females per cage). During this time females were deprived of sugar solution to increase hunger levels and maximize the biting rate. Water was provided to prevent dehydration, but removed 6 hours prior to the start of the experiment.

Experimental design. The three experimental blocks were carried out in February, March and April 2018 respectively. Twelve days after infection, coinciding with the acute phase of the *P. relictum* infection in canaries[55], birds

were placed individually into compartmentalized cages designed for physically separating their two legs. For birds in the "exposed" group, at 6:00 pm, 45–50 uninfected female mosquitoes were added to each compartment (left and right) for 180 minutes. Unexposed birds were placed under the same experimental conditions but without mosquitoes. At the end of the mosquito exposure period (9:00 pm), a red lamp was used to capture mosquitoes and five microliters of blood was collected from the medial metatarsal vein of each leg. Three independent drops of blood were then smeared onto three different microscope slides for each of the samples. Blood fed mosquitoes were placed individually into a numbered plastic tube covered with a mesh. Food was provided in the form of a cotton pad soaked in a 10% sugar solution placed on top of each tube. Mosquitoes were dissected 7 to 8 days later and the number of *Plasmodium* oocysts in their midgut counted with the aid of a binocular microscope[74]. Haematin excreted at the bottom of each plastic tube was quantified as an estimate of the female's blood meal size[74].

The intensity of bird infection (parasitaemia) was determined visually by counting the number of infected red blood cells per 3000 erythrocytes in randomly chosen fields on the blood smears[73]. The three replicate were used to calculate an average parasitaemia (mean ± SE) for each leg of each bird. The legs of each bird were then classified as either the lower infected leg (LIL) or higher infected leg (HIL). All slides were examined by the same experimenter, and parasitaemia was used as a proxy for transmissible stage (gametocytes) production because parasitaemia and gametocytaemia are strongly positively correlated in this system (see Fig. 2 in[55]).

Statistical analyses. Analyses were carried out using the R statistical package (v. 3.4.1, http://www.cran.r-project.org/).

Repeatability estimation. The repeatability estimation of the parasite load between observers, in the case of samples from humans, and between slides within the same extremities, in the case of birds, were obtained using the intra-class correlation coefficient (package rptR[75]).

Asymmetry of parasite densities. The presence of directional and/or fluctuating asymmetry (DA and FA, respectively), while taking sampling or measurement variation into account, for avian and human malaria system respectively, was tested by the restricted maximum-likelihood (REML) estimation of a mixed regression model where we fitted the models to the repeated measurements of right and left parasite density sides[76]. Side (right or left) was included as fixed effects in the models and log-transformed trait values (gametocyte number or parasitaemia) as response variables, individual identity and side were fitted as a random effects. This procedure allows the separation of measurement error from bilateral asymmetry analysis[76]. The presence of DA was assessed by the F-test of the fixed effect (side[76]), with degrees of freedom corrected for statistical dependence by Satterthwaite formulae[77]. We used random intercepts and the fixed slopes (estimated within individuals) to estimate the variation in individual trait value and the individual FA, respectively. The significance of FA was then calculated by performing a likelihood ratio test (LRT) comparing two models: the original full model and a reduced model without the side as a random effect[76].

The presence of antisymmetry was excluded after inspection of the distributions of the right (R) minus left (L) side values for gametocyte densities and parasitaemias in humans and birds, respectively (Kolmogorov-Smirnov test, Human: D = 0,229, P = 0,057; Bird D = 0,199, P = 0,260[53,78]). As our results indicated the existence of fluctuating asymmetry in human and avian malaria system (see Results), we calculated an individual asymmetry index (AI) as the unsigned right-left difference ($|R\text{-}L|$) between the average of trait values across the two (human) or three (bird) replicate counts of each individual[53,79]. Trait size dependence was examined with a non-parametric test of association (Spearman test) between unsigned AI values ($|R-L|$) and $(R+L)/2$. These analyses revealed positive trait size dependence for both human and avian malaria system (human: S = 2956, p = 0.0009, Rho = 0.55; bird: 384, $p < 0.0001$, Rho = 0.83). We therefore calculated a new asymmetry index with a correction for size-dependence ($AIcor = |R-L|/(R+L)/2$ [63,65]).

The part of variance explained by difference in counts between observers or repeated slides, for humans and birds respectively, was estimated from a mixed model with observers or repeated slides as a fixed factor, and side (left, right), nested in individual, as a random factor (MuMin package[80]). The part of variance explained by both heterogeneities between host and by FA were estimated from the maximal model described previously to test the presence of DA or FA.

Parasite transmission to mosquito vector. Mixed effects models were used to analyze the effect of bird leg infection class (LIL or HIL) on the mosquito blood meal rate (proportion of females that had taken a blood meal), blood meal size and *Plasmodium* transmission to the vector. The explanatory variables leg class (LIL or HIL) and haematin (when it was appropriate) were fitted as fixed factors. Individual, nested within experimental block, was fitted as a random factor. Blood meal rate and infection prevalence (oocyst presence/absence) were analyzed using GLMM with a binomial error distribution (lme4 package[81]). Blood meal size and mosquito infection intensity (number of oocysts) were analyzed using lmer with normal error distribution. For the analysis of infection intensity, only individuals that developed ≥ 1 oocyst were included. Maximal models, including all higher-order interactions, were simplified by eliminating non-significant terms and interactions to establish a minimal model[82]. Non-significant interactions and terms were removed step by step according to their significance using either a likelihood ratio test or an F test[83]. The significant Chi-square or F values given in the text are for the minimal model, whereas non-significant values correspond to those obtained before the deletion of the variable from the model.

Ethics statement. Human blood samples were collected prior to treatment with a dose of artemether–lumefantrine according to National Malaria Control Programme recommendation and after written informed consent was obtained from the parent(s) or guardian(s). Ethical clearance was provided by the national ethics committee

of Burkina Faso (no. 2018-9-118) and the institutional committee of the Institut de Recherche en Sciences de la Santé (no. A06-2018/CEIRES). The methods used in this study were performed in accordance with the relevant guidelines of the national ethics committee of Burkina Faso and the institutional committee of the Institut de Recherche en Sciences de la Santé. The animal care and protocols used in the avian malaria study was approved by the Ethical Committee of the Vaud Canton veterinary authority, Switzerland (authorization number 1730.4). The methods used in this study were performed in accordance with the relevant guidelines and regulations of the Vaud Canton veterinary authority (Switzerland).

Data availability

All data supporting the conclusions of this paper are available on the Dryad website: https://doi.org/10.5061/dryad.6djh9w0z5.

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References

1. WHO | World malaria report 2019. *WHO*, https://www.who.int/publications-detail/world-malaria-report-2019.
2. Bhatt, S. *et al*. The effect of malaria control on *Plasmodium falciparum* in Africa between 2000 and 2015. *Nature*. **526**, 207–211 (2015).
3. Weill, M. *et al*. Comparative genomics: Insecticide resistance in mosquito vectors. *Nature* **423**, 136–137 (2003).
4. Ranson, H. & Lissenden, N. Insecticide resistance in African anopheles mosquitoes: A worsening situation that needs urgent action to maintain malaria control. *Trends Parasitol.* **32**, 187–196 (2016).
5. Price, R. N. *et al*. Mefloquine resistance in *Plasmodium falciparum* and increased pfmdr1 gene copy number. *Lancet Lond. Engl.* **364**, 438–447 (2004).
6. Ariey, F. *et al*. A molecular marker of artemisinin-resistant *Plasmodium falciparum* malaria. *Nature* **505**, 50–55 (2014).
7. Carter, R. Transmission blocking malaria vaccines. *Vaccine* **19**, 2309–2314 (2001).
8. Carter, R. & Chen, D. H. Malaria transmission blocked by immunisation with gametes of the malaria parasite. *Nature* **263**, 57 (1976).
9. Doumbo, O. K., Niaré, K., Healy, S. A. & Duffy, I. S. and P. E. Malaria transmission-blocking vaccines: Present status and future perspectives. In: *Towards Malaria Elimination-A Leap Forward*. IntechOpen. (2018).
10. Acquah, F. K., Adjah, J., Williamson, K. C. & Amoah, L. E. Transmission-blocking vaccines: old friends and new prospects. *Infect. Immun.* **87**, e00775–18 (2019).
11. Wu, Y., Sinden, R. E., Churcher, T. S., Tsuboi, T. & Yusibov, V. Chapter three - Development of malaria transmission-blocking vaccines: From concept to product. in *Advances in Parasitology* (eds. Rollinson, D. & Stothard, J. R.) vol. 89 109–152 (Academic Press, 2015).
12. Bompard, A. *et al*. Evaluation of two lead malaria transmission blocking vaccine candidate antibodies in natural parasite-vector combinations. *Sci. Rep.* **7**, 1–9 (2017).
13. Mathias, D. K. *et al*. A Small molecule glycosaminoglycan mimetic blocks *Plasmodium* invasion of the mosquito midgut. *PLOS Pathog.* **9**, e1003757 (2013).
14. Niu, G. *et al*. Targeting mosquito FREP1 with a fungal metabolite blocks malaria transmission. *Sci. Rep.* **5**, 14694 (2015).
15. Dong, Y., Manfredini, F. & Dimopoulos, G. Implication of the mosquito midgut microbiota in the defense against malaria parasites. *PLoS Pathog* **5**, e1000423 (2009).
16. Cirimotich, C. M., Ramirez, J. L. & Dimopoulos, G. Native microbiota shape insect vector competence for human pathogens. *Cell Host Microbe* **10**, 307–310 (2011).
17. Hughes, G. L., Rivero, A. & Rasgon, J. L. *Wolbachia* can enhance *Plasmodium* infection in mosquitoes: Implications for malaria control? *PLOS Pathog.* **10**, e1004182 (2014).
18. Atkinson, C. T., Woods, K. L., Dusek, R. J., Sileo, L. S. & Iko, W. M. Wildlife disease and conservation in Hawaii: pathogenicity of avian malaria (*Plasmodium relictum*) in experimentally infected iiwi (*Vestiaria coccinea*). *Parasitology* **111**(Suppl), S59–69 (1995).
19. Atkinson, C. T. & Samuel, M. D. Avian malaria *Plasmodium relictum* in native Hawaiian forest birds: epizootiology and demographic impacts on apapane *Himatione sanguinea*. *J. Avian Biol.* **41**, 357–366 (2010).
20. Ewen, J. G. *et al*. Establishment of exotic parasites: the origins and characteristics of an avian malaria community in an isolated island avifauna. *Ecol. Lett.* **15**, 1112–1119 (2012).
21. Ichimori, K. Correlation of mosquito size, blood meal size and malarial oocyst production. *Med. Entomol. Zool.* **40**, 81–85 (1989).
22. Jeffery, G. M. Blood meal volume *in Anopheles quadrimaculatus*, *A. albimanus* and *Aedes aegypti*. *Exp. Parasitol.* **5**, 371–375 (1956).
23. Bradley, J. *et al*. Predicting the likelihood and intensity of mosquito infection from sex specific *Plasmodium falciparum* gametocyte density. *eLife* **7**, e34463 (2018).
24. Da, D. F. *et al*. Experimental study of the relationship between *Plasmodium* gametocyte density and infection success in mosquitoes; implications for the evaluation of malaria transmission-reducing interventions. *Exp. Parasitol.* **149**, 74–83 (2015).
25. Ouédraogo, A. L. *et al*. Dynamics of the human infectious reservoir for malaria determined by mosquito feeding assays and ultrasensitive malaria diagnosis in Burkina Faso. *J. Infect. Dis.* **213**, 90–99 (2016).
26. Koepfli, C. & Yan, G. *Plasmodium* gametocytes in field studies: Do we measure commitment to transmission or detectability? *Trends Parasitol.* **34**, 378–387 (2018).
27. Slater, H. C. *et al*. The temporal dynamics and infectiousness of subpatent *Plasmodium falciparum* infections in relation to parasite density. *Nat. Commun.* **10**, 1433 (2019).
28. Drew, D. R. & Reece, S. E. Development of reverse-transcription PCR techniques to analyse the density and sex ratio of gametocytes in genetically diverse *Plasmodium chabaudi* infections. *Mol. Biochem. Parasitol.* **156**, 199–209 (2007).
29. Gruenberg, M. *et al*. qRT-PCR versus IFA-based quantification of male and female gametocytes in low-density *Plasmodium falciparum* infections and their relevance for transmission. *J. Infect. Dis.* **221**(4), 598–607 (2020).
30. O'Meara, W. P., Collins, W. E. & McKenzie, F. E. Parasite prevalence: A static measure of dynamic infections. *Am. J. Trop. Med. Hyg.* **77**, 246–249 (2007).
31. Färnert, A. *Plasmodium falciparum* population dynamics: only snapshots in time? *Trends Parasitol.* **24**, 340–344 (2008).
32. Schneider, P. *et al*. Adaptive periodicity in the infectivity of malaria gametocytes to mosquitoes. *Proc. Biol. Sci.* **285**, (2018).
33. Pigeault, R., Caudron, Q., Nicot, A., Rivero, A. & Gandon, S. Timing malaria transmission with mosquito fluctuations. *Evol. Lett.* **2**, 378–389 (2018).
34. Isaïa, J., Rivero, A., Glaizot, O., Christe, P. & Pigeault, R. Last-come, best served? Mosquito biting order and *Plasmodium* transmission. *bioRxiv* **031625** (2020).
35. Huff, C. G. & Bloom, W. A malarial parasite infecting all blood and blood-forming cells of birds. *J. Infect. Dis.* **57**, 315–336 (1935).
36. Janse, C. J. *et al*. *In vitro* formation of ookinetes and functional maturity of *Plasmodium berghei* gametocytes. *Parasitology* **91**, 19–29 (1985).
37. Douglas, R. G., Amino, R., Sinnis, P. & Frischknecht, F. Active migration and passive transport of malaria parasites. *Trends Parasitol.* **31**, 357–362 (2015).

38. Boyd, M. F., Stratman-Thomas, W. K. & Kitchen, S. F. On the relative susceptibility of *Anopheles quadrimaculatus* to *Plasmodium vivax* and *Plasmodium falciparum*. *Am. J. Trop. Med. Hyg.* **s1-15**, 485–493 (1935).
39. Muirhead-Thomson, R. C. & Mercier, E. C. Factors in malaria transmission by *Anopheles albimanus* in Jamaica. *Ann. Trop. Med. Parasitol.* **46**, 103–116 (1952).
40. Smalley, M. E., Abdalla, S. & Brown, J. The distribution of *Plasmodium falciparum* in the peripheral blood and bone marrow of Gambian children. *Trans. R. Soc. Trop. Med. Hyg.* **75**, 103–105 (1981).
41. Pritsch, M. *et al*. Stability of gametocyte-specific Pfs25-mRNA in dried blood spots on filter paper subjected to different storage conditions. *Malar. J.* **11**, 138 (2012).
42. Pichon, G., Awono-Ambene, H. P. & Robert, V. High heterogeneity in the number of *Plasmodium falciparum* gametocytes in the bloodmeal of mosquitoes fed on the same host. *Parasitology* **121**(Pt 2), 115–120 (2000).
43. Gaillard, F. O., Boudin, C., Chau, N. P., Robert, V. & Pichon, G. Togetherness among *Plasmodium falciparum* gametocytes: interpretation through simulation and consequences for malaria transmission. *Parasitology* **127**, 427–435 (2003).
44. Sinden, R. E. Sexual development of malarial parasites. in *Advances in Parasitology* (eds. Baker, J. R. & Muller, R.) vol. 22 153–216 (Academic Press, 1983).
45. Ouedraogo, J., Guiguemde, T. & Gbary, A. Etude comparative de la densite parasitaire de *Plasmodium falciparum* dans le sang capillaire et dans le sang veineux chez des porteurs asymptomatiques (région de Bobo-Dioulasso, Burkina Faso). *Médecine D'Afrique Noire* **38**, 601–605 (1991).
46. Njunda, A., Assob, N., Nsagha, S., Mokenyu, M. & Kwenti, E. Comparison of capillary and venous blood using blood film microscopy in the detection of malaria parasites: A hospital based study. *Sci. J. Microbiol.* **2**, 89–94 (2013).
47. Kast, K. *et al*. Evaluation of *Plasmodium falciparum* gametocyte detection in different patient material. *Malar. J.* **12**, 438 (2013).
48. Mischlinger, J. *et al*. Use of capillary blood samples leads to higher parasitemia estimates and higher diagnostic sensitivity of microscopic and molecular diagnostics of malaria than venous blood samples. *J. Infect. Dis.* **218**, 1296–1305 (2018).
49. Sandeu, M. M. *et al*. Do the venous blood samples replicate malaria parasite densities found in capillary blood? A field study performed in naturally-infected asymptomatic children in Cameroon. *Malar. J.* **16**, 345 (2017).
50. Lehane, A. *et al*. Comparison on simultaneous capillary and venous parasite density and genotyping results from children and adults with uncomplicated malaria: a prospective observational study in Uganda. *BMC Infect. Dis.* **19**, 559 (2019).
51. Meibalan, E. *et al*. *P. falciparum* gametocyte density and infectivity in peripheral blood and skin tissue of naturally infected parasite carriers. *J Infect Dis. jiz680* (2019).
52. van den Berghe, L., Chardome, M. & Peel, E. Supériorité des préparations de scarification du derme sur les préparations de sang périphérique pour le diagnostic de malaria. *Instit Med Trop* **9**, 553–562 (1952).
53. Palmer, A. R. & Strobeck, C. Fluctuating asymmetry analyses revisited. in *Developmental instability: causes and consequences* 279–319 (2003).
54. Rivero, A. & Gandon, S. Evolutionary ecology of avian malaria: Past to present. *Trends Parasitol.* **34**(8), 712–726 (2018).
55. Pigeault, R. *et al*. Avian malaria: a new lease of life for an old experimental model to study the evolutionary ecology of *Plasmodium*. *Phil Trans R Soc B* **370**, 20140300 (2015).
56. Alonso, P. L. *et al*. A Research agenda to underpin malaria eradication. *PLOS Med.* **8**, e1000406 (2011).
57. Dearsly, A. L., Sinden, R. E. & Self, I. A. Sexual development in malarial parasites: gametocyte production, fertility and infectivity to the mosquito vector. *Parasitology* **100**, 359–368 (1990).
58. Sattabongkot, J., Maneechai, N. & Rosenberg, R. *Plasmodium vivax*: Gametocyte infectivity of naturally infected Thai adults. *Parasitology* **102**(Pt 1), 27–31 (1991).
59. Gaye, A. *et al*. Infectiousness of the human population to *Anopheles arabiensis* by direct skin feeding in an area hypoendemic for malaria in Senegal. *Am. J. Trop. Med. Hyg.* **92**, 648–652 (2015).
60. Ouédraogo, A. L. *et al*. Substantial contribution of submicroscopical *Plasmodium falciparum* gametocyte carriage to the infectious reservoir in an area of seasonal transmission. *PLoS ONE* **4**, (2009).
61. Morlais, I. *et al*. *Plasmodium falciparum* mating patterns and mosquito infectivity of natural isolates of gametocytes. *PLOS ONE* **10**, e0123777 (2015).
62. Dawes, E. J., Zhuang, S., Sinden, R. E. & Basáñez, M.-G. The temporal dynamics of *Plasmodium* density through the sporogonic cycle within Anopheles mosquitoes. *Trans. R. Soc. Trop. Med. Hyg.* **103**, 1197–1198 (2009).
63. Churcher, T. S. *et al*. Probability of transmission of malaria from mosquito to human is regulated by mosquito parasite density in naïve and vaccinated hosts. *PLOS Pathog.* **13**, e1006108 (2017).
64. Nacher, M. Does the shape of *Plasmodium falciparum* gametocytes have a function? *Med. Hypotheses* **62**, 618–619 (2004).
65. Nixon, C. P. *Plasmodium falciparum* gametocyte transit through the cutaneous microvasculature: A new target for malaria transmission blocking vaccines? *Hum. Vaccines Immunother.* **12**, 3189–3195 (2016).
66. Neveu, G. *et al*. *Plasmodium falciparum* gametocyte-infected erythrocytes do not adhere to human primary erythroblasts. *Sci. Rep.* **8**, 1–11 (2018).
67. Barraclough, R. K., Duval, L., Talman, A. M., Ariey, F. & Robert, V. Attraction between sexes: male-female gametocyte behaviour within a *Leucocytozoon toddi* (Haemosporida). *Parasitol. Res.* **102**, 1321–1327 (2008).
68. Jovani, R. *et al*. Double gametocyte infections in apicomplexan parasites of birds and reptiles. *Parasitol. Res.* **94**, 155–157 (2004).
69. Paul, R. E. L., Bonnet, S., Boudin, C., Tchuinkam, T. & Robert, V. Aggregation in malaria parasites places limits on mosquito infection rates. *Infect. Genet. Evol.* **7**, 577–586 (2007).
70. Dekker, T. *et al*. Selection of biting sites on a human host by *Anopheles gambiae s.s., An. arabiensis* and *An. quadriannulatus*. *Entomol. Exp. Appl.* **87**, 295–300 (1998).
71. Braack, L. *et al*. Biting behaviour of African malaria vectors: 1. where do the main vector species bite on the human body. *Parasit. Vectors* **8**, 76 (2015).
72. Bompard, A. *et al*. High *Plasmodium* infection intensity in naturally infected malaria vectors in Africa. *bioRxiv* **780064** (2019).
73. Valkiunas, G. *Avian Malaria Parasites and other Haemosporidia*. (CRC Press, 2004). Boca Raton
74. Vézilier, J., Nicot, A., Gandon, S. & Rivero, A. Insecticide resistance and malaria transmission: infection rate and oocyst burden in *Culex pipiens* mosquitoes infected with *Plasmodium relictum*. *Malar. J.* **9**, 379 (2010).
75. Nakagawa, S. & Schielzeth, H. Repeatability for gaussian and non-gaussian data: a practical guide for biologists. *Biol. Rev. Camb. Philos. Soc.* **85**, 935–956 (2010).
76. Dongen, S. V. Molenberghs & Matthysen. The statistical analysis of fluctuating asymmetry: REML estimation of a mixed regression model. *J. Evol. Biol.* **12**, 94–102 (1999).
77. Verbeke, G. & Molenberghs, G. *Linear Mixed Models for Longitudinal Data*. (Springer-Verlag, 2000).
78. Palmer, A. R. & Strobeck, C. Fluctuating asymmetry: Measurement, analysis, patterns. *Annu. Rev. Ecol. Syst.* **17**, 391–421 (1986).
79. Lazić, M. M., Kaliontzopoulou, A., Carretero, M. A. & Crnobrnja-Isailović, J. Lizards from urban areas are more asymmetric: Using fluctuating asymmetry to evaluate environmental disturbance. *PLOS ONE* **8**, e84190 (2013).
80. Barto, N. K. *MuMIn: Multi-model inference*. (2014).
81. Bates, D., Mächler, M., Bolker, B. & Walker, S. Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* **067**, (2015).
82. Crawley, M. J. *The R Book*. (John Wiley & Sons, 2012). Chichester.
83. Bolker, B. M. *Ecological Models and Data in R*. (Princeton University Press, 2008). Princeton and Oxford.

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Author contributions
Contributed to experiment design and conception: R.P., J.I., T.L., P.C.; contributed materials/samples: R.P., J.I., T.L., S.R.Y., A.C., J.B.O., K.R.D.; analyzed data : R.P.; contributed to paper writing: R.P., J.I., T.L., S.R.Y., A.C., P.C. All authors reviewd the manuscript.

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Chapter Three

Last-come, best served? Mosquito biting order and *Plasmodium* transmission

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Last-come, best served? Mosquito biting order and *Plasmodium* transmission

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A pervasive characteristic of parasite infections is their tendency to be overdispersed. Understanding the mechanisms underlying this overdispersed distribution is of key importance as it may impact the transmission dynamics of the pathogen. Although multiple factors ranging from environmental stochasticity to inter-individual heterogeneity may explain parasite overdispersion, parasite infection is also overdispersed in an inbred host population maintained under laboratory conditions, suggesting that other mechanisms are at play. Here, we show that the aggregated distribution of malaria parasites within mosquito vectors is partially explained by a temporal heterogeneity in parasite infectivity triggered by the bites of mosquitoes. Parasite transmission tripled between the mosquito's first and last blood feed in a period of only 3 h. Surprisingly, the increase in transmission is not associated with an increase in parasite investment in production of the transmissible stage. Overall, we highlight that *Plasmodium* is capable of responding to the bites of mosquitoes to increase its own transmission at a much faster pace than initially thought and that this is partly responsible for overdispersed distribution of infection. We discuss the underlying mechanisms as well as the broader implications of this plastic response for the epidemiology of malaria.

1. Introduction

A ubiquitous feature of parasite infections is their tendency to be overdispersed [1–4]. In other words, in a natural population of hosts, the majority of individuals tend to harbour few or no parasites, while a few hosts harbour the vast majority of the parasite population. This pattern has been observed in a wide range of diseases, from viruses and fungal parasites of plants [5,6] to protozoan and metazoan parasites of humans [7,8].

Previous work has shown that the overdispersed pattern of parasites among hosts can have important consequences for disease dynamics [9,10]. Overdispersion reduces the deleterious effects of parasites on host populations and increases the intensity of density-dependent suppression of parasite population growth (e.g. mating probability, intra- and inter-specific competition [11,12]). Another property emerging from parasite overdispersion is the effect on parasite transmission. The small fraction of heavily infected individuals may act as super-spreaders, playing a large role in disease transmission [13–15]. In many host–parasite systems, 20% of hosts are responsible for 80% of new infections [16,17]. In vector-borne diseases, parasite overdispersion has been observed both in vertebrate hosts and in vector populations [18–22]. Despite this, studies have mainly focused on the epidemiological consequences of parasite overdispersion for the host, rather than for the vectors [17,23,24]. Yet, for many of these diseases, key traits determining the transmission dynamics of the

pathogen (e.g. lifespan, the length of the parasite's extrinsic incubation period) may depend on the intensity of parasite infection in the vector [25–29].

Anderson & Gordon identified environmental stochasticity as the prime cause of overdispersion in parasite populations [30]. Environmental stochasticity refers to both the physical parameters of the environment and the differences in host susceptibility resulting from behavioural differences, genetic factors or past experiences of infection. The mechanisms underlying the aggregated distribution of parasites in vector populations remain rarely explored and little understood.

Plasmodium parasites are known for being the aetiological agents of malaria and for their devastating effects on human populations. These vector-borne parasites also infect many other terrestrial vertebrate species, including other mammals, reptiles and birds. The life cycle of this parasite is the same in hosts of all taxa. When the mosquito vectors take a blood meal on an infected host, they ingest the parasite's transmissible stages (female and male gametocytes). After sexual reproduction of the parasite, the motile zygotes penetrate the wall of the mosquito midgut and start developing into oocysts, which in turn produce the sporozoites. These sporozoites, once in the mosquito's salivary glands, can be transmitted to a new vertebrate host. There is abundant evidence that the distribution of oocysts, the most commonly quantified parasite stage in mosquitoes, is highly overdispersed [8,31–33]. The simplest explanation for this aggregated distribution of oocysts is that vectors vary in susceptibility to *Plasmodium* infection according to their genetic background or to their physiological status [8,34,35]. Polymorphism in mosquito immune genes is strongly associated with natural resistance to *Plasmodium* [34,36], and ageing tends to decrease the susceptibility of vectors to *Plasmodium* infection [35]. Puzzlingly, however, oocyst overdispersion is also common under controlled laboratory conditions in highly inbred, and therefore physiologically and genetically homogeneous, mosquito populations [8,31,33]. This suggests that factors other than the genetic or physiological variations between mosquitoes may contribute to the aggregated distribution of oocysts in vectors.

Spatial aggregation of gametocytes in vertebrate blood could be partially responsible for the aggregated distribution of oocysts in mosquitoes. Recent work has shown that gametocyte density can change by more than 0.4-fold between blood collected from different human body parts ([37], but see [38]). Although the direct connection between spatial heterogeneity in vertebrate blood and overdispersion in mosquitoes has never been made, it has been reported that *Plasmodium* gametocytes show an aggregated distribution within mosquitoes that recently fed on a human host [39].

The aggregated distribution of *Plasmodium* parasites within mosquitoes could also be due to within-host *temporal* variation in parasite density and/or infectivity. Under this scenario, mosquitoes feeding during the high parasite density/infectivity phase would become more heavily infected than those feeding during the low density/infectivity phase. *Plasmodium* parasite density and/or infectivity in the vertebrate host can indeed vary over relatively short time scales. A recent study found that rodent malaria *Plasmodium chabaudi* gametocytes are twice as infective at night despite being less numerous in the blood [40]. A periodic late afternoon increase in parasitaemia is also observed in the avian malaria system [41]. Such temporal variation may be a function of changes in the physiological, nutritional or immunological condition of the host [42–44]. It may, however, also be an adaptive parasite strategy aimed at maximizing its own transmission [41,45]. Recent work has shown that host parasitaemia increases a few days after a mosquito blood feeding bout, suggesting that *Plasmodium* may be capable of adjusting its transmission strategy by responding plastically to the temporal fluctuations in vector availability [41,45]. These results, however, are not able to explain the aggregated distribution of parasites among mosquitoes feeding within a short feeding bout typically lasting a few hours.

Here, we test whether *Plasmodium* is able to respond plastically to the bites of mosquitoes at a much more rapid pace than initially thought. More specifically, we test whether there is a pattern in the oocyst load of mosquitoes feeding within a short (3 h) time interval: do the bites of the first mosquitoes increase the infectivity of the parasite such that mosquitoes biting later end up infected with more oocysts? To test this hypothesis, we use the avian malaria system, the only currently available animal experimental system that allows working with a parasite recently isolated from the wild (*Plasmodium relictum*), with its natural mosquito vector (*Culex pipiens* [46,47]). Specifically, we carry out a series of experiments designed to answer two main questions: (i) Is oocystaemia correlated with mosquito biting order? In other words, do mosquitoes biting first have a lower intensity of infection than those biting later on? and (ii) Is this due to a temporal increase in the parasitaemia/gametocytaemia in the birds as a result of mosquito bites?

2. Material and methods

(a) Parasite and mosquito

Three experiments were carried out using three different isolates of *P. relictum* (lineage SGS1). The parasite strain used in the first block of the first experiment was isolated from an infected great tit (*Parus major*) in 2015. The strain used in the second experiment was isolated from an infected great tit in 2018. The strain used in the second block of the first experiment and in the third experiment was isolated from an infected house sparrow (*Passer domesticus*) in 2019. All strains were maintained through regular passages across our stock canaries (*Serinus canaria*) using intraperitoneal (i.p.) injections until the beginning of the experiment.

All experiments were conducted with *C. pipiens* mosquitoes collected in Lausanne (Switzerland) in August 2017, and maintained in the insectary since. Mosquitoes were reared using standard protocols [48]. We used females 7–13 days after emergence, which had not had prior access to blood. Mosquitoes were maintained on glucose solution (10%) since their emergence and were starved (but provided with water to prevent dehydration) for 24 h before the experiment.

(b) Experimental design

To investigate the impact of mosquito bite-driven plasticity on *Plasmodium* transmission, three experiments were carried out in which infected birds (which had no previous exposure to haematophagous invertebrates) were exposed to mosquitoes for 3 h (18.00–21.00) and mosquitoes were sampled at regular intervals thereafter (different protocols for the three experiments, see below). To investigate the impact of vector bites on parasite population growth, the parasitaemia (number of parasites in the blood) and gametocytaemia (number of mature gametocytes in the blood) of vertebrate hosts exposed or not (control) to mosquitoes were measured just before and just after the

mosquito exposure period using blood smears [49] (electronic supplementary material, table S1). Although parasitaemia and gametocytaemia are highly correlated in this system (see fig. 2 in [46], and electronic supplementary material, table S1), we measured both variables in order to control for potential changes in conversion rates (density of gametocytes relative to the total number of parasites).

All experiments were carried out using domestic canaries (*S. canaria*). Birds were inoculated by intraperitoneal injection of 100 µl of an infected blood pool (day 0). The blood pool was made with a 1 : 1 mixture of phospate-buffered saline (PBS) and blood sampled from 2–4 canaries infected with the parasite three weeks before the experiment.

(i) Experiment 1: oocyst burden and mosquito biting order: batch experiment

Two experimental blocks were carried out with two different isolates of *P. relictum*, using 14 and 5 infected birds, respectively. On days 11–13 post-infection, corresponding to the acute phase of infection, blood was sampled from each bird at 17.45. Straight after blood sampling, birds were individually placed in experimental cages (L 40 × W 40 × H 40 cm).

At 18.00, eight and three haphazardly chosen birds, from blocks 1 and 2, respectively, were exposed to four successive batches of 25 ± 3 uninfected female mosquitoes. Each mosquito batch was left in the cage for 45 min before being taken out and replaced by a new batch (i.e. batch 1 ($T_{0\text{min}}$), batch 2 ($T_{45\text{min}}$), batch 3 ($T_{90\text{min}}$) and batch 4 ($T_{135\text{min}}$)). Blood-fed mosquitoes from both batches were counted and individually placed in numbered plastic tubes (30 ml) covered with mesh and containing a cotton pad soaked in 10% glucose solution. At the end of the last mosquito exposure session (21.00), a second blood sample was taken from each bird. A red lamp was used to capture blood-fed mosquitoes without disturbing the birds and the mosquitoes. Control (unexposed) birds were placed in the same experimental conditions but without mosquitoes.

Tubes containing the blood-fed mosquitoes were kept in standard insectary conditions to obtain an estimate of the blood meal size and the level of infection (infection prevalence and oocyst burden). For this purpose, 7–8 days post blood meal, the females were taken out of the tubes and the amount of haematin excreted at the bottom of each tube was quantified as an estimate of the blood meal size [48]. Females were then dissected, and the number of *Plasmodium* oocysts in their midgut counted with the aid of a binocular microscope [48].

(ii) Experiment 2: oocyst burden and mosquito biting order: individual monitoring

To obtain a finer measurement of the impact of mosquito biting order on oocyst burden, a second experiment was carried out with the same protocol as described above, except that the birds (four of the eight infected birds) were exposed to a single batch of 100 uninfected mosquitoes for 3 h (18.00–21.00). Female mosquitoes were continuously observed and individually removed from the cages immediately after blood feeding in order to record the order of biting of each female.

(iii) Experiment 3: mosquito biting order and density of parasites ingested

A third experiment was carried out to investigate whether the total number of parasites in the blood meal, immediately after the blood feeding, fluctuated during the feeding bout. Two infected birds were individually placed in experimental cages and exposed to a single batch of 100 mosquitoes for 3 h (18.00–21.00). Mosquitoes were removed from the cages immediately after blood feeding. The order of biting of each female was recorded and every second mosquito collected was either immersed immediately in liquid nitrogen to quantify the number of parasites ingested by quantitative polymerase chain reaction (qPCR) or stored in an individual plastic tube and dissected one week later to count the number of oocysts in the midgut.

(c) Molecular analyses

The quantification of parasites contained within the blood meal was carried out using qPCR with a protocol adapted from [50]. Briefly, DNA was extracted from blood-fed females using standard protocols (Qiagen DNeasy 96 blood and tissue kit). For each individual, we conducted two qPCRs: one targeting the nuclear 18S ribosomal DNA (rDNA) gene of *Plasmodium* (primers 18sPlasm7 5′-AGCCTGAGAAATAGCTACCACATCTA-3′, 18sPlasm8 5′-TGTTATTTCTTGTCACTACCTCTCTTCTTT-3′) and the other targeting the 18S rDNA gene of the bird (primers 18sAv7 5′-GAAACTCGCAATGGCTCATTAAATC-3′, 18sAv8 5′-TATTAGCTCTAGAATTACCACAGTTATCCA-3′). All samples were run in triplicate (Bio-Rad CFX96™ Real-Time System). Samples with a threshold C_t value higher than 35 were considered uninfected. Parasite number was calculated with relative quantification values (RQ). RQ can be interpreted as the fold-amount of the target gene (*Plasmodium* 18S rDNA) with respect to the amount of the reference gene (bird 18S rDNA) and are calculated as $2^{-(C_t18S\ Plasmodium\ -\ C_t18s\ Bird)}$. For convenience, RQ values were standardized by $\times 10^4$ factor and log-transformed.

(d) Statistical analyses

Analyses were carried out using the R statistical package (v. 3.4.1). Data were analysed separately for each experiment and each experimental block. Blood meal rate, blood meal size, infection prevalence, oocyst burden (where only individuals that developed ≥ 1 oocyst were included) and quantity of parasites contained within the blood meal (which may depend on which bird mosquitoes fed on) were analysed, fitting bird as a random factor into the models using *lmer*, *glmer* or *glmer.nb* (package: lme4 [51]) according to whether the errors were normally (haematin quantity, and quantity of parasites contained within the blood meal), binomially (blood meal rate and infection prevalence) or negative binomially distributed (oocyst burden). Blood meal size (when it was not a response variable) and mosquito batches (experiment 1) or mosquito biting order (experiments 2 and 3) were used as fixed factors. Parasitaemia and gametocytaemia of birds were analysed using *lmer* with bird fitted as a random factor into the models to account for temporal pseudo-replication. Times of day (17.45 and 21.00) and bird group (exposed to mosquito bites or control) were used as fixed factors.

The different statistical models built to analyse the data are described in electronic supplementary material, table S2. Maximal models, including all higher-order interactions, were simplified by sequentially eliminating non-significant terms and interactions to establish a minimal model [52]. The significance of the explanatory variables was established using a likelihood ratio test [53]. The significant chi-squared values given in the text are for the minimal model, whereas non-significant values correspond to those obtained before the deletion of the variable from the model. *A posteriori* contrasts were carried out by aggregating factor levels and by testing the fit of the simplified model using a likelihood ratio test [52].

3. Results

(a) Experiment 1: oocyst burden and mosquito biting order: batch experiment

In this experiment, birds were exposed to four successive batches of 25 ± 3 uninfected mosquitoes. Each mosquito

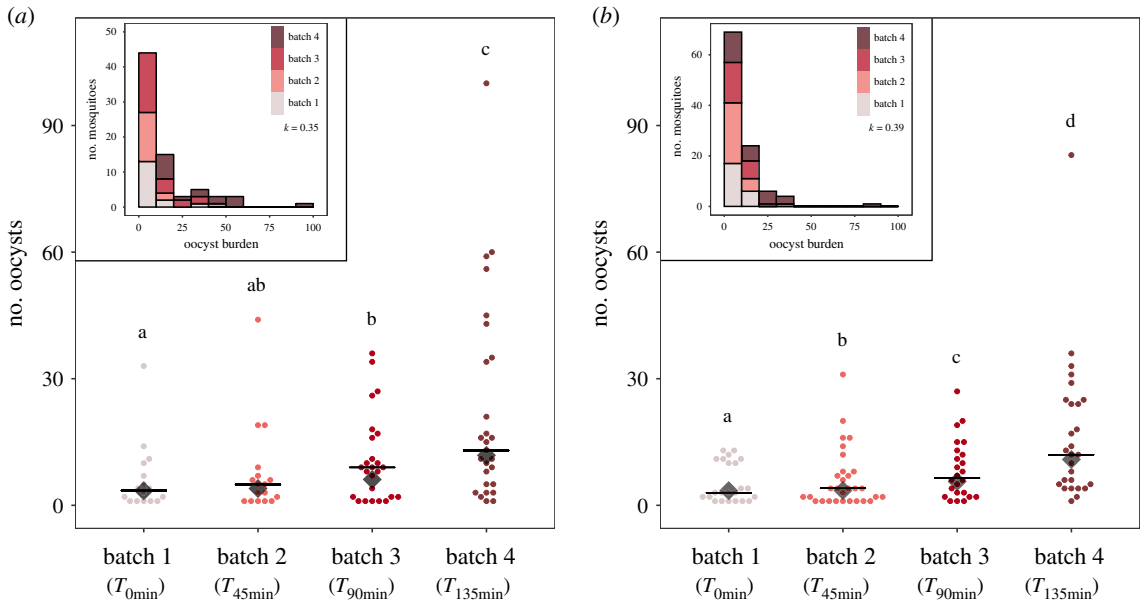

Figure 1. Experiment 1: impact of mosquito batch order on *Plasmodium* transmission. Number of oocysts in the midgut of *Plasmodium*-infected mosquitoes according to mosquito batch. Each mosquito batch was left in contact with birds for 45 min (batch 1 (T_{0min}), batch 2 (T_{45min}), batch 3 (T_{90min}) and batch 4 (T_{135min})). Birds were infected either by a *P. relictum* lab strain (experimental block 1, *a*) or by a *P. relictum* strain freshly collected in the field (experimental block 2, *b*). Black horizontal lines represent medians and black diamonds represent geometric means. Levels not connected by the same letter are significantly different. Histograms in each panel show the distribution of oocyst burden in mosquitoes in the experimental blocks 1 (*a*) and 2 (*b*); the colours represent the mosquito batches (from 1 to 4). *k*, clumping parameter. (Online version in colour.)

batch was kept in the cage for 45 min before being replaced with a new batch (batch 1 (T_{0min}), batch 2 (T_{45min}), batch 3 (T_{90min}) and batch 4 (T_{135min})). The blood meal rate (i.e. proportion of blood-fed mosquitoes) and the haematin quantity, a proxy for blood meal size, were similar for all batches (mean ± s.e., blood meal rate: batch 1: 19% ± 6, batch 2: 23% ± 8, batch 3: 29% ± 4 and batch 4: 31% ± 3, model 1: $\chi^2 = 5.90$, $p = 0.116$; haematin excreted (ng): batch 1: 17.58 ± 1.60, batch 2: 18 ± 1.76, batch 3: 17.34 ± 1.54 and batch 4: 18.29 ± 1.8, model 2: $\chi^2 = 3.55$, $p = 0.314$). Although mosquitoes from batches 3 and 4 tended to have a higher infection prevalence (proportion of mosquitoes containing at least one oocyst in the midgut; batch 3: 64.4% ± 11.9 and batch 4: 78.2% ± 8.6) than those from batches 1 and 2 (batch 1: 56.7% ± 15 and batch 2: 56.7% ± 19.4), the difference in prevalence between the different batches was not statistically significant (model 3: $\chi^2 = 2.74$, $p = 0.433$). The overall distribution of oocyst burden across batches was highly overdispersed (figure 1*a*; mean ± s.e., variance-to-mean ratio (VMR) = 11.48 ± 3.37, clumping parameter $k = 0.35$ [52]). Oocyst burden increased with mosquito batch (geometric mean: batch 1: 3.41 ± 3.04, batch 2: 3.99 ± 3.25, batch 3: 6.13 ± 3.36 and batch 4: 11.84 ± 3.53, model 4: $\chi^2 = 35.283$, $p < 0.0001$; figure 1*a*). Females from batch 4 had almost twice as many oocysts as those from batch 3 (contrast analyses: batch 4/batch 3: $\chi^2 = 11.02$, $p < 0.001$) and three times more than females from batches 1 and 2 (batch 4/batch 2: $\chi^2 = 17.95$, $p < 0.001$, batch 4/batch 1: $\chi^2 = 19.31$, $p < 0.0001$; figure 1*a*). No significant difference was, however, observed between mosquitoes from batches 1 and 2 (contrast analyses: batch 1/batch 2: $\chi^2 = 0.15$, $p = 0.697$) or between mosquitoes from batches 2 and 3 (batch 2/batch 3: $\chi^2 = 2.29$, $p = 0.129$; figure 1*a*). When the analysis was re-run removing outliers (threshold: third quartile + 1.5 × interquartile range), the mosquito biting order still had a significant effect on the oocyst burden (model 5: $\chi^2 = 12.43$, $p = 0.006$, geometric mean without outliers: batch 1: 3.41 ± 3.04, batch 2: 3.43 ± 2.82, batch 3: 6.13 ± 3.36, batch 4: 7.52 ± 2.82). Haematin quantity exhibited no significant association with the oocyst burden (model 4: $\chi^2 = 0.02$, $p = 0.875$).

The increase in *Plasmodium* oocyst burden with mosquito batch was not explained by an increase in total parasite or gametocyte burden in the birds' peripheral blood. The parasitaemia and gametocytaemia of exposed birds remained roughly constant throughout the experiment (parasitaemia: model 6: $\chi^2 = 0.39$, $p = 0.529$; gametocytaemia: model 7: $\chi^2 = 0.02$, $p = 0.877$; electronic supplementary material, table S1) and were similar between exposed and unexposed (control) birds (parasitaemia: model 6: $\chi^2 = 0.29$, $p = 0.5907$; gametocytaemia: model 7: $\chi^2 = 0.60$, $p = 0.4364$; electronic supplementary material, table S1).

To test the repeatability of our results, a second experimental block, with a new *P. relictum* strain freshly collected in the field, was performed. The results of block 2 fully confirmed those of the first block. The blood meal rate and the quantity of haematin excreted by mosquitoes were similar for all batches (blood meal rate: batch 1: 50% ± 11, batch 2: 45% ± 17, batch 3: 38% ± 3 and batch 4: 43% ± 3, model 8: $\chi^2 = 1.77$, $p = 0.621$; haematin excreted (ng): batch 1: 24.78 ± 1.46, batch 2: 26.43 ± 1.66, batch 3: 24.88 ± 1.96 and batch 4: 26.65 ± 1.42, model 9: $\chi^2 = 1.14$, $p = 0.766$). The difference in infection prevalence between the different batches was not statistically significant (model 10: $\chi^2 = 5.64$, $p = 0.130$) although mosquitoes from batches 2, 3 and 4 tended to have a higher prevalence (mean ± s.e., batch 2: 73.1% ± 7.0, batch 3: 68.6% ± 7.9 9 and batch 4: 71.1% ± 7.5) than those from batch 1 (mean ± s.e., batch 1: 51.1% ± 7.5). The distribution of oocyst burden in mosquitoes was overdispersed (figure 1*b*; mean ± s.e., VMR = 11.40 ± 5.66, $k = 0.39$) and we observed a significant increase in oocyst burden with mosquito batch order (model 11: $\chi^2 = 34.34$, $p < 0.0001$; geometric mean: batch 1: 3.48 ± 2.69, batch 2: 3.52 ± 2.95, batch 3: 5.63 ± 2.72 and batch 4: 10.87 ± 2.75, all contrast analyses were significant; figure 1*b*). When the analysis was re-run by

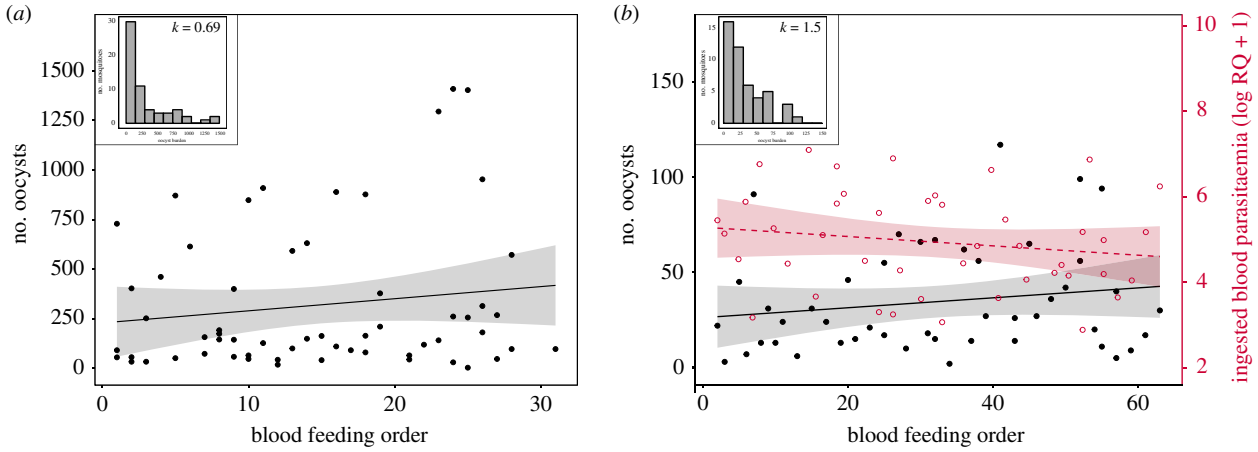

Figure 2. Effect of individual mosquito blood feeding order on the number of parasites ingested and on the intensity of infection. (*a*) Relationship between oocyst burden and mosquito biting order (experiment 2). (*b*) Relationship between the number of parasites ingested (red open dots and red dashed line), or the oocyst burden (black dots and black solid line), and the mosquito biting order (experiment 3). Each point represents one blood-fed mosquito. Shaded areas on either side of the regression line represent 95% confidence intervals. Histograms show the distribution of oocyst burden in mosquitoes in experiment 2 (*a*) and 3 (*b*). k, clumping parameter. (Online version in colour.)

removing outliers, the mosquito biting order still had a significant effect on the oocyst burden (model 12: $\chi^2 = 19.307$, $p = 0.0002$; geometric mean without outliers: batch 1: 3.48 ± 2.69, batch 2: 3.26 ± 2.77, batch 3: 5.63 ± 2.72 and batch 4: 8.13 ± 2.39). A significant positive correlation between haematin and oocyst burden was found (model 11: $\chi^2 = 4.46$, $p = 0.03$). As in the previous experimental block, the vertebrate host parasitaemia and gametocytaemia remained constant throughout the experiment (parasitaemia: model 13: $\chi^2 = 1.29$, $p = 0.256$; gametocytaemia: model 14: $\chi^2 = 0.88$, $p = 0.349$, respectively) and were similar between exposed and unexposed (control) birds (parasitaemia: model 13: $\chi^2 = 2.44$, $p = 0.118$; gametocytaemia: model 14: $\chi^2 = 2.45$, $p = 0.117$, respectively; electronic supplementary material, table S1).

(b) Experiment 2: oocyst burden and mosquito biting order: individual monitoring

To obtain a finer measurement of the impact of mosquito biting order on parasite transmission, infected birds were exposed to 100 mosquitoes for 3 h (18.00–21.00) and mosquitoes were individually removed from the cages immediately after blood feeding. Haematin quantity and infection prevalence were independent of the mosquito biting order (haematin excreted (ng) = 24.91 ± 1.26, model 13: $\chi^2 = 2.44$, $p = 0.118$; infection prevalence = 98.6%, model 16: $\chi^2 = 0.83$, $p = 0.363$). The distribution of oocyst burdens across all mosquitoes was highly overdispersed (mean ± s.e., VMR = 90.26 ± 41.53, $k = 0.69$; figure 2*a*). Biting order was a significant explanatory factor of oocyst burden: mosquitoes that bit later showed higher oocyst burden than mosquitoes that bit earlier (model 17: $\chi^2 = 8.28$, $p = 0.004$; figure 2*a*). Haematin quantity and oocyst burden were significantly positively correlated (model 17: $\chi^2 = 19.151$, $p < 0.001$). As in the first experiment, vertebrate host parasitaemia and gametocytaemia remained constant throughout the experiment (parasitaemia: model 18: $\chi^2 = 2.03$, $p = 0.154$; gametocytaemia: model 19: $\chi^2 = 0.13$, $p = 0.718$, respectively) and were similar between exposed and unexposed (control) birds (parasitaemia: model 18: $\chi^2 = 0.98$, $p = 0.321$; gametocytaemia: model 19: $\chi^2 = 0.12$, $p = 0.731$; electronic supplementary material, table S1).

(c) Experiment 3: number of parasites ingested and mosquito biting order

The first two experiments showed an increase in the oocyst burden with the order of mosquito bites but did not show a corresponding increase in parasite density in the peripheral blood of the vertebrate hosts (measured from blood samples). We carried out a third experiment to determine whether the total number of parasites in the blood meal fluctuated during the feeding bout. The amount of parasite ingested by the mosquitoes remained roughly constant throughout the exposure period (model 20: $\chi^2 = 1.54$, $p = 0.215$; figure 2*b*). The haematin quantity and the infection prevalence (oocyst stage) were also independent of the mosquito biting order (haematin excreted (ng) = 10.38 ± 0.76, model 21: $\chi^2 = 1.89$, $p = 0.169$; infection prevalence: 98%, model 22: $\chi^2 = 0.37$, $p = 0.545$). In contrast, the distribution of oocyst burden across all mosquitoes was still overdispersed (mean ± s.e., VMR = 15.03 ± 1.86, $k = 1.5$; figure 2*b*) and was significantly associated with mosquito biting order (model 23: $\chi^2 = 6.45$, $p = 0.011$; figure 2*b*). As in experiments 1 and 2, mosquitoes that bit later showed higher oocyst burden than mosquitoes that bit earlier (figure 2*b*). Haematin quantity had no effect on the oocyst burden (model 23: $\chi^2 = 3.77$, $p = 0.052$).

4. Discussion

Overdispersed distribution of vector-borne parasites in vertebrate and invertebrate host populations has important consequences for parasite transmission and disease control strategies [16,28,54]. Parasite overdispersion is driven by multiple factors ranging from population processes to inter-individual heterogeneity in susceptibility and parasite exposure [55–57]. Here, using three different isolates of *P. relictum*, we provide evidence that the aggregated distribution of malaria parasites within mosquito vectors may also be explained by mosquito biting order. On average, $10 \pm 3\%$ of the variation in oocyst burden was explained by biting order: mosquitoes that bite first end up with a lower intensity of infection than those that bite later on. This fluctuation in *Plasmodium* infectivity may reflect an adaptive strategy of parasites to optimize transmission.

The abundance of invertebrate vectors fluctuates at time scales ranging from days to years [40,58,59]. Previous studies have shown that malaria parasites have evolved two different and complementary transmission strategies to cope with both short- (circadian) and long-(seasonal) term fluctuations in mosquito activity. *Plasmodium* adopts an unconditional strategy whereby within-host parasitaemia and/or gametocyte infectivity varies daily, coinciding with the activity levels of its vector [41,42], but also a plastic strategy, allowing parasite growth to increase after exposure to mosquito bites [41,45,60]. This plastic strategy allows the parasite to react to daily and seasonal fluctuations in mosquito abundance by increasing its overall parasitaemia in the vertebrate blood [41,45].

In this study, we demonstrate that the plastic response of *Plasmodium* is much faster than initially thought [41,45]. When vertebrate hosts were exposed to mosquito bites during a short period of time (3 h), the parasite burden in mosquitoes increased gradually with their biting order. The density of parasites within the mosquito midgut tripled between the first and the last blood-fed mosquito. Although the biting order of the mosquitoes cannot be decoupled from the biting time (these two parameters are obviously highly correlated), the increase in the intensity of infection in such a short period of time suggests that the effect observed here cannot be explained solely by circadian fluctuation in parasite density in vertebrate blood. Many mosquito species exhibit a circadian rhythm in the host-biting activity but stochastic environmental factors such as variations in temperature, wind or humidity drastically affect the abundance of mosquitoes from one day to another [61–63]. Therefore, the association between an unconditional strategy (circadian fluctuation) and a quick plastic response to mosquito bites may allow malaria parasites to fine-tune investment in transmission according to the presence of mosquitoes.

Interestingly, this adaptive hypothesis involving an active parasite response to mosquito bites is not mediated by an increase in either parasite replication rate or gametocyte production: parasitaemia and gametocytaemia of birds exposed to mosquitoes were not different before and after mosquito probing. This result was confirmed by monitoring the number of parasites ingested by the mosquitoes immediately after the blood meal, throughout the exposure period. These results contrast with those obtained in recent studies [41,45], where the increase in oocyst burden observed in mosquitoes fed on a host a few days after the host was exposed to vector bites was correlated with an increase in parasitaemia and gametocytaemia. Our study suggests that malaria parasites may be employing an alternative strategy that allows them to react rapidly to the bites. One possibility is that *Plasmodium* may be reacting to mosquito bites by altering the physiological state of the gametocytes to render them more infectious. It was suggested as early as 1966 [64] that malaria parasite infectivity is not only due to the number of gametocytes in the blood but also to their physiological state. This prediction was recently experimentally confirmed by a study carried out with a rodent malaria parasite: *P. chabaudi* gametocytes were twice as infective at night despite being less numerous in the blood [40]. The mechanisms underlying gametocyte infectivity remain poorly understood. Although we know that gametocytes go through several stages (from one to eight depending on the species of *Plasmodium* [65]) of development before reaching the 'mature' stage, we do not know whether the mature stage is systematically infectious. The mechanism by which malaria parasites accelerate the rate of maturation and/or infectivity of gametocytes in response to mosquito bites should be explored.

The response of the vertebrate host to mosquito bites could also enhance parasite transmission from the vertebrate host to the invertebrate vector by two non-exclusive mechanisms: (i) increased infectivity and/or survival of parasites in vector midgut and (ii) modified susceptibility of mosquitoes to infection. *Plasmodium* abundance varies drastically during its journey within the mosquito, which is partly intertwined with the kinetics of blood digestion [32]. Within seconds of ingestion by the mosquito, the drop in temperature and the rise in pH, associated with the presence of xanthurenic acid, trigger gametocyte activation and differentiation into gametes [64–66]. Studies on ookinete production have revealed that in addition to mosquito-derived xanthurenic acid, there are a series of undefined blood-derived factors ingested by mosquitoes that act as significant sources of gametocyte activation [67,68]. Indeed, numerous host blood-derived compounds remain or become active during mosquito blood digestion. Complement components, vertebrate antibodies and regulator factor H may impact gametocytes-to-zygote and zygote-to-ookinetes stages transition and survival [69–71]. Several studies also showed that ingested vertebrate-derived factors negatively impact mosquito microbiota (e.g. complement cascade [72]) and their peritrophic matrix (e.g. chitinase [71,73]), both of which are known to play a key role in the mosquito refractoriness to *Plasmodium* infection [74]. The concentration of these vertebrate-derived compounds in the ingested blood and, ultimately, their impact on parasite infectivity and/or vector susceptibility might progressively increase with the number of bites and thus explain the increase in oocyst density with mosquito biting order.

Our study did not determine whether the increase in oocyst burden with mosquito biting order was mediated by a plastic response of the parasite or by a response of the vertebrate host to mosquito exposure. Further work comparing the transcriptome of vertebrate hosts and parasites before and after mosquito exposure would be a useful first step. It would be also relevant to determine how biting order affects infection intensity with a membrane-feeding assay. This method would allow manipulative experiments with inactivation of the serum and cross-manipulation of infected and uninfected blood samples. Another important point that remains unknown is whether the progressive increase in parasite burden observed in mosquitoes was the result of a localized or a systemic phenomenon within the bird. If the gradual increase in parasite density was mediated by a local response of the parasite or vertebrate host, one would expect to observe this effect only among mosquitoes biting the same area of the body. Conversely, if the effect was due to a more general host response (e.g. increased blood levels of stress hormones [75]), then the relationship between mosquito biting order and parasite burden would be observed regardless of the body area bitten.

Here, we report a higher oocyst burden than is found in the field [76,77]. All experimental infections carried out in the laboratory (e.g. human and avian) produce oocyst burdens that are substantially larger than those found in the field because infections are carried out under optimal conditions (e.g. no host defensive behaviour, optimal temperature and humidity). We also report strong variation in oocyst burdens

between the different experiments. In experiment 2, the average parasite density in mosquitoes was more than 10 times higher than those observed in the other experiments. It was unlikely that these differences were due to variations in the susceptibility of mosquitoes to malaria infection. All mosquitoes used in the experiments were from the same population collected from the field in 2017 and since maintained under laboratory conditions. The experiments were carried out using different isolates of *P. relictum*. However, contrary to what might be expected, the isolates generating the highest oocyst burdens in mosquitoes had a similar parasitaemia and gametocytaemia to those generating lower oocyst burdens (electronic supplementary material, table S1). This suggests that parameters other than those measured in this study are responsible for the transmission rate or the development of *Plasmodium* in mosquitoes (e.g. sex ratio [78]). Interestingly, an effect of blood meal size on oocyst burden was observed, but only in some experiments. The positive relationship between blood meal size and the intensity of infection in mosquitoes seems to be mediated by the parasite density within the vertebrate host: the lower the parasite load within the vertebrate host, the larger the bloodmeal size effect seems to be.

In summary, we provide evidence that the overdispersion of parasite burden observed in mosquitoes fed on the same infected host is partly explained by a temporal heterogeneity in *Plasmodium* infectivity resulting from the biting order of mosquitoes. These results show that the parasite is either directly or indirectly capable of responding to the bites of mosquitoes to increase its own transmission over much shorter time scales than previously thought. Further work is required to elucidate whether these two strategies are complementary and, particularly, to elucidate the underlying mechanisms. Despite recent progress toward disease control, the number of malaria cases has increased in several countries. The efficacy of control strategies is continually challenged and threatened by the evolution of insecticide [79] and drug [80] resistances. To overcome these issues, the development of innovative therapeutic approaches is necessary and urgent. Understanding the mechanisms allowing *Plasmodium* to increase transmission in response to mosquito bites could lead to the development of new pharmaceutical approaches to control malaria transmission.

Ethics. This study was approved by the Ethical Committee of the Vaud Canton veterinary authority, authorization number 1730.4.

Data accessibility. All data supporting the conclusions of this paper are available on the Dryad Digital Repository: https://dx.doi.org/10.5061/dryad.ns1rn8pps [81].

Authors' contributions. A.R. and R.P. conceived the study and all authors elaborated the experimental design. R.P. and J.I. performed the experiments and analysed the data. R.P. and J.I. wrote the first draft of the manuscript, and all authors contributed substantially to revision.

Competing interests. We declare we have no competing interests.

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References

1. Shaw DJ, Dobson AP. 1995 Patterns of macroparasite abundance and aggregation in wildlife populations: a quantitative review. *Parasitology* **111**, S111–S133. (doi:10.1017/S0031182000075855)
2. Shaw DJ, Grenfell BT, Dobson AP. 1998 Patterns of macroparasite aggregation in wildlife host populations. *Parasitology* **117**, 597–610. (doi:10.1017/S0031182098003448)
3. Lord CC, Barnard B, Day K, Hargrove JW, McNamara JJ, Paul RE, Trenholme K, Woolhouse ME. 1999 Aggregation and distribution of strains in microparasites. *Phil. Trans. R. Soc. Lond. B* **354**, 799–807. (doi:10.1098/rstb.1999.0432)
4. Poulin R. 2013 Explaining variability in parasite aggregation levels among host samples. *Parasitology* **140**, 541–546. (doi:10.1017/S0031182012002053)
5. Ruiz L, Janssen D, Martín G, Velasco L, Segundo E, Cuadrado IM. 2006 Analysis of the temporal and spatial disease progress of *Bemisia tabaci*-transmitted cucurbit yellow stunting disorder virus and cucumber vein yellowing virus in cucumber. *Plant Pathol.* **55**, 264–275. (doi:10.1111/j.1365-3059.2006.01316.x)
6. Grogan LF, Phillott AD, Scheele BC, Berger L, Cashins SD, Bell SC, Puschendorf R, Skerratt LF, Fenton A. 2016 Endemicity of chytridiomycosis features pathogen overdispersion. *J. Anim. Ecol.* **85**, 806–816. (doi:10.1111/1365-2656.12500)
7. Bundy DAP, Anderson RM, Thresh JM. 1988 Population ecology of intestinal helminth infections in human communities. *Phil. Trans. R. Soc. Lond. B* **321**, 405–420. (doi:10.1098/rstb.1988.0100)
8. Medley GF, Sinden RE, Fleck S, Billingsley PF, Tirawanchai N, Rodriguez MH. 1993 Heterogeneity in patterns of malarial oocyst infections in the mosquito vector. *Parasitology* **106**, 441–449. (doi:10.1017/S0031182000076721)
9. Anderson RM, May RM. 1978 Regulation and stability of host-parasite population interactions: I. Regulatory processes. *J. Anim. Ecol.* **47**, 219–247. (doi:10.2307/3933)
10. May RM, Anderson RM. 1978 Regulation and stability of host-parasite population interactions: II. Destabilizing processes. *J. Anim. Ecol.* **47**, 249–267. (doi:10.2307/3934)
11. Anderson RM. 1978 The regulation of host population growth by parasitic species. *Parasitology* **76**, 119–157. (doi:10.1017/S0031182000047739)
12. Jaenike J. 1996 Population-level consequences of parasite aggregation. *Oikos* **76**, 155–160. (doi:10.2307/3545757)
13. Poulin R. 1993 The disparity between observed and uniform distributions: a new look at parasite aggregation. *Int. J. Parasitol.* **23**, 937–944. (doi:10.1016/0020-7519(93)90060-C)
14. Lloyd-Smith JO, Schreiber SJ, Kopp PE, Getz WM. 2005 Superspreading and the effect of individual variation on disease emergence. *Nature* **438**, 355–359. (doi:10.1038/nature04153)
15. Martin LB *et al.* 2019 Extreme competence: keystone hosts of infections. *Trends Ecol. Evol.* **34**, 303–314. (doi:10.1016/j.tree.2018.12.009)
16. Woolhouse MEJ *et al.* 1997 Heterogeneities in the transmission of infectious agents: implications for the design of control programs. *Proc. Natl Acad. Sci. USA* **94**, 338–342. (doi:10.1073/pnas.94.1.338)
17. Cooper L *et al.* 2019 Pareto rules for malaria super-spreaders and super-spreading. *Nat. Commun.* **10**, 3939. (doi:10.1038/s41467-019-11861-y)
18. Das PK, Subramanian S, Manoharan A, Ramaiah KD, Vanamail P, Grenfell BT, Bundy DA, Michael E. 1995 Frequency distribution of *Wuchereria bancrofti* infection in the vector host in relation to human host: evidence for density dependence. *Acta Trop.* **60**, 159–165. (doi:10.1016/0001-706X(95)00123-V)
19. Churcher TS, Ferguson NM, Basáñez M-G. 2005 Density dependence and overdispersion in the transmission of helminth parasites. *Parasitology* **131**, 121–132. (doi:10.1017/S0031182005007341)
20. Churcher TS, Bousema T, Walker M, Drakeley C, Schneider P, Ouédraogo AL, Basáñez M-G. 2013 Predicting mosquito infection from *Plasmodium*

falciparum gametocyte density and estimating the reservoir of infection. *eLife* **2**, e00626. (doi:10.7554/eLife.00626)

21. Paul REL, Bonnet S, Boudin C, Tchuinkam T, Robert V. 2007 Aggregation in malaria parasites places limits on mosquito infection rates. *Infect. Genet. Evol.* **7**, 577–586. (doi:10.1016/j.meegid.2007.04.004)
22. Wetten S, Collins RC, Vieira JC, Marshall C, Shelley AJ, Basáñez M-G. 2007 Vector competence for *Onchocerca volvulus* in the *Simulium* (*Notolepria*) *exiguum* complex: cytoforms or density-dependence? *Acta Trop.* **103**, 58–68. (doi:10.1016/j.actatropica.2007.05.009)
23. Bousema T, Griffin JT, Sauerwein RW, Smith DL, Churcher TS, Takken W, Ghani A, Drakeley C, Gosling R. 2012 Hitting hotspots: spatial targeting of malaria for control and elimination. *PLoS Med.* **9**, e1001165. (doi:10.1371/journal.pmed.1001165)
24. Paull SH, Song S, McClure KM, Sackett LC, Kilpatrick AM, Johnson PT. 2012 From superspreaders to disease hotspots: linking transmission across hosts and space. *Front. Ecol. Environ.* **10**, 75–82. (doi:10.1890/110111)
25. Basáñez MG, Townson H, Williams JR, Frontado H, Villamizar NJ, Anderson RM. 1996 Density-dependent processes in the transmission of human onchocerciasis: relationship between microfilarial intake and mortality of the simuliid vector. *Parasitology* **113**, 331–355. (doi:10.1017/S003118200006649X)
26. Dawes EJ, Zhuang S, Sinden RE, Basáñez M-G. 2009 The temporal dynamics of *Plasmodium* density through the sporogonic cycle within *Anopheles* mosquitoes. *Trans. R. Soc. Trop. Med. Hyg.* **103**, 1197–1198. (doi:10.1016/j.trstmh.2009.03.009)
27. Dawes EJ, Churcher TS, Zhuang S, Sinden RE, Basáñez M-G. 2009 *Anopheles* mortality is both age- and *Plasmodium*-density dependent: implications for malaria transmission. *Malar. J.* **8**, 228. (doi:10.1186/1475-2875-8-228)
28. Courtenay O, Peters NC, Rogers ME, Bern C. 2017 Combining epidemiology with basic biology of sand flies, parasites, and hosts to inform leishmaniasis transmission dynamics and control. *PLoS Pathog.* **13**, e1006571. (doi:10.1371/journal.ppat.1006571)
29. Gutiérrez-López R, Martínez-de la Puente J, Gangoso L, Yan J, Soriguer R, Figuerola J. 2019 Experimental reduction of host *Plasmodium* infection load affects mosquito survival. *Scient. Rep.* **9**, 8782. (doi:10.1038/s41598-019-45143-w)
30. Anderson RM, Gordon DM. 1982 Processes influencing the distribution of parasite numbers within host populations with special emphasis on parasite-induced host mortalities. *Parasitology* **85**, 373–398. (doi:10.1017/S0031182000055347)
31. Sinden RE *et al.* 2007 Progression of *Plasmodium berghei* through *Anopheles stephensi* is density-dependent. *PLoS Pathog.* **3**, e195. (doi:10.1371/journal.ppat.0030195)
32. Vaughan JA. 2007 Population dynamics of *Plasmodium* sporogony. *Trends Parasitol.* **23**, 63–70. (doi:10.1016/j.pt.2006.12.009)
33. Pigeault R, Vézilier J, Nicot A, Gandon S, Rivero A. 2015 Transgenerational effect of infection in *Plasmodium*-infected mosquitoes. *Biol. Lett.* **11**, 20141025. (doi:10.1098/rsbl.2014.1025)
34. Harris C, Lambrechts L, Rousset F, Abate L, Nsango SE, Fontenille D, Morlais I, Cohuet A, Schneider DS. 2010 Polymorphisms in *Anopheles gambiae* immune genes associated with natural resistance to *Plasmodium falciparum*. *PLoS Pathog.* **6**, e1001112. (doi:10.1371/journal.ppat.1001112)
35. Pigeault R, Nicot A, Gandon S, Rivero A. 2015 Mosquito age and avian malaria infection. *Malar. J.* **14**, 383. (doi:10.1186/s12936-015-0912-z)
36. Riehle MM *et al.* 2006 Natural malaria infection in *Anopheles gambiae* is regulated by a single genomic control region. *Science* **312**, 577–579. (doi:10.1126/science.1124153)
37. Pigeault R, Isaïa J, Yerbanga RS, Dabiré KR, Ouédraogo J-B, Cohuet A, Lefèvre T, Christe P. 2020 Different distribution of malaria parasite in left and right extremities of vertebrate hosts translates into differences in parasite transmission. *Scient. Rep.* **10**, 10183. (doi:10.1038/s41598-020-67180-6)
38. Meibalan E. *et al.* 2019 *Plasmodium falciparum* gametocyte density and infectivity in peripheral blood and skin tissue of naturally infected parasite carriers in Burkina Faso. *J. Infect. Dis.* **2018**, jiz680. (doi:10.1093/infdis/jiz680)
39. Pichon G, Awono-Ambene HP, Robert V. 2000 High heterogeneity in the number of *Plasmodium falciparum* gametocytes in the bloodmeal of mosquitoes fed on the same host. *Parasitology* **121**, 115–120. (doi:10.1017/S0031182099006277)
40. Schneider P, Rund SSC, Smith NL, Prior KF, O'Donnell AJ, Reece SE. 2018 Adaptive periodicity in the infectivity of malaria gametocytes to mosquitoes. *Proc. R. Soc. B* **285**, 20181876. (doi:10.1098/rspb.2018.1876)
41. Pigeault R, Caudron Q, Nicot A, Rivero A, Gandon S. 2018 Timing malaria transmission with mosquito fluctuations. *Evol. Lett.* **2**, 378–389. (doi:10.1002/evl3.61)
42. Mideo N, Reece SE, Smith AL, Metcalf CJE. 2013 The Cinderella syndrome: why do malaria-infected cells burst at midnight? *Trends Parasitol.* **29**, 10–16. (doi:10.1016/j.pt.2012.10.006)
43. Prior KF *et al.* 2018 Timing of host feeding drives rhythms in parasite replication. *PLoS Pathog.* **14**, e1006900. (doi:10.1371/journal.ppat.1006900)
44. Prior KF, Rijo-Ferreira F, Assis PA, Hirako IC, Weaver DR, Gazzinelli RT, Reece SE. 2020 Periodic parasites and daily host rhythms. *Cell Host Microbe* **27**, 176–187. (doi:10.1016/j.chom.2020.01.005)
45. Cornet S, Nicot A, Rivero A, Gandon S, Vernick KD. 2014 Evolution of plastic transmission strategies in avian malaria. *PLoS Pathog.* **10**, e1004308. (doi:10.1371/journal.ppat.1004308)
46. Pigeault R, Vézilier J, Cornet S, Zélé F, Nicot A, Perret P, Gandon S, Rivero A. 2015 Avian malaria: a new lease of life for an old experimental model to study the evolutionary ecology of *Plasmodium*. *Phil. Trans. R. Soc. B* **370**, 20140300. (doi:10.1098/rstb.2014.0300)
47. Rivero A, Gandon S. 2018 Evolutionary ecology of avian malaria: past to present. *Trends Parasitol.* **34**, 712–726. (doi:10.1016/j.pt.2018.06.002)
48. Vézilier J, Nicot A, Gandon S, Rivero A. 2010 Insecticide resistance and malaria transmission: infection rate and oocyst burden in *Culex pipiens* mosquitoes infected with *Plasmodium relictum*. *Malar. J.* **9**, 379. (doi:10.1186/1475-2875-9-379)
49. Valkiunas G. 2004 *Avian malaria parasites and other haemosporidia*. Boca Raton, FL: CRC Press.
50. Cornet S, Nicot A, Rivero A, Gandon S, Ebert D. 2013 Malaria infection increases bird attractiveness to uninfected mosquitoes. *Ecol. Lett.* **16**, 323–329. (doi:10.1111/ele.12041)
51. Bates D, Mächler M, Bolker B, Walker S. 2015 Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* **67**, 1–48. (doi:10.18637/jss.v067.i01)
52. Crawley MJ. 2012 *The R book*. Chichester: John Wiley & Sons.
53. Bolker BM. 2008 *Ecological models and data in R*. Princeton, NJ: Princeton University Press.
54. Churcher TS *et al.* 2017 Probability of transmission of malaria from mosquito to human is regulated by mosquito parasite density in naïve and vaccinated hosts. *PLoS Pathog.* **13**, e1006108. (doi:10.1371/journal.ppat.1006108)
55. Wassom DL, Dick TA, Arnason N, Strickland D, Grundmann AW. 1986 Host genetics: a key factor in regulating the distribution of parasites in natural host populations. *J. Parasitol.* **72**, 334–337. (doi:10.2307/3281615)
56. Rosà R, Pugliese A. 2002 Aggregation, stability, and oscillations in different models for host-macroparasite interactions. *Theor. Popul. Biol.* **61**, 319–334. (doi:10.1006/tpbi.2002.1575)
57. Hansen F, Jeltsch F, Tackmann K, Staubach C, Thulke H-H. 2004 Processes leading to a spatial aggregation of *Echinococcus multilocularis* in its natural intermediate host *Microtus arvalis*. *Int. J. Parasitol.* **34**, 37–44. (doi:10.1016/j.ijpara.2003.10.003)
58. Barrozo RB, Schilman PE, Minoli SA, Lazzari CR. 2004 Daily rhythms in disease-vector insects. *Biol. Rhythm Res.* **35**, 79–92. (doi:10.1080/09291010412331313250)
59. Lalubin F, Delédevant A, Glaizot O, Christe P. 2013 Temporal changes in mosquito abundance (*Culex pipiens*), avian malaria prevalence and lineage composition. *Parasites Vectors* **6**, 307. (doi:10.1186/1756-3305-6-307)
60. Billingsley PF, Snook LS, Johnston VJ. 2005 Malaria parasite growth is stimulated by mosquito probing. *Biol. Lett.* **1**, 185–189. (doi:10.1098/rsbl.2004.0260)
61. Ngowo HS, Kaindoa EW, Matthiopoulos J, Ferguson HM, Okumu FO. 2017 Variations in household microclimate affect outdoor-biting behaviour of malaria vectors. *Wellcome Open Res.* **2**, 102. (doi:10.12688/wellcomeopenres.12928.1)

62. Chao S, Zhu D, Dixon D, Khater E, Xue R-D. 2019 Diel activity patterns of adult female mosquitoes (Diptera: Culicidae) determined by a novel rotated trap in northeastern Florida, USA. *J. Vector Ecol.* **44**, 149–153. (doi:10.1111/jvec.12339)
63. Sallam MF, Pereira RM, Batich C, Koehler P. 2019 Factors affecting short-range host-seeking for the yellow fever mosquito (Diptera: Culicidae). *J. Med. Entomol.* **56**, 609–616. (doi:10.1093/jme/tjy230)
64. Hawking F, Worms MJ, Gammage K, Goddard PA. 1966 The biological purpose of the blood-cycle of the malaria parasite *Plasmodium cynomolgi*. *Lancet* **288**, 422–424. (doi:10.1016/S0140-6736(66)92722-X)
65. Gautret P, Motard A. 1999 Periodic infectivity of *Plasmodium* gametocytes to the vector. A review. *Parasite (Paris)* **6**, 103–111.
66. Billker O, Lindo V, Panico M, Etienne AE, Paxton T, Dell A, Rogers M, Sinden RE, Morris HR. 1998 Identification of xanthurenic acid as the putative inducer of malaria development in the mosquito. *Nature* **392**, 289–292. (doi:10.1038/32667)
67. Sinden RE, Butcher GA, Billker O, Fleck SL. 1996 Regulation of infectivity of *Plasmodium* to the mosquito vector. *Adv. Parasitol.* **38**, 53–117. (doi:10.1016/S0065-308X(08)60033-0)
68. Arai M, Billker O, Morris HR, Panico M, Delcroix M, Dixon D, Ley SV, Sinden RE. 2001 Both mosquito-derived xanthurenic acid and a host blood-derived factor regulate gametogenesis of *Plasmodium* in the midgut of the mosquito. *Mol. Biochem. Parasitol.* **116**, 17–24. (doi:10.1016/S0166-6851(01)00299-7)
69. Margos G, Navarette S, Butcher G, Davies A, Willers C, Sinden RE, Lachmann PJ. 2001 Interaction between host complement and mosquito-midgut-stage *Plasmodium berghei*. *Infect. Immun.* **69**, 5064–5071. (doi:10.1128/IAI.69.8.5064-5071.2001)
70. Simon N, Lasonder E, Scheuermayer M, Kuehn A, Tews S, Fischer R, Zipfel PF, Skerka C, Pradel G. 2013 Malaria parasites co-opt human factor H to prevent complement-mediated lysis in the mosquito midgut. *Cell Host Microbe* **13**, 29–41. (doi:10.1016/j.chom.2012.11.013)
71. Pakpour N, Riehle MA, Luckhart S. 2014 Effects of ingested vertebrate-derived factors on insect immune responses. *Curr. Opin. Insect Sci.* **3**, 1–5. (doi:10.1016/j.cois.2014.07.001)
72. Khattab A, Barroso M, Miettinen T, Meri S, Ribeiro JMC. 2015 *Anopheles* midgut epithelium evades human complement activity by capturing factor H from the blood meal. *PLoS Negl. Trop. Dis.* **9**, e0003513. (doi:10.1371/journal.pntd.0003513)
73. Di Luca M, Romi R, Severini F, Toma L, Musumeci M, Fausto AM, Mazzini M, Gambellini G, Musumeci S. 2007 High levels of human chitotriosidase hinder the formation of peritrophic membrane in anopheline vectors. *Parasitol. Res.* **100**, 1033–1039. (doi:10.1007/s00436-006-0372-z)
74. Boissière A *et al.* 2012 Midgut microbiota of the malaria mosquito vector *Anopheles gambiae* and interactions with *Plasmodium falciparum* infection. *PLoS Pathog.* **8**, e1002742. (doi:10.1371/journal.ppat.1002742)
75. Dhondt AA, Dobson AP. 2017 Stress hormones bring birds, pathogens and mosquitoes together. *Trends Parasitol.* **33**, 339–341. (doi:10.1016/j.pt.2017.01.001)
76. Hogg JC, Hurd H. 1997 The effects of natural *Plasmodium falciparum* infection on the fecundity and mortality of *Anopheles gambiae s. l.* in north east Tanzania. *Parasitology* **114**, 325–331. (doi:10.1017/S0031182096008542)
77. Bompard A *et al.* 2020 High *Plasmodium* infection intensity in naturally infected malaria vectors in Africa. *Int. J. Parasitol.* **50**, 985–996. (doi:10.1016/j.ijpara.2020.05.012)
78. Bradley J *et al.* 2018 Predicting the likelihood and intensity of mosquito infection from sex specific *Plasmodium falciparum* gametocyte density. *eLife* **7**, e34463. (doi:10.7554/eLife.34463)
79. Ranson H, Lissenden N. 2016 Insecticide resistance in African *Anopheles* mosquitoes: a worsening situation that needs urgent action to maintain malaria control. *Trends Parasitol.* **32**, 187–196. (doi:10.1016/j.pt.2015.11.010)
80. Ariey F *et al.* 2014 A molecular marker of artemisinin-resistant *Plasmodium falciparum* malaria. *Nature* **505**, 50–55. (doi:10.1038/nature12876)
81. Isaïa J, Rivero A, Glaizot O, Christe P, Pigeault R. 2020 Data from: Last-come, best served? Mosquito biting order and *Plasmodium* transmission. Dryad Digital Repository. (doi:10.5061/dryad.ns1rn8pps)

Chapter Four

Variations in the mosquito larval environment: effect on the within-vector dynamics of *Plasmodium* and mosquito immunity

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Authors' contributions. All authors conceived the study and elaborated the experimental design. J.I. and M.S.S.G. performed the experiments and analyzed the data. J.I. wrote the first draft of the manuscript, and all authors contributed substantially to revision.

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Variations in the mosquito larval environment: effect on the within-vector dynamics of *Plasmodium* and mosquito immunity

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Abstract

What factors shape variation in transmission rate of parasites is major question in the study of host-parasite interactions. One source of variation known to affect a vectors' ability to transmit pathogens to a new host is the quality of the habitat in which it grew. While most studies have focused on the impact of habitat quality on parasite prevalence and intensity, little is known about its impact on the extrinsic incubation period (EIP). The EIP corresponds to the developmental time of the parasite inside the vector, from the blood meal ingestion to the invasion of salivary glands. Here, we used the avian malaria system to investigate whether manipulation of the vector's environment quality (i.e. nutritional stress) during larval development influences both the vector propensity to become infected and the EIP. Mosquito larvae were subjected to either of two treatments: a high-diet treatment receiving a standard maintenance diet, and a low-diet treatment receiving 25% of the standard diet. Larval nutritional stress seemed to have no impact on the probability to become infected. However, females reared under nutritional stress had reduced fecundity and a weaker immune system. One explanation would be that stressed females trade investment in reproduction for immune defences to overcome parasitic infection. This seems unlikely however, as we found no differences in parasite intensities between the two treatments. Our results highlight that variation in the quality of the vector larval environment affects mosquito life history traits and could impact their ability to support infection. Transmission outcomes may partly rely on trade-offs occurring within the mosquito vector between investment in reproduction, survival and immune system. Future analyses could assess the extent to which larval nutritional stress affects EIP of the parasite.

Keywords: Larval nutritional stress, Vector, *Plasmodium,* EIP, Immune system, Transmission

Introduction

Vector-borne diseases (VBDs) are responsible every year for more than 700,000 deaths and more than 80% of the world population lives in areas exposed to at least one vector-borne pathogen (WHO 2017). These diseases are caused by parasites, viruses and bacteria that are transmitted by vectors which are mostly hematophagous arthropods. Vector-borne diseases are ecologically complex, since the pathogen must evolve and cope with both within-host and -vector environments, which may also interact with each other (Combes 2020)

The transmission dynamic of vector-borne pathogens depend on the individual vectorial capacity (IC) of the vector, *i.e.* its ability to transmit pathogens (Rivero *et al.* 2010), defined as:

$$IC = \frac{a^2 bce^{-gn}}{g}$$

This equation is based on several parameters: ***a*** refers to the vector feeding behaviour and is defined as the number of bites on a focal host (per vector, per day); ***b*** refers to the probability that a host becomes infected from an infectious bite, defined by a combination of host susceptibility to infection and vector infectiousness; ***c*** is the vector's susceptibility to infection; ***g*** is the death rate of the vector; and **n** the extrinsic incubation period of the pathogen (i.e. number of days required for the vector to become infectious after being infected; EIP)(Rivero *et al.* 2010).

The impact of environmental variation on some parameters of the vectorial capacity has been extensively studied (Ferguson & Read 2002; Muturi *et al.* 2012; Breaux *et al.* 2014; Murdock *et al.* 2016; Lefevre *et al.* 2018). Most studies focused on the extent to which such variations might affect the probability of the vector to become infected and resist the infection. For instance, variations in the habitat quality of the vector, such as larval competition, food availability and quality are known to impact the prevalence and/or intensity of pathogen infection within the vector as well as vector fecundity, immunity and longevity (Ferguson & Read 2002; Alto *et al.* 2008; Muturi *et al.* 2012; Breaux *et al.* 2014; Hien *et al.* 2016; Yan *et al.* 2021). Under environmental stress, resource allocation into the

immune system to fight off pathogen infection might result in reduced investment in other fitness traits such as survival or growth rate (Tripet *et al.* 2008; Shaw *et al.* 2018). Comparatively, little attention has been given to the impact of such variations on the time required for the vector to become infectious after an infection, i.e. the extrinsic incubation period of the pathogen (Ohm *et al.* 2018). The EIP is a key component of the IC as it is linked with the propensity of the vector to become infectious and interacts directly with its longevity, thereby determining the number of vectors that will have lived long enough to be able to transmit the pathogen (Lefevre *et al.* 2018; Ohm *et al.* 2018).

Most studies on the factors influencing the EIP focused on the effects of temperature (Ohm *et al.* 2018). For instance, for both the human malaria parasite *Plasmodium falciparum* and the dengue virus, shifts in EIP were observed in response to mean temperature variation (Carrington *et al.* 2013) as well as daily temperature fluctuations (Beck-Johnson *et al.* 2017). In the current climate change context, such studies give a better understanding of current and future risks of vector-borne diseases transmission (Murdock *et al.* 2016; Beck-Johnson *et al.* 2017). Nevertheless, many other factors can disturb the environment of vectors and affect their potential for transmission. Variations in the quality of the larval environment of *Anopheles stephensi* mosquitoes have been reported to affect their capacity to transmit malaria parasites (Moller-Jacobs *et al.* 2014; Shapiro *et al.* 2016). Reduced larval food increases adult mortality rate and causes a delay in human malaria parasite development and in the time required for mosquitoes to become infectious (Shapiro *et al.* 2016). This could be explained by an adaptive response of the parasite, which would extend its EIP to reduce vector mortality, thereby enhancing the chances that the vector survives the infection long enough to transmit it. The extent to which a pathogen could plastically shift the EIP when its transmission potential is compromised and spare its vector deserves consideration.

The aim of this study was to investigate whether manipulation of the vector's environmental quality might underpin both the vector propensity to become infected and the time to become infectious. To test this hypothesis, we used a natural avian malaria system with a parasite recently

isolated from the wild (*Plasmodium relictum*) and its natural mosquito vector *Culex pipiens* (Glaizot *et al.* 2012; Pigeault *et al.* 2015). We imposed a nutritional stress on the vector larvae and tested whether it influenced both (i) the temporal dynamics of *Plasmodium* within their vectors, capturing both the mosquito intensity of infection and the time required to become infectious (EIP), and (ii) to what extent this affected the mosquito's fecundity and immune system.

Materials and Methods

Haemosporidian parasite

The experiment was carried out using a *P. relictum* (lineage SGS1) strain isolated from infected house sparrows (*Passer domesticus)* in December 2020 on the campus of the University of Lausanne, Switzerland (46°31'25.607''N 6°34'40.714''E). The strain was maintained through regular passages across our stock canaries (*Serinus canaria*) using intraperitoneal injections until the beginning of the experiment (Pigeault *et al.* 2015). Seven canaries were experimentally inoculated by intraperitoneal injections of 150 µl of an infected blood pool from our infected canary stock. The dynamic of infection was monitored every two days from day 5 to day 20 post-infection by measuring parasitaemia (*i.e.* the number of asexual and sexual stages of the parasite in the vertebrate host blood) and gametocytaemia (*i.e.* the number of sexual stages of the parasite in the vertebrate host blood) both by microscopic examination of Giemsa stained blood-smears and by qPCR (see Molecular analyses).

Mosquito rearing

The *C. pipiens* mosquito population used in this experiment was initiated from wild clutches collected on the campus of the University of Lausanne in August 2017, and was maintained in the lab since then and reared using standard protocols (Vézilier *et al.* 2010). Two cohorts were generated: (i) High-diet treatment (HD), receiving *ad libithum* food, composed by TetraMin Junior fish pellets, Schweizer Classic rabbit pellets and JBL Novo Malawi fish flakes (1:1:1 ratio), which corresponds to the standard maintenance diet, and (ii) Low-diet treatment (LD), which corresponds to 25% of the

standard diet. These two treatments were based on the results of a pilot study to ensure that the low-diet treatment would generate nutritional stress without killing all the larvae. Mosquito egg clutches (n=25) were obtained by feeding 50 females on one healthy bird (*Serinus canaria*). Two and three thousand first-instar larvae were collected for the high- and low-diet treatment respectively and placed in 400 mL of water in tanks at a constant density of 100 individuals. Larvae from the LD treatment were collected three days earlier than larvae from the HD treatment to adjust for the slower developmental time expected under nutritional stress and to match the time of adult's emergence in both treatments for the experiment. To ensure that each larva in each tank received the same amount of food according to its treatment, larvae were counted every 2 days to adjust food quantities both to the number of surviving larvae and their larval stage. Tank water was changed on feeding days to avoid food accumulation and bacterial growth on the water surface. Larval mortality was monitored daily until adult emergence. Emerged adult mosquitoes were then maintained on a 10% glucose solution.

Mosquito blood meal and dissections

Twenty-four hours before the infected blood meal, uninfected mated female mosquitoes were marked with different fluorescent powders to distinguish between treatments (Vézilier *et al.* 2012) and starved but provided with water to prevent dehydration.

Twelve days after infection (i.e. peak of infection), birds were exposed individually to 50 uninfected mated female mosquitoes of both treatments for three hours (6-9 p.m.). At the end of the experiment, blood-fed females were sorted by microscope examination of their colour, counted and kept individually in plastic tubes under standard laboratory conditions (25°C – 70% RH) with 10% *ad libitum* glucose.

Every two days starting from day 4 to day 20 post-blood meal (pbm), 2 ± 1 mosquitoes were haphazardly sampled per treatment and per bird to monitor the dynamic of oocysts formation and sporozoites production as well as the mosquito innate immune response. Each mosquito was dissected to (i) count the number of oocysts in their midgut with a binocular microscope, (ii) quantify

the transmissible sporozoites in their head/thorax using real-time quantitative PCR (see molecular analyses below) and (iii) measure the number of circulating haemocytes (i.e. immune cells involved in anti-pathogen activity) within the mosquito haemolymph using a haemocytometer. On day 5 pbm, when mosquitoes had digested the blood, the amount of haematin excreted at the bottom of each tube was quantified as a proxy for blood meal size and females were transferred to a new tube containing 2.5 ml of water for oviposition. Egg rafts were photographed to count the number of eggs laid as a measure of mosquito fecundity, using ImageJ.

Molecular analyses

Real-time quantitative PCR was used to quantify both the number of parasites in the vertebrate host blood (i.e. parasitaemia) and the number of *Plasmodium* sporozoites (head/thorax) in the mosquito. The amount of parasites in the bird's blood was quantified following a protocol adapted from Cornet *et al.* (2013). DNA was extracted using Qiagen DNeasy Blood and Tissue kit, following manufacturer protocol. For each bird, two qPCRs were carried out: one targeting the nuclear 18s rDNA gene of *Plasmodium* (Primers 18sPlasm7 5'-AGCCTGAGAAATAGCTACCACATCTA-3', 18sPlasm8 5'-TGTTATTTCTTGTCACTACCTCTCTTCTTT-3') and the other targeting the 18s rDNA gene of the bird (Primers 18sAv7 5'-GAAACTCGCAATGGCTCATTAAATC-3', 18sAv8 5'-TATTAGCTCTAGAATTACCACAGTTATCCA-3'). All samples were run in triplicates on a Bio-Rad CFX96^{TM} Real-Time System. Samples with a threshold Ct value higher than 35 were considered uninfected. The number of parasites was calculated as relative quantification values (RQ). RQ can be interpreted as the fold-amount of target gene (*Plasmodium* 18s rDNA) with respect to the amount of the reference gene (Bird18s rDNA) and is calculated as $2^{-(Ct18s\ Plasmodium - Ct18s\ Bird)}$.

Quantification of sporozoites. Molecular work for the quantification of the sporozoites is still in progress for this study. The head and thorax of each sample was flash frozen in liquid nitrogen and ground using beads. DNA was then extracted using the Qiagen DNeasy 96 blood and tissue kit following the manufacturer instructions with one modification: samples were incubated overnight.

For each mosquito, two qPCRs were carried out: one targeting the mtDNA cytb gene of *Plasmodium* (Primers L4050Plasmo 5'-GCTTTATGTATTGTATTTATAC-3', H4121Plasmo 5'-GACTTAAAAGATTTGGATAG-3', Probe TexasRed-CYTB-BHQ2 5'- CCTTTAGGGTATGATACAGC-3') and the other targeting the CQ11 nuclear gene of *C. pipiens* mosquitoes (Primers 1725-F 5'-GCGGCCAAATATTGAGACTT-3', 1726-R 5'- CGTCCTCAAACATCCAGACA-3', Probe FAM-CQ11-BHQ1 5'-GGAACATGTTGAGCTTCGGK-3'). All samples were run in triplicates (QuantStudio 6 and 7 Pro Real-Time PCR Systems). Samples with a threshold Ct value higher than 35 for the parasite were considered uninfected. The number of parasites was calculated as relative quantification values (RQ). RQ can be interpreted as the fold-amount of target gene (*Plasmodium* CYTB) with respect to the amount of the reference gene (*Cx. pipiens* CQ11) and was calculated as $2^{-(Ct\text{CYTB } Plasmodium - Ct\text{CQ11 } Cx.\ pipiens)}$.

Statistical analyses

We tested the effect of larval nutritional stress on different mosquito life history and infection-related traits. Each trait was analysed separately. All analyses were performed using the R software (version 4.2.1). The mosquito body size, number of eggs, blood meal size, oocyst burden and haemocyte concentration were analysed using linear mixed models, as implemented in the *lmer* function from the R package *lme4* (Bates *et al.* 2015). The influence of larval nutritional state on eggs prevalence (i.e. proportion of mosquitoes that laid eggs) and blood meal rate (i.e. proportion of blood-fed mosquitoes) was analysed using generalized linear mixed models, as implemented in the *glmer* function of *lme4*. All models included the bird as a random factor. Larval nutritional treatment, haematine, oocyst burden and wing length (to normalize for mosquito body size) were fitted as fixed factors, including all interactions. The models were then simplified by sequentially eliminating non-significant interactions and terms to establish a minimal model. The significance of the explanatory variables was tested using likelihood ratio tests (Crawley 2012). The sample sizes included in each analysis and the final models are described in **Table S1**.

Results

Effect of larval nutritional stress on adult mosquito fecundity.

Food treatments had an impact on some mosquito life history traits. Larval nutritional stress affected female wing length, measured as a proxy of the body size (model 1, $X^2 = 275.96$, $p < 0.001$). High diet females had significantly larger wings (mean ± se, 3.78 ± 0.014 mm) than low diet females (3.31 ± 0.015 mm). Larval nutritional stress also impacted some but not all aspects of the fecundity of adult female mosquitoes. The probability of a female laying eggs was not impacted by the larval nutritional stress (model 2, $X^2 = 1.19$, $p=0.27$) nor by the blood meal size (model 2, $X^2 = 0.32$, $p = 0.57$) or the body size (model 2, $X^2 = 1.26$, $p = 0.26$). The number of eggs laid by a female was not explained directly by the treatment (model 3, $X^2 = 0.87$, $p = 0.35$, **Figure 1**) but by both the blood meal size (model 3, $X^2 = 17.681$, $p < 0.001$) and the body size (model 3, $X^2 = 7.3$, $p = 0.007$). The majority of the variation is explained by mosquito body size and blood meal size, which in turn are explained by treatment. High diet females were bigger, took larger blood meals and laid more eggs.

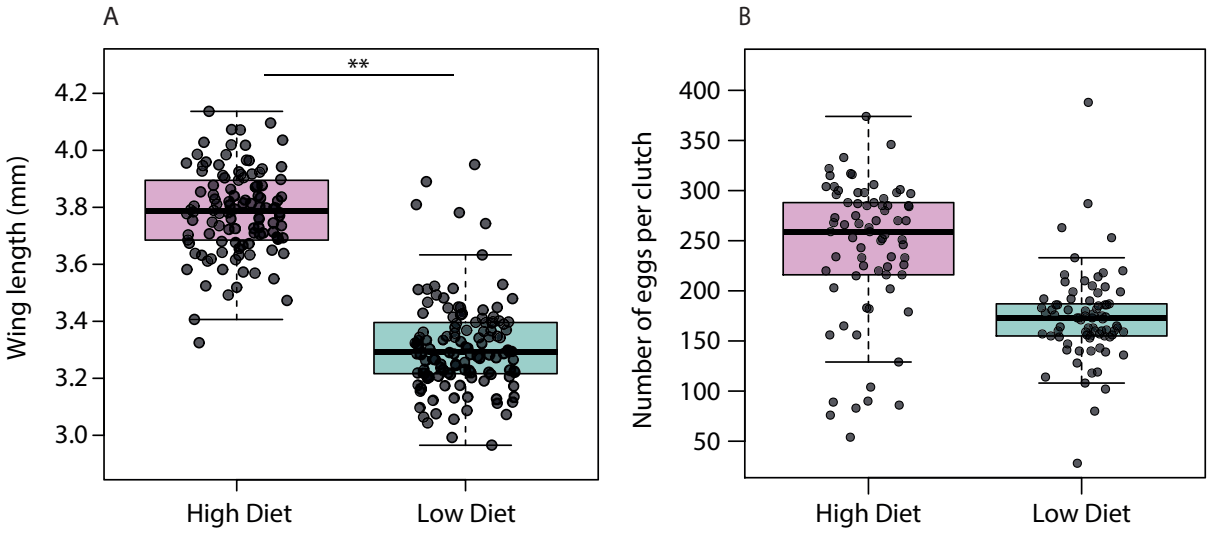

Figure 1: **Effect of larval nutritional stress on mosquito life history traits**. (A) Wing length, a proxy for body size, of females' mosquito reared on the High Diet treatment (pink) or the Low Diet treatment (blue). Significance level: **, $p < 0.01$. (B) Number of eggs per clutch per females in High-food treatment (pink) or Low-diet treatment (blue). Each point represents one female mosquito.

Effect of larval nutritional stress on pathogen infectivity.

There was no difference in blood meal rate (i.e. proportion of blood-fed mosquitoes) between the treatments (LD: 43%, HD: 39%, model 5, $X^2 = 1.34$, $p = 0.25$). The haematin quantity, measured as a proxy for blood meal size, varied significantly with the female body size (model 5, $X^2 = 34.38$, $p < 0.001$) rather than the mosquito treatment (model 5, $X^2 = 0.137$, $p = 0.71$). The bigger the female mosquitoes were, the larger blood meal size they took.

The temporal dynamic of oocyst production within the mosquitoes was consistent across the treatments (**Figure 2A**). When focusing on the peak of oocyst infection, on day 6 post blood meal, even though mosquitoes reared in LD conditions tended to hold slightly more oocysts (mean ± se, LD mosquitoes: 321.78 ± 82.5 oocysts) than mosquitoes reared in HD conditions (HD mosquitoes: 245.87 ± 77.6 oocysts), the difference in oocyst burden between the two treatments was not statistically significant (model 6, $X^2 = 0.5$, $p = 0.48$, **Figure 2B**).

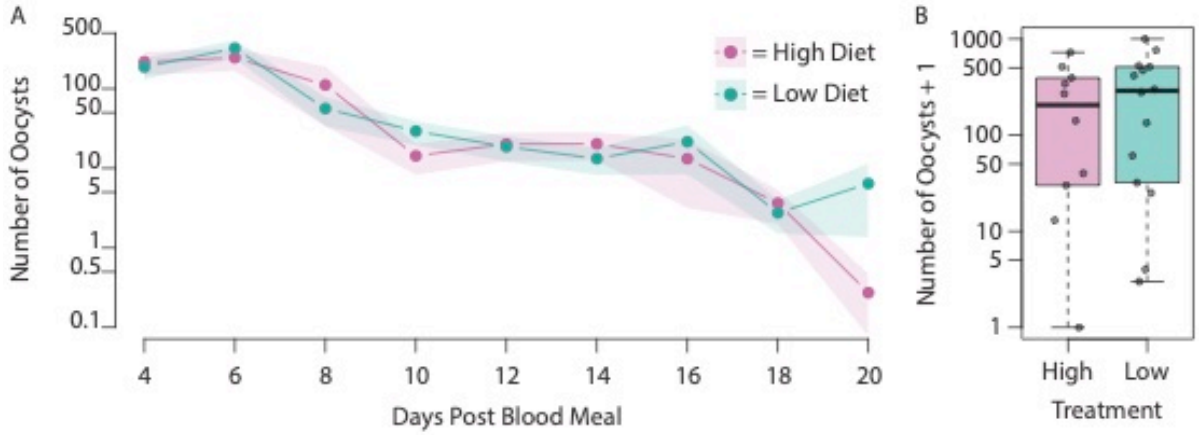

Figure 2: **Relationship between larval nutritional stress and oocyst burden.** (A) Mean oocyst count over the course of infection for the High-diet treatment (HD, pink) or the Low-diet treatment (LD, green). (B) Impact of larval nutritional stress on the oocyst burden at the peak of infection, day 6 pbm. Note the logarithmic scale of the y axes.

Effect of larval nutritional stress on extrinsic incubation period (EIP).

Data on sporozoites quantity is not available yet. Molecular work for the quantification of sporozoites is currently in progress.

Effect of larval nutritional stress on mosquito immunity to infection.

At peak of oocyst infection, on day 6 post blood meal, even though mosquitoes reared in high food conditions tended to harboured slightly more haemocytes in their haemolymph (11.16 ± 3.38 hc/µl) than mosquitoes reared in low food conditions (4.7 ± 1.8 hc/µl) the difference between the two treatments was not statistically significant (model 7, $X^2 = 0.87$, p = 0.35). Haemocytes concentration was also not associated with the body size of the mosquito (model 7, $X^2 = 2.31$, p=0.13 or the oocyst burden (model 7, $X^2 = 19$, p=0.66).

Discussion

Understanding what factors shape parasite dynamics in vectors is a major topic of interest in host-parasite interactions and may contribute to the control of vector-borne diseases. The aim of this study was to investigate how variations in the vector's environment quality affect some components of the vectorial capacity and thus transmission outcome. We induced a nutritional stress during the larval development of the major vector of the avian malaria parasite and found that such variations affect both the fecundity and the immunity of the mosquitoes with no effect on their intensity of infection. Unfortunately, regarding the EIP, we cannot conclude at this time as analyses are still in progress.

Mosquito larvae are aquatic invertebrates and the conditions they experience during their development are known to shape their adult life history traits through carry-over effects (Araújo *et al.* 2012; Takken *et al.* 2013; Breaux *et al.* 2014; Moller-Jacobs *et al.* 2014; Vantaux *et al.* 2016; Bataillard *et al.* 2020). Manipulating the quality of the larval environment by inducing a nutritional stress did not affect the propensity of females to lay eggs but affected their fecundity. Clutch size of females reared under low food conditions were reduced by up to 30% compared to females reared under high food conditions. This result is in line with previous studies that demonstrated a reduction in fecundity associated to larval nutritional stress (Takken *et al.* 2013; Moller-Jacobs *et al.* 2014; Vantaux *et al.*

2016). One explanation may be that reduced fecundity was associated with reduced blood meal size due to smaller individual size. Females under nutritional stress might trade investment in reproduction for survival as well as for investment in immune defences to overcome parasitic infections (Sheldon & Verhulst 1996; Hajkazemian *et al.* 2021). This way, the vector ensures minimum reproduction while limiting potential costs of infection.

Mosquito propensity to infect further hosts relies partly on its ability to support an infection without this being detrimental to its own survival (Hajkazemian *et al.* 2021). Contrary to our expectations, nutritional stress at larval stage did not appear to affect the infectivity of *Plasmodium* in the adult mosquitoes. High diet and low diet females had similar oocyst burdens in their midgut at the peak of infection. In addition, low diet females at the peak of infection tend to had a lower number of haemocytes circulating in their haemolymph compared to the high diet ones, suggesting that the immune response might be less important in the low diet females. The hypothesis that females under nutritional stress might trade investment in reproduction for investment in immune defences to overcome parasitic infections seems unlikely.

Female mosquitoes take a blood meal to get the nutrients essential to develop their eggs. Recently, studies have shown that the development of the oocyst stage relies on non-competitive strategies in mosquito resources exploitation: parasites exploit the surplus of nutritional resources that was not invested in oogenesis (Costa *et al.* 2018; Werling *et al.* 2019). Even though diet-stressed females take smaller blood meals, parasite might exploit the same amount of "surplus" resources. This is consistent with our results as the negative effect found on the fecundity of females belonging to low food conditions was not associated with higher oocyst burdens.

The negative carry-over effect of food limitation at the larval stage on adult immunity does not allow us to conclude that nutrient-deficient females are more susceptible to *Plasmodium* infection. Previous studies showed that larval poor nutritional status effectively reduced haemocyte numbers in adult mosquitoes, but enhanced fat body immune factors, known to show activities

against pathogens (e.g. AMPs), as a compensation for this reduction (Hillyer & Christensen 2005; Telang *et al.* 2012). Haemocyte-mediated immune response is one of the most efficient response against pathogens trough phagocytic, lytic and melanization pathways (Hillyer & Christensen 2005; Kumar *et al.* 2018). However, *Plasmodium* seems to be relatively unaffected by the anti-parasitic mosquito response at the oocyst stage (Blandin & Levashina 2007). Oocyst are encysted at the midgut barrier and release thousands of sporozoites trough the hemocoel that will actively invade the salivary glands within a few hours. Through the haemolymph flow, sporozoite degradation seems to be a rapid immune process that drastically impairs the salivary glands invasion (Hillyer *et al.* 2007). Sporozoites stages might be a better target for the mosquito immune systems. The extent to which variations in the numbers of circulating haemocytes are associated with within-vector dynamics of sporozoites remains to be investigated in our study as molecular studies are still ongoing.

A negative carry-over effect of food limitation at the larval stage is expected on the EIP of *Plasmodium*. For instance, for the human malaria parasite, the time to 50% of the maximum sporozoites prevalence within the mosquito population was delayed by 2 days for individuals reared in low food conditions (Shapiro *et al.* 2016). Slowing down the time to invasion of the salivary glands might be adaptive for the parasite, as it spares the vector and enhances its chance of surviving the infection long enough to re-transmit it. For parasites infecting mosquitoes in better conditions, a delay of two days in EIP might provide additional opportunity for transmission, which could have strong consequences in terms of disease outbreaks.

The ability for a vector to transmit a pathogen between vertebrate hosts, i.e. vectorial capacity, is sensitive to perturbations (Lefèvre *et al.* 2013; Cator *et al.* 2020). In this study we highlighted that variations in the habitat quality of mosquito larvae may affect both their life history traits and their ability to acquire and resist infection. The conclusions of this study are consistent with results from recent studies: transmission outcomes rely on multiple interaction between the host, the vector, the parasite and the environment they face (Lefèvre *et al.* 2013; Lefevre *et al.* 2018; Cansado-

Utrilla *et al.* 2021). All are intertwined and evolve in response to one another. In the current context, where the vector is a promising target for vector-borne disease control programmes (Powell 2019; Wilson *et al.* 2020; Oke *et al.* 2022), there is an urgent need to better understand the relative contributions of each actor in the tripartite interaction in transmission-related traits. How do they trade-off and respond to disturbances and to what extent this might affect the vectorial capacity?

Limitations. Beyond these preliminary results, some limitations of our study need to be considered. In this version, the analyses on the extrinsic incubation remain to be carried out. Furthermore, we cannot exclude the fact that, under nutritional stress, larvae were competing with each other for food, implying that we might have selected the most competitive individuals, since the larvae were reared at a constant density of 100 individuals per 400mL of water. To counteract this effect, a second experimental block was performed recently where the larvae were individually reared and fed to get rid of intraspecific competition. The results of this second experimental block are currently being processed. Future studies should be conducted to elucidate whether interactions between multiple stressors that reflect more accurately the current environment of larvae could shape mosquito competence.

References

Alto, B.W., Lounibos, L.P., Mores, C.N. & Reiskind, M.H. (2008). Larval competition alters susceptibility of adult *Aedes* mosquitoes to dengue infection. *Proceedings of the Royal Society B: Biological Sciences*, 275, 463–471.

Araújo, M. da-Silva, Gil, L.H.S. & e-Silva, A. de-Almeida. (2012). Larval food quantity affects development time, survival and adult biological traits that influence the vectorial capacity of *Anopheles darlingi* under laboratory conditions. *Malaria Journal*, 11, 261.

Bataillard, D., Christe, P. & Pigeault, R. (2020). Impact of field-realistic doses of glyphosate and nutritional stress on mosquito life history traits and susceptibility to malaria parasite infection. *Ecology and Evolution*, 10, 5079–5088.

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software*, 67(1), 1–48.

Beck-Johnson, L.M., Nelson, W.A., Paaijmans, K.P., Read, A.F., Thomas, M.B. & Bjørnstad, O.N. (2017). The importance of temperature fluctuations in understanding mosquito population dynamics and malaria risk. *Royal Society Open Science*, 4, 160969.

Blandin, S.A. & Levashina, E.A. (2007). Phagocytosis in mosquito immune responses. *Immunological Reviews*, 219, 8–16.

Breaux, J.A., Schumacher, M.K. & Juliano, S.A. (2014). What does not kill them makes them stronger: larval environment and infectious dose alter mosquito potential to transmit filarial worms. *Proceedings of the Royal Society B: Biological Sciences*, 281, 20140459.

Cansado-Utrilla, C., Zhao, S.Y., McCall, P.J., Coon, K.L. & Hughes, G.L. (2021). The microbiome and mosquito vectorial capacity: rich potential for discovery and translation. *Microbiome*, 9, 111.

Carrington, L.B., Armijos, M.V., Lambrechts, L. & Scott, T.W. (2013). Fluctuations at a Low Mean Temperature Accelerate Dengue Virus Transmission by *Aedes aegypti*. *PLOS Neglected Tropical Diseases*, 7, e2190.

Cator, L.J., Johnson, L.R., Mordecai, E.A., El Moustaid, F., Smallwood, T.R.C., LaDeau, S.L., *et al.* (2020). The Role of Vector Trait Variation in Vector-Borne Disease Dynamics. *Frontiers in Ecology and Evolution*, 8, 189.

Combes, C. (2020). *The Art of Being a Parasite*. University of Chicago Press.

Cornet, S., Nicot, A., Rivero, A. & Gandon, S. (2013). Malaria infection increases bird attractiveness to uninfected mosquitoes. *Ecology Letters*, 16, 323–329.

Costa, G., Gildenhard, M., Eldering, M., Lindquist, R.L., Hauser, A.E., Sauerwein, R., *et al.* (2018). Non-competitive resource exploitation within mosquito shapes within-host malaria infectivity and virulence. *Nature Communications*, 9, 3474.

Crawley, M.J. (2012). *The R Book*. John Wiley & Sons.

Ferguson, H.M. & Read, A.F. (2002). Genetic and environmental determinants of malaria parasite virulence in mosquitoes. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 269, 1217–1224.

Glaizot, O., Fumagalli, L., Iritano, K., Lalubin, F., Rooyen, J.V. & Christe, P. (2012). High Prevalence and Lineage Diversity of Avian Malaria in Wild Populations of Great Tits (*Parus major*) and Mosquitoes (*Culex pipiens*). *PLOS ONE*, 7, e34964.

Hajkazemian, M., Bossé, C., Mozūraitis, R. & Emami, S.N. (2021). Battleground midgut: The cost to the mosquito for hosting the malaria parasite. *Biology of the Cell*, 113, 79–94.

Hien, D.F. d S., Dabiré, K.R., Roche, B., Diabaté, A., Yerbanga, R.S., Cohuet, A., *et al.* (2016). Plant-Mediated Effects on Mosquito Capacity to Transmit Human Malaria. *PLOS Pathogens*, 12, e1005773.

Hillyer, J.F., Barreau, C. & Vernick, K.D. (2007). Efficiency of salivary gland invasion by malaria sporozoites is controlled by rapid sporozoite destruction in the mosquito haemocoel. *International Journal for Parasitology*, 37, 673–681.

Hillyer, J.F. & Christensen, B.M. (2005). Mosquito phenoloxidase and defensin colocalize in melanization innate immune responses. *Journal of Histochemistry and Cytochemistry*, 53, 689–698.

Kumar, A., Srivastava, P., Sirisena, P., Dubey, S.K., Kumar, R., Shrinet, J., *et al.* (2018). Mosquito Innate Immunity. *Insects*, 9, 95.

Lefevre, T., Ohm, J., Dabiré, K.R., Cohuet, A., Choisy, M., Thomas, M.B., *et al.* (2018). Transmission traits of malaria parasites within the mosquito: Genetic variation, phenotypic plasticity, and consequences for control. *Evolutionary Applications*, 11, 456–469.

Lefèvre, T., Vantaux, A., Dabiré, K.R., Mouline, K. & Cohuet, A. (2013). Non-Genetic Determinants of Mosquito Competence for Malaria Parasites. *PLoS Pathog*, 9, e1003365.

Moller-Jacobs, L.L., Murdock, C.C. & Thomas, M.B. (2014). Capacity of mosquitoes to transmit malaria depends on larval environment. *Parasites Vectors*, 7, 593.

Murdock, C.C., Sternberg, E.D. & Thomas, M.B. (2016). Malaria transmission potential could be reduced with current and future climate change. *Scientific Reports*, 6, 27771.

Muturi, E.J., Blackshear Jr., M. & Montgomery, A. (2012). Temperature and density-dependent effects of larval environment on *Aedes aegypti* competence for an alphavirus. *Journal of Vector Ecology*, 37, 154–161.

Ohm, J.R., Baldini, F., Barreaux, P., Lefevre, T., Lynch, P.A., Suh, E., *et al.* (2018). Rethinking the extrinsic incubation period of malaria parasites. *Parasites & Vectors*, 11.

Oke, C.E., Ingham, V.A., Walling, C.A. & Reece, S.E. (2022). Vector control: agents of selection on malaria parasites? *Trends in Parasitology*. In Press.

Pigeault, R., Vézilier, J., Cornet, S., Zélé, F., Nicot, A., Perret, P., *et al.* (2015). Avian malaria: a new lease of life for an old experimental model to study the evolutionary ecology of Plasmodium. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 370, 20140300.

Powell, J.R. (2019). An Evolutionary Perspective on Vector-Borne Diseases. *Frontiers in Genetics*, 10, 1266.

Rivero, A., Vézilier, J., Weill, M., Read, A.F. & Gandon, S. (2010). Insecticide Control of Vector-Borne Diseases: When Is Insecticide Resistance a Problem? *PLOS Pathogens*, 6, e1001000.

Shapiro, L.L.M., Murdock, C.C., Jacobs, G.R., Thomas, R.J. & Thomas, M.B. (2016). Larval food quantity affects the capacity of adult mosquitoes to transmit human malaria. *Proceedings of the Royal Society B: Biological Sciences*, 283, 20160298.

Shaw, D.K., Tate, A.T., Schneider, D.S., Levashina, E.A., Kagan, J.C., Pal, U., *et al.* (2018). Vector Immunity and Evolutionary Ecology: The Harmonious Dissonance. *Trends in Immunology*, 39, 862–873.

Sheldon, B.C. & Verhulst, S. (1996). Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. *Trends in Ecology & Evolution*, 11, 317–321.

Takken, W., Smallegange, R.C., Vigneau, A.J., Johnston, V., Brown, M., Mordue-Luntz, A.J., *et al.* (2013). Larval nutrition differentially affects adult fitness and *Plasmodium* development in the malaria vectors *Anopheles gambiae* and *Anopheles stephensi*. *Parasites & Vectors*, 6, 345.

Telang, A., Qayum, A.A., Parker, A., Sacchetta, B.R. & Byrnes, G.R. (2012). Larval nutritional stress affects vector immune traits in adult yellow fever mosquito *Aedes aegypti* (*Stegomyia aegypti*). *Medical and Veterinary Entomology*, 26, 271–281.

Tripet, F., Aboagye-Antwi, F. & Hurd, H. (2008). Ecological immunology of mosquito–malaria interactions. *Trends in Parasitology*, 24, 219–227.

Vantaux, A., Lefèvre, T., Cohuet, A., Dabiré, K.R., Roche, B. & Roux, O. (2016). Larval nutritional stress affects vector life history traits and human malaria transmission. *Scientific Reports*, 6, 36778.

Vézilier, J., Nicot, A., Gandon, S. & Rivero, A. (2010). Insecticide resistance and malaria transmission: infection rate and oocyst burden in *Culex pipiens* mosquitoes infected with *Plasmodium relictum*. *Malaria Journal*, 9, 379.

Vézilier, J., Nicot, A., Gandon, S. & Rivero, A. (2012). Plasmodium infection decreases fecundity and increases survival of mosquitoes. *Proceedings of the Royal Society B: Biological Sciences*, 279, 4033–4041.

Werling, K., Shaw, W.R., Itoe, M.A., Westervelt, K.A., Marcenac, P., Paton, D.G., *et al.* (2019). Steroid Hormone Function Controls Non-competitive *Plasmodium* Development in *Anopheles*. *Cell*, 177, 315-325.e14.

World health statistics 2017: monitoring health for the SDGs, Sustainable Development Goals. (2017) *Geneva: World Health Organization*. Licence: CC BY-NC-SA 3.0 IGO.

Wilson, A.L., Courtenay, O., Kelly-Hope, L.A., Scott, T.W., Takken, W., Torr, S.J., *et al.* (2020). The importance of vector control for the control and elimination of vector-borne diseases. *PLOS Neglected Tropical Diseases*, 14, e0007831.

Yan, J., Kibech, R. & Stone, C.M. (2021). Differential effects of larval and adult nutrition on female survival, fecundity, and size of the yellow fever mosquito, *Aedes aegypti*. *Frontiers in Zoology*, 18, 10.

Table S1: Description of statistical models used in the study. "N" gives the sample size. "Maximal Model" includes the complete set of explanatory variables. "Minimal model" gives the model containing only the significant variables and their interactions. Square brackets indicate variables fitted as random factors. Curly brackets indicate the error structure used (n: normal errors, b: binomial errors).

Variable of interest	Resp. variable	Model Nb.	N	Maximal model	Minimal model	Rsubroutine [err struct.]
Body size	Wing length	1	235	Treatment + (1|Ring)	Treatment + (1|Ring)	lmer{n}
Eggs prevalence	cbind(eggs,no_eggs)	2	197	Treatment + Hematine + Body_size + (1|Ring)	1 + (1|Ring)	glmer{b}
Number of eggs	Nb_eggs	3	143	Treatment + Hematine + Body_size + (1|Ring)	Hematine + Body_size + (1|Ring)	lmer{n}
Blood meal rate	Bm_rate	4	14	Treatment + (1|Ring)	1 + (1|Ring)	glmer{b}
Blood meal size	Hematine	5	235	Treatment + Body_size + (1|Ring)	Body_size + (1|Ring)	lmer{n}
Oocyst burden - Day 6 pbm	log(Oocyst+1)	6	23	Treatment + Hematine + Body_size + (1|Ring)	1 + (1|Ring)	lmer{n}
[Hemocytes] - Day 6 pbm	log(HcConcentration)	7	23	Treatment + Oocyst + Body_size + (1|Ring)	1 + (1|Ring)	lmer{n}

General discussion

The general purpose of my thesis was to investigate the traits that underpin the dynamics of *Plasmodium* malaria parasite within its mosquito vector and the extent to which this can drive heterogeneity in pathogen transmission. I have shown that both inter-individual and intra-individual heterogeneity in parasite dynamics within the host influence the resulting development of the parasite within the vector. Furthermore, I have highlighted that *Plasmodium* might have developed plastic strategies to adjust its own development in order to maximize its transmission both within its vertebrate host and within the vector.

Plastic strategies involved in transmission from the vertebrate host to the vector

Transmission to the mosquito vector is dependent on the presence of mature gametocytes in the vertebrate host blood when the vector blood feeds. In chapter **one, two** and **three** I have studied how heterogeneity of infection both between and within-vertebrate hosts impact the transmission. There is evidence that variations of *Plasmodium* intensities shape the resulting mosquito infection rate. Specifically, we have shown that both spatial (**chapter two;** Pigeault *et al.* 2020) and temporal (**chapter three;** Isaïa *et al.* 2020) heterogeneity exist at the intra-individual host level. *Plasmodium* is non-homogeneously distributed within each bird, and is able to increases its transmission to the vector at a short time scale. This is mediated either by accumulative mosquito bites or by host responses to the content of mosquito saliva.

Nevertheless, and contrary to our predictions, we did not always find a positive relationship at the inter-individual level between host parasitaemia and mosquito oocyst burden. This is quite surprising, as parasitaemia in the avian malaria system was shown to be positively associated with the intensity of mosquito infection at the oocyst stage both in terms of prevalence and intensity (Pigeault

et al. 2015). To assess to which extent this was the case, I combined all the data I generated in my thesis across all experiments. I analyzed the relationship between parasitaemia and the resulting oocyst burden in mosquitoes (**Figure 1**). While parasitaemia was a good predictor of oocyst burden for low values (ranging from 0 to 5% of infected red blood cells), there was no clear relationship for higher parasitaemia (ranging from 5 up to 20% of infected red blood cells). There thus seems to be a threshold beyond which parasitaemia no longer predicts oocyst burden in the mosquito. In the avian malaria system, this threshold appears to be around 5% of infected red blood cells, which might correspond to an optimal parasitaemia to ensure transmission. The marginal profitability of increased parasitemia may decrease beyond this threshold. In addition, additional mechanisms may be triggered above 5% of parasitemia, which could explain why the positive relationship with oocyst burden disappears. A similar pattern is found in the relationship between oocyst and sporozoite burden within the mosquito (Pollitt *et al.* 2013), indicating that there might be optimal densities at different stages of the life cycle of *Plasmodium*. Even tough proximal mechanisms remain unknown, this might result from one or several adaptive strategies evolved by *Plasmodium*.

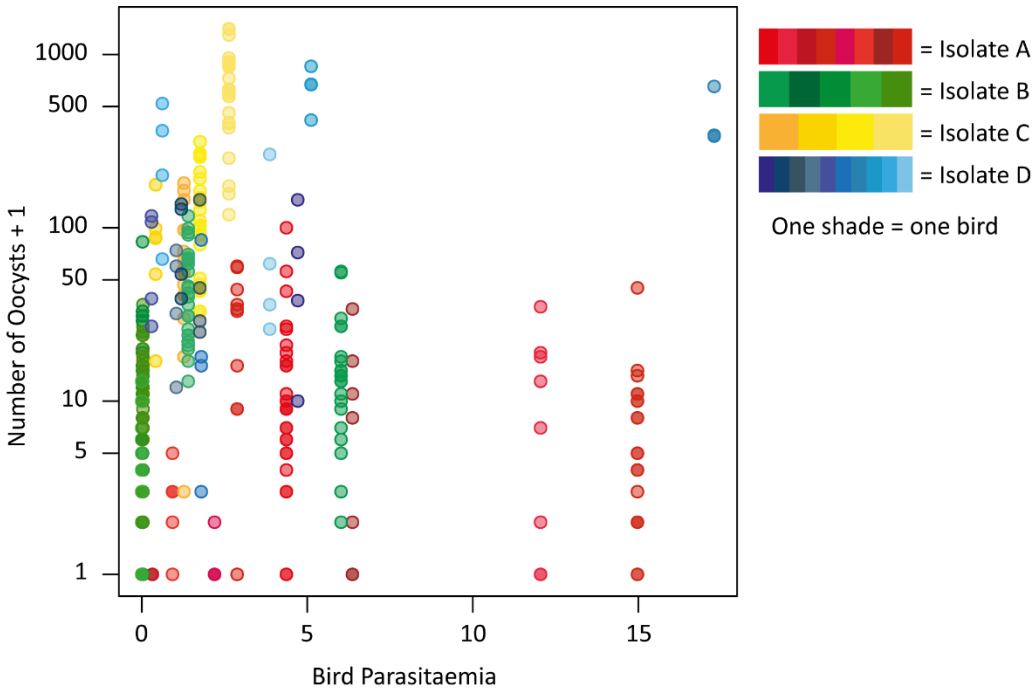

Figure 1: Relationship between parasitemia in the bird and oocyst burden in the mosquito across all datasets generated during my PhD. Each point is one mosquito. The four colours represent four different *Plasmodium* isolates from the wild, and each shade corresponds to a different bird.

The trade-off in resource allocation between within-host growth and survival (i.e. the conversion rate of asexual to sexual gametocytes) and reproduction (i.e. the gametocytes sex ratio) is expected to be solved via phenotypic plasticity (Pigliucci 2001; Meyers & Bull 2002; Reece *et al.* 2009; Mideo & Reece 2012; Carter *et al.* 2014). Over the recent years, it has been shown that *Plasmodium* is able to detect variations in its environment and plastically alter its traits related to transmission (Paul *et al.* 2002; Pollitt *et al.* 2011; Carter *et al.* 2014; Cornet *et al.* 2014). For example, one cue that can be detected under high levels of infection is increased levels of erythropoietin (the hormone triggering red blood cells production) in response to anemia associated to strong immune response (Paul *et al.* 2002). Based on that, one hypothesis is that *Plasmodium* might increase its investment into male gametocytes , the main target of the host immune response, to ensure that a female gamete will encounter a male gamete within the mosquito blood meal (Paul *et al.* 2002; West *et al.* 2002; Ramiro *et al.* 2011). Indeed, the number of future zygotes might be affected, as host antibodies affect the motility of male gametes, which could explain why higher parasitaemia does not necessarily lead to high oocyst burden. Another explanation could be that gametocytes infectivity is variable (Hawking *et al.* 1966). The maturation process of gametocytes remains unclear, but a study on rodent malaria highlighted that gametocytes were more infective, despite being less numerous in the blood at night (Schneider *et al.* 2018). There thus seems to be plastic variability in gametocytes infectivity. Beyond a certain threshold in the number of gametocytes (or parasitaemia), their infectivity might be impaired as the profitability for transmission to the mosquito decreases. *Plasmodium* might combine one or more of these strategies to fine-tune its transmission to the vector at a short time scale.

Several future directions can be taken to obtain a better a picture of the relationship between parasitaemia and mosquito infection rate. I tried to infect canaries with two diluted doses of parasite, however I got overlapping parasitaemia due to the strong variation in parasite development between birds. The protocol needs to be improved but would allow both to test a wider range of parasitaemia and increase the mosquito sample size, thus providing a better understanding of the extent to which beyond a certain threshold, host parasitaemia is no longer a good predictor of vector infection rate.

Furthermore, measuring the number of ex-flagellating gametes within the mosquito midgut right after a blood meal would allow us to measure gametocyte sex ratio and to test to what extent the parasite modifies it according to the host's parasitemia.

Plastic strategies involved in transmission from the vector to the vertebrate host

The strategies used by *Plasmodium* to maximize its fitness within its mosquito vector has received little attention. In my thesis, I highlighted that *Plasmodium* might plastically trade its investment between growth and survival (oocyst production) and transmission (sporozoites formation) within its vector. Indeed, within-vector environment is also a challenging environment for *Plasmodium.* It experiences successive life-stage transitions (gametocytes, ookinetes, oocyst, sporozoites) and goes through intense density variations (Vaughan 2007; Poudel *et al.* 2008; Churcher *et al.* 2010). Parasite overcrowding can be costly for the mosquitoes because it can alter fitness-related traits such as its survival and fecundity (Vézilier *et al.* 2012; Hajkazemian *et al.* 2021). Two non-exclusive strategies used by *Plasmodium* to prevent overcrowding and the resulting fitness cost of its vector have been reported in the literature: reducing its own population (e.g. by apoptosis; Al-Olayan *et al.* 2002; Reece *et al.* 2011) and shifting its development time (Habtewold *et al.* 2021). **In chapter one**, I showed that high levels of parasitaemia lead to a shorter extrinsic incubation period, which is the time required for the parasite to invade the mosquito salivary glands. This accelerated development probably took place between the ookinetes-to-oocyst transition. It could be due either to competition for resources (both between parasites clones and for mosquito resources) and/or to an adaptive strategy to favour its transmission when there is a risk of death for the vector. However, these results are in contrast with those on the rodent malaria. Indeed, in case of high intensities, *Plasmodium* might enter a dormancy-like stage, thereby slowing down oocyst growth and maturation (Habtewold *et al.* 2021). **In chapter 4,** I induced a nutritional stress during the mosquito development to lower its immune response in order to investigate the effect of reduced immunity on oocyst developmental time when the mosquito

is in poor condition. Results are not yet available but they will certainly give us more information about such hypothesis. Even though the relationship between high infection intensity and development speed of oocyst remains unclear, this supports the idea that *Plasmodium* has evolved plastic strategies to maximize its fitness not only within the host, but also within the vector.

Improving measures of transmission?

From the vertebrate host to the vector. The most widely used proxy for whether an individual is infected and infectious is gametocytes density in one blood sample. However, I have shown in **chapter two** that *Plasmodium* is non-homogeneously distributed within its vertebrate host. The differences in parasite load were observed between the extremities of both human and birds hosts. Therefore, an estimation of gametocyte density from only one blood sample, as is commonly measured, could provide unreliable estimate of the infectivity of gametocyte carriers.

From the vector to the vertebrate host. The most widely used measure of infection within the mosquito is the number of oocysts in the midgut. This is a quick and cheap way of finding out whether an individual is infected and to which extent. Therefore, most studies use it as sole proxy for the propensity of a vector to infect future hosts. Even if it is assumed that oocyst burden is a good predictor of sporozoite burden (Miura *et al.* 2019), the relationship is not so clear (Pollitt *et al.* 2013) and depends on the *Plasmodium*-vector systems. It would be interesting to consider these two measures to get a better estimate of the infectious potential of the mosquito. Another key parameter to assess transmission that would be worth measuring in addition to oocyst burden is the extrinsic incubation period of the parasite inside the vector (Ohm *et al.* 2018). This parameter determines the time required for the vector to become infectious. However, destructive methods commonly used to monitor the infection in a mosquito population limits sample sizes and prevents individual tracking of *Plasmodium* within the mosquito over the course of infection. To overcome this, I tried to develop non-invasive methods to follow the course of infection within mosquitoes. I tried to detect *Plasmodium* DNA in the sugar cottons on which mosquitoes feed as they are known to release

sporozoites via their saliva. However, our results were not reliable over the time, and the protocol for our model study, *Culex pipiens* – *Plasmodium relictum* remains to be improved (Brugman *et al.* 2018; Gutiérrez-López *et al.* 2019; Ramírez *et al.* 2019; Guissou *et al.* 2021).

General conclusion

In this thesis, I investigated what sources create heterogeneity of infection in vectors (**Figure 2A**) and the extent to which the resulting heterogeneity in their competence-related traits (directly related to individual vectorial capacity, **Figure 2B**) could influence the potential intensity of parasite transmission. I emphasized that within-vector *Plasmodium* dynamics might be influenced in different ways by both the vertebrate host and vector factors and could underlie the resulting transmission intensity. *Plasmodium* is a plastic parasite, able to evolve sophisticated strategies in order to optimize its transmission both from the vertebrate host to the vector, and from the vector to the vertebrate host. Furthermore, it might combine several of these strategies throughout the overall infection path. One of the key messages of this thesis is the need to establish a framework considering several measures of the vector infection, such as oocyst burden, sporozoites burden and EIP, to have a complete understanding of the transmission dynamics. Predicting when and how the within-vector dynamics vary in terms of intensity but also temporally under varying conditions, might be useful data that can be incorporated into epidemiological models to better understand and predict malaria transmission dynamics.

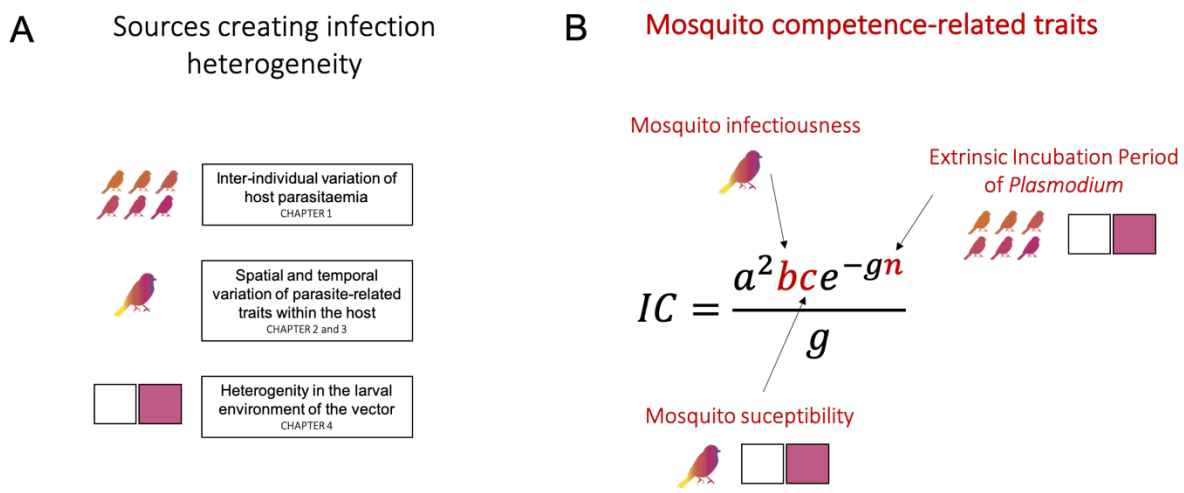

Figure 2: (A) Summary of the factors studied in this thesis that create heterogeneity of infection in vectors (B) in different traits directly related to the mosquito competence and thus the vectorial capacity. The individual vectorial capacity (IC) is defined by several parameters: ***a*** refers to the feeding behaviour of the vector (number of bites on a focal host (per vector, per day)); ***b*** stands for the probability that a host becomes infected from an infectious bite, defined by a combination of host susceptibility to infection and vector infectiousness; ***c*** is the vector's susceptibility to infection; ***g*** is the mortality rate of the vector; and ***n*** the extrinsic incubation period of the pathogen (i.e. number of days required for the vector to become infectious; EIP).

References

Al-Olayan, E.M., Williams, G.T. & Hurd, H. (2002). Apoptosis in the malaria protozoan, Plasmodium berghei: a possible mechanism for limiting intensity of infection in the mosquito. *International Journal for Parasitology*, 32, 1133–1143.

Brugman, V.A., Kristan, M., Gibbins, M.P., Angrisano, F., Sala, K.A., Dessens, J.T., *et al.* (2018). Detection of malaria sporozoites expelled during mosquito sugar feeding. *Sci Rep*, 8, 1–9.

Carter, L.M., Schneider, P. & Reece, S.E. (2014). Information use and plasticity in the reproductive decisions of malaria parasites. *Malaria Journal*, 13, 115.

Churcher, T.S., Dawes, E.J., Sinden, R.E., Christophides, G.K., Koella, J.C. & Basáñez, M.-G. (2010). Population biology of malaria within the mosquito: density-dependent processes and potential implications for transmission-blocking interventions. *Malaria Journal*, 9, 311.

Cornet, S., Nicot, A., Rivero, A. & Gandon, S. (2014). Evolution of Plastic Transmission Strategies in Avian Malaria. *PLoS Pathogens*, 10, e1004308.

Guissou, E., Waite, J.L., Jones, M., Bell, A.S., Suh, E., Yameogo, K.B., *et al.* (2021). A non-destructive sugar-feeding assay for parasite detection and estimating the extrinsic incubation period of Plasmodium falciparum in individual mosquito vectors. *Sci Rep*, 11, 9344.

Gutiérrez-López, R., Martínez-de la Puente, J., Gangoso, L., Yan, J., Soriguer, R. & Figuerola, J. (2019). Experimental reduction of host Plasmodium infection load affects mosquito survival. *Sci Rep*, 9, 8782.

Habtewold, T., Sharma, A.A., Wyer, C.A.S., Masters, E.K.G., Windbichler, N. & Christophides, G.K. (2021). Plasmodium oocysts respond with dormancy to crowding and nutritional stress. *Sci Rep*, 11, 3090.

Hajkazemian, M., Bossé, C., Mozūraitis, R. & Emami, S.N. (2021). Battleground midgut: The cost to the mosquito for hosting the malaria parasite. *Biology of the Cell*, 113, 79–94.

Hawking, F., Worms, M.J., Gammage, K. & Goddard, P.A. (1966). The Biological Purpose of the Blood-Cycle of the Malaria Parasite Plasmodium cynomolgi. *The Biological Purpose of the Blood-Cycle of the Malaria Parasite Plasmodium cynomolgi.*, 422–4.

Isaïa, J., Rivero, A., Glaizot, O., Christe, P. & Pigeault, R. (2020). Last-come, best served? Mosquito biting order and Plasmodium transmission. *Proceedings of the Royal Society B: Biological Sciences*, 287, 20202615.

Meyers, L.A. & Bull, J.J. (2002). Fighting change with change: adaptive variation in an uncertain world. *Trends in Ecology & Evolution*, 17, 551–557.

Mideo, N. & Reece, S.E. (2012). Plasticity in parasite phenotypes: evolutionary and ecological implications for disease. *Future Microbiology*, 7, 17–24.

Miura, K., Swihart, B.J., Deng, B., Zhou, L., Pham, T.P., Diouf, A., *et al.* (2019). Strong concordance between percent inhibition in oocyst and sporozoite intensities in a Plasmodium falciparum standard membrane-feeding assay. *Parasites & Vectors*, 12, 206.

Ohm, J.R., Baldini, F., Barreaux, P., Lefevre, T., Lynch, P.A., Suh, E., *et al.* (2018). Rethinking the extrinsic incubation period of malaria parasites. *Parasites & Vectors*, 11.

Paul, R.E.L., Brey, P.T. & Robert, V. (2002). Plasmodium sex determination and transmission to mosquitoes. *Trends in Parasitology*, 18, 32–38.

Pigeault, R., Isaïa, J., Yerbanga, R.S., Dabiré, K.R., Ouédraogo, J.-B., Cohuet, A., *et al.* (2020). Different distribution of malaria parasite in left and right extremities of vertebrate hosts translates into differences in parasite transmission. *Scientific Reports*, 10, 10183.

Pigeault, R., Vézilier, J., Cornet, S., Zélé, F., Nicot, A., Perret, P., *et al.* (2015). Avian malaria: a new lease of life for an old experimental model to study the evolutionary ecology of Plasmodium. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 370, 20140300.

Pigliucci, M. (2001). *Phenotypic Plasticity: Beyond Nature and Nurture*. JHU Press.

Pollitt, L.C., Churcher, T.S., Dawes, E.J., Khan, S.M., Sajid, M., Basáñez, M.-G., *et al.* (2013). Costs of crowding for the transmission of malaria parasites. *Evolutionary Applications*, 6, 617–629.

Pollitt, L.C., Mideo, N., Drew, D.R., Schneider, P., Colegrave, N. & Reece, S.E. (2011). Competition and the Evolution of Reproductive Restraint in Malaria Parasites. *The American Naturalist*, 177, 358–367.

Poudel, S.S., Newman, R.A. & Vaughan, J.A. (2008). Rodent Plasmodium Population Dynamics of Early Sporogony within Anopheles stephensi Mosquitoes. *para*, 94, 999–1008.

Ramírez, A.L., van den Hurk, A.F., Mackay, I.M., Yang, A.S.P., Hewitson, G.R., McMahon, J.L., *et al.* (2019). Malaria surveillance from both ends: concurrent detection of Plasmodium falciparum in saliva and excreta harvested from Anopheles mosquitoes. *Parasites & Vectors*, 12, 355.

Ramiro, R.S., Alpedrinha, J., Carter, L., Gardner, A. & Reece, S.E. (2011). Sex and Death: The Effects of Innate Immune Factors on the Sexual Reproduction of Malaria Parasites. *PLOS Pathogens*, 7, e1001309.

Reece, S.E., Pollitt, L.C., Colegrave, N. & Gardner, A. (2011). The Meaning of Death: Evolution and Ecology of Apoptosis in Protozoan Parasites. *PLOS Pathogens*, 7, e1002320.

Reece, S.E., Ramiro, R.S. & Nussey, D.H. (2009). SYNTHESIS: Plastic parasites: sophisticated strategies for survival and reproduction? *Evolutionary Applications*, 2, 11–23.

Schneider, P., Rund, S.S.C., Smith, N.L., Prior, K.F., O'Donnell, A.J. & Reece, S.E. (2018). Adaptive periodicity in the infectivity of malaria gametocytes to mosquitoes. *Proceedings of the Royal Society B: Biological Sciences*, 285, 20181876.

Vaughan, J.A. (2007). Population dynamics of Plasmodium sporogony. *Trends in Parasitology*, 23, 63–70.

Vézilier, J., Nicot, A., Gandon, S. & Rivero, A. (2012). Plasmodium infection decreases fecundity and increases survival of mosquitoes. *Proceedings of the Royal Society B: Biological Sciences*, 279, 4033–4041.

West, S.A., Smith, T.G., Nee, S. & Read, A.F. (2002). FERTILITY INSURANCE AND THE SEX RATIOS OF MALARIA AND RELATED HEMOSPORORIN BLOOD PARASITES. *Journal of Parasitology*, 88, 258–263.

Appendix One

Impact of host stress on the replication rate of *Plasmodium*: Take it easy to avoid malaria relapse

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AUTHOR'S CONTRIBUTIONS

RP, OG and PC designed the study. RP, RDPA, MB and JI performed the experiments. RP and RDPA analyzed the data. RP wrote the first draft of the manuscript with input from all authors.

CONFLICT OF INTEREST

The authors have no conflict of interest.

ANIMAL ETHICS

This study was approved by the Ethical Committee of the Vaud Canton veterinary authority, authorization number 1730.4.

DATA AVAILABILITY STATEMENT

Data supporting the results are stored in Figshare website (https://figshare.com/s/099861f53eb25f9a4eb5). A public DOI will be generated after acceptance of this manuscript.

ABSTRACT

1. Malaria is widespread throughout the world and affects many animal species. Although the origin of this vector-borne disease was discovered more than a century ago, several aspects of the within-host infection dynamic are still poorly understood. Among these aspects, the factors triggering parasite relapses and recrudescences - episodes of brief increase in parasite number following a period when the parasite was either absent or present at very low levels in the blood - have still not been clearly identified. Yet, they may contribute significantly to overall infection rates in vertebrate host population.
2. Here, we investigated whether artificial or natural increases in stress hormone levels in chronically infected birds can impact the replication rate of *Plasmodium relictum* and transmission to its natural vector, the mosquito *Culex pipiens*. Our results provide evidence that increased levels of corticosterone, either induced by oral ingestion or caused by handling stress, can trigger malaria relapses. However, we did not observe any effect on the transmission rate of the parasite to the mosquito vector.
3. Further, while it has been previously shown that vertebrate host exposure to mosquito may also induce a relapse, here we suggest that this phenomenon could be mediated by the stress induced by mosquito bites.
4. Our study is a first fundamental step in understanding the mechanisms underlying malaria relapses. It remains to be ascertained whether this feature extends to other malaria system and in particular to human malaria.

KEYWORDS

Avian malaria, Corticosterone, Oocyst burden, *Plasmodium*, Relapses, Within-host infection dynamic

1. INTRODUCTION

Malaria is caused by a vector-borne parasite genus, *Plasmodium*, which infects not only human but also many other vertebrate hosts, including reptiles, birds and non-human primates. After parasite transmission from a mosquito vector, the intensity of infection in the vertebrate host's peripheral blood changes drastically over time. Following an initial acute phase, characterized by a high number of parasites in the blood, the infection is either cleared or reaches a chronic phase, where the parasite burden stabilizes at a low level for several months or years (Ashley & White, 2014; Bishop et al., 1938; Ishtiaq, 2021; Telford, 1989). In some *Plasmodium* species, the chronic phase of infection may be interrupted by several episodes of intense, but brief, increase in parasite replication rate (i.e. malaria recurrences, (Applegate, 1971; Cogswell, 1992; Ishtiaq, 2021)) and ultimately by an increase in parasite infectiousness to invertebrate vectors (Cornet, Nicot, et al., 2014). These bouts of asexual replication rate may be due to relapse or to recrudescence. While the former results from the activation of dormant liver-stage parasites, called hypnozoites (Zanghi & Vaughan, 2021), the latter is due to incomplete elimination of erythrocytic stages either in the blood or present in extra-vascular compartments (Markus, 2018; Mayor et al., 2009; Popovici et al., 2019). In practice, it is difficult to determine whether a significant increase in parasitaemia (i.e. percentage of erythrocytes infected) during the chronic stage of infection is due to a relapse or a recrudescence. In the following, the term relapse will therefore be used to encompass both cases.

Relapses may significantly contribute to overall infection rates in vertebrate host populations and is an important source of malaria transmission (Commons et al., 2020; Taylor et al., 2019; N. J. White, 2011). For instance, in Papua New Guinea, malaria relapses contribute to approximately 50% of the overall number of *P. vivax* infections (Betuela et al., 2012). The periodicity of malaria relapses is not clearly defined and seems to vary according to parasite species (e.g. (Cogswell, 1992)), geographical area (e.g. (Battle et al., 2014)), host medical treatment (Taylor et al., 2019) but also seems to depend on the number of sporozoites (i.e. transmissible stage) injected by the mosquito

(Warren et al., 1974). Nevertheless, in temperate region, a seasonal pattern seems to emerge. In primate (human and non-human), avian and saurian malaria, relapses are most often observed in spring or early summer, irrespective of when the vertebrate host was originally infected (Applegate & Beaudoin, 1970; Cosgrove et al., 2008; Huldén et al., 2008; Schall, 1996; M. T. White et al., 2016), which suggests that the parasite reacts to changes in its environment. These seasonal relapse may be viewed as a strategy that allows the parasite to match the annual dynamics of vector populations (Cornet, Nicot, et al., 2014). In temperate zones, mosquito vectors are only present for a few months (Lalubin et al., 2013) and parasites may benefit from using environmental cues to coincide with the occurence of mosquitoes in the environment (Applegate & Beaudoin, 1970; Cornet, Nicot, et al., 2014; Valkiunas, 2004). However, nonseasonal relapses may also occur at any time of the year without clear connection with seasonal change in the environment (Valkiunas, 2004).

The mechanisms that trigger relapses are not well understood. For more than half a century, various authors have hypothesised that host hormonal systems could function as cues for parasite (Applegate & Beaudoin, 1970; Cornelius et al., 2014; Gross, 1947; Valkiunas, 2004). The increase in the amount of sex hormones in the blood during the breeding season, which correlates with the increase in day length in spring and thus with the presence of mosquitoes, has often been advanced as a potential trigger of seasonal relapses. But, to date, there is no empirical evidence confirming a role of reproductive hormones in malaria relapses (Applegate & Beaudoin, 1970; Eisen, 2000; Pearson, 2002; Slowinski et al., 2022). Conversely, studies, mainly carried out with avian malaria system, showed that stress-related hormonal changes may increase host parasitemia during both acute and chronic stage of infection (Applegate, 1970, 1971; Names et al., 2021; Schoenle et al., 2019). Since the concentration of stress hormones increases during the reproductive period in many organisms (Eggermann et al., 2013; Lormée et al., 2003; Reedy et al., 2014), it has been postulated that these hormones play a key role for seasonal relapses (Applegate, 1970, 1971). Variations in stress-related hormones levels could also be involved in non-seasonal relapses since it has been shown that malaria infection intensity might be impacted by hosts' exposition to stressful conditions (e.g. nutritional

stress, pollution, artificial light at night, (Becker et al., 2020; Bichet et al., 2013; Cornet, Bichet, et al., 2014). The mechanisms by which variation in stress hormone concentration might induce hypnozoite activation or impact *Plasmodium* replication rate has not been clearly identified, but chronic and acute stress may have detrimental effects on both host's adaptive and innate immune function (Bourgeon & Raclot, 2006; Cain & Cidlowski, 2017; Gao et al., 2017).

In addition to indirect signals from the environment (i.e. host hormones), it has been suggested that malaria parasites may fine-tune their investment in replication rate by using direct cues from vectors themselves to best match their phenology and abundance. Several studies have indeed shown that *Plasmodium* responded plastically to mosquito blood feeding bout by increasing parasitaemia ((Billingsley et al., 2005; Cornet, Nicot, et al., 2014; Lawaly et al., 2012; Pigeault et al., 2018) but see (Shutler et al., 2005)). This phenomenon is particularly significant during the chronic stage of infection (Cornet, Nicot, et al., 2014; Pigeault et al., 2018) and could be one of the mechanisms triggering seasonal but also nonseasonal relapses. While it is not excluded that *Plasmodium* directly detects mosquito cues (e.g. proteins) present in the saliva injected during the blood meal (Huldén & Huldén, 2011), the response of *Plasmodium* to mosquito bites could, as suggested by (Dhondt & Dobson, 2017), be mediated by the stress response of the vertebrate host. Exposure to blood-sucking insect may generate stress (Arsenopoulos et al., 2017; Kavaliers & Colwell, 1996; Martínez-de la Puente et al., 2011) and this physiological response, through its effect on host's immune system (Cain & Cidlowski, 2017; Gao et al., 2017), might underlie the increase in *Plasmodium* replication rates observed a few days after mosquito bites (Dhondt & Dobson, 2017).

In the present study, we investigated under controlled conditions whether an acute increase in stress-related hormone triggers malaria relapses. Then, we tested whether the increase in parasite burden, observed in vertebrate blood a few days after mosquito bites, could be mediated by the physiological stress response of the vertebrate host. For this purpose, we increased, either artificially or naturally, the stress hormone concentration of chronically infected host during a short period of

time and monitored the impact on *Plasmodium* replication rate and transmission to mosquito vectors. Thereafter, we explored the association between mosquito bites and the vertebrate blood stress hormone concentrations to determine whether stress associated with mosquito exposure might be sufficient to induce relapse. Avian malaria was used as a system to investigate these questions. Avian malaria is the oldest experimental system for investigating the life cycle of *Plasmodium* parasites (Rivero & Gandon, 2018) and particularly to study relapses (Applegate, 1970, 1971; Applegate & Beaudoin, 1970; Cornet, Nicot, et al., 2014; Pigeault et al., 2018). Further, avian malaria is the only currently available animal experimental system that allows working with a parasite recently isolated from the wild (*Plasmodium relictum*), and with its natural mosquito vector (*Culex pipiens*).

2- MATERIALS AND METHODS

2-1 Biological system

Plasmodium relictum SGS1 is the most common and prevalent parasite lineage causing avian malaria in Europe and is known to induce a long chronic phase marked by sudden events of relapses (Valkiunas, 2004). The parasite strain used in the first and second experiment was isolated from two infected House sparrows (*Passer domesticus*) captured in January 2019 and December 2020. The strains were maintained through regular passages across our stock canaries (*Serinus canaria*) using intraperitoneal injections until the experiments.

All experiments were carried out using domestic canaries (*Serinus canaria*). Prior to the experiments, a small amount (ca. 3-5 µL) of blood was collected from the medial metatarsal vein of each bird to ensure that they were free from any haemosporidian infection (Hellgren et al., 2004), for further details see Appendix 1, section 1).

Experiments were conducted with *Culex pipiens* mosquitoes (the main vector of *Plasmodium relictum* in Europe, Glaizot *et al*, 2012) collected in Lausanne (Switzerland, 46°31’25.607”N 6°34’40.714”E) in August 2017, and maintained in the insectary since. Mosquitoes were reared using

standard protocols (25 °C ± 1 °C, 70 ± 5% RH and with 12L:12D photoperiod (Vézilier et al., 2010). We used females 7-13 days after emergence, which have not previously had access to blood. Mosquitoes were maintained on glucose solution (10%) since their emergence and were starved (but provided with water to prevent dehydration) for 24h before the experiments.

2-2 Experiment 1: Impact of artificial increase of corticosterone concentration in vertebrate blood on *Plasmodium* replication rate and transmission

Eighteen birds were infected by intraperitoneal injection of 150µL of an infected blood pool (day 0). The blood pool was made of a 1:1 mixture of PBS and blood pool sampled from eight canaries that had been similarly infected two weeks before the experiment. After experimental infection, blood samples from the medial metatarsal vein (2-3µL) was sampled in all birds to monitor parasitaemia during the acute (10 **dpi**, i.e. **d**ay **p**ost-**i**nfection) and the chronic pre-experimental stage of infection (35, 47 dpi). One individual died day 25 post-infection. The remaining were assigned to two different experimental groups: "CORT" (n=9) and "Control" (n=8). To that end, we ranked the individuals by decreasing parasitaemia level (measured by quantitative PCR (qPCR) during the acute stage of infection, i.e. 10 dpi). We randomly assigned one of the two individuals with the highest parasitaemia levels to each experimental group. We repeated this for the two individuals with the next highest ranks until all individuals were assigned to a treatment group.

To study the impact of an artificial increase of corticosterone (the main glucocorticoid in birds) on *Plasmodium* replication rate during the chronic infection stage, we carried out an experiment consisting of four experimental days separated by three days interval. Due to the high number of manipulations, all experimental days were split into two blocks corresponding to two consecutive days. Eight individuals were used in the first experimental block (experimental days: 52, 55, 58 and 61 dpi) and nine birds for the second block (experimental days: 53, 56, 59 and 62 dpi). At 5:45 PM of each experimental day, birds ingested either 20µL of corticosterone solution (*CORT group*, 20 µg of

corticosterone diluted in 20µL of DMSO, dose per body mass ≈ 1 µg/g, (Breuner et al., 1998)) or 20µL of DMSO (*control group*). A 2.5mL syringe was used to inject the solutions directly into birds' beak. The ingestion of 20µg of corticosterone solution induced a rapid and strong increase in corticosterone concentration in bird's blood (average increase of 46.4 ng/ml ± 16 ng/ml compared to an increase of 2.17 ng/ml ± 0.45 ng/ml in the control group, see Appendix 1, section 2). At 6:00 PM, birds were immobilized in PVC tubes (length 18 cm, diameter 4.5 cm) covered by a mesh at both ends (see Fig. S1 in (Cornet et al., 2013) and placed in individual cages (L40 x W40 x H40 cm) in the dark for two hours. At 8:00 PM, blood samples from the medial metatarsal vein (ca. 2-3 µL) were taken from all individuals to quantify parasitaemia by qPCR. A red lamp was used to capture the birds and collect the blood samples.

To explore the impact of corticosterone ingestion on *Plasmodium* transmission from vertebrate host to mosquitoes, on the fourth experimental day (61/62 dpi), 60 ± 5 uninfected female mosquitoes were added in each cage at 7:00 PM (i.e. one hour after corticosterone or DMSO ingestion). After the blood sample has been taken from all birds at 8:00 PM, blood-fed mosquitoes were counted and individually placed in numbered plastic tubes (30 ml) covered with a mesh and with a cotton pad soaked in a 10% glucose solution. Tubes were kept in standard insectary conditions for one week. Females were then taken out of the tubes and the amount of haematin excreted at the bottom of each tube was quantified as an estimate of the blood meal size (Vézilier *et al*, 2010). Females were dissected, and the number of *Plasmodium* oocysts in their midgut counted with the aid of a binocular microscope (Vézilier *et al*, 2010).

2-3 Experiment 2: Impact of hosts' stress induces by handling on *Plasmodium* replication rate and transmission

In addition to the effect associated with the artificial increase of host corticosterone concentration on *Plasmodium* replication rate (see results section), the first experiment also suggested an additional

effect associated to the stress induced by host handling. In the second experiment we therefore explored this issue. For this purpose, eighteen individuals were infected and their parasitaemia was monitored during the acute and chronic stage of infection as described above. However, in order to minimize the hosts' stress induced by handling before the experiment, only one blood sample was taken during the chronic pre-experimental stage of infection (35 dpi). Individuals were then assigned to two different experimental groups: “handling” (n=9) and “control” (n=9) using the same procedure as described above.

While control individuals were not manipulated, birds belonging to the “handling” group were captured at days 52 or 53, 55 or 56 and 58 or 59 post-infection (experimental days were split into two blocks as described above) and handled and restrained in the same way as in Experiment 1 to generate stress. To avoid a confounding effect between handling stress and blood loss, no blood sample was taken during this period. Then, to explore the impact of handling stress on the dynamics of *Plasmodium* replication rate and transmission to mosquitoes, on day 61 or 62 dpi, all birds were captured, immobilized in PVC tubes and placed, at 6pm, in the dark in individual cages. One hour later, all individuals were exposed for one hour to 60 ± 5 uninfected female mosquitoes. At the end of the exposure session, a blood sample (ca. 2-3 µL) was taken from the medial metatarsal vein of all individuals to quantify parasitaemia by qPCR. Blood-fed female mosquitoes were counted, isolated, and dissected as described above.

Finally, for both corticosterone and handling experiments, blood samples were taken on all birds at day 65 and 75 post-infection to measure their parasitaemia. The chronic infection period was therefore divided into three different stages: the chronic pre-experimental stage (35-47 dpi), the chronic experimental stage (52-62 dpi) and the chronic post-experimental stage (65-75 dpi). At the end of the experiment all individuals were treated to clear infection with a dose of commercially

available Malarone, a mix of atovaquone/proguanil at a concentration of 1.08mg in 40 µl (Jenkins *et al*, 2015).

2-4 Experiment 3: Effect of mosquito bites on corticosterone concentration in birds' blood

Eighteen uninfected individuals were used for this experiment, from which nine were exposed to uninfected mosquito bites ("exposed") and the remaining nine were used as "control". To obtain baseline or near baseline corticosterone levels prior to mosquito exposure, blood samples were collected from the brachial vein of all birds within three minutes of capture (Wingfield & Romero, 2011). Immediately after the blood sampling, birds were immobilized in PVC tube covered by a mesh at both ends and placed in individual cages in the dark at 6:00 PM. After one hour of acclimatization, 60 ± 5 mosquitoes were released in each cage containing an "exposed" bird. One hour later (i.e. 8:00 PM), a new blood samples was taken from the brachial vein of all birds to quantify corticosterone concentrations. Then, the number of blood-fed mosquitoes in each cage was counted. Total corticosterone from plasma samples was quantified using enzyme immunoassay kit from Enzo Life Sciences (Corticosterone ELISA Kit, cat. No. ADI-900-097, Lausen, Switzerland) following the manufacturer's instructions. Samples were run in duplicate and randomized across plates. Concentration values were extrapolated from standard curves and all samples felt within the standard curves. Inter-plate variation was 1.3%.

2-5 Molecular analyses

The quantification of blood parasitaemia was carried out using qPCR with a protocol adapted from Cornet et al, 2013. Briefly, DNA was extracted from the blood using a standard protocol (Qiagen DNeasy 96 blood and tissue kit). For each individual, we conducted two qPCRs: one targeting the nuclear 18s rDNA gene of *Plasmodium* (Primers 18sPlasm7 5'-AGCCTGAGAAATAGCTACCACATCTA-3',

18sPlasm8 5'-TGTTATTTCTTGTCACTACCTCTCTTCTTT-3') and the other targeting the 18s rDNA gene of the bird (Primers 18sAv7 5'-GAAACTCGCAATGGCTCATTAAATC-3', 18sAv8 5'-TATTAGCTCTAGAATTACCACAGTTATCCA-3'). All samples were run in triplicate (Bio-Rad CFX96TM Real-Time System). Parasite number was calculated with relative quantification values (RQ). RQ can be interpreted as the fold-amount of target gene (*Plasmodium* 18s rDNA) with respect to the amount of the reference gene (Bird18s rDNA) and are calculated as $2^{-(Ct18s\ Plasmodium - Ct18s\ Bird)}$. For convenience, RQ values were standardized by $\times 10^4$ factor and log-transformed (log(x, base = exp(1)), (Cornet et al., 2013)).

2.6- Statistical analysis

Analyses were carried out using the R statistical package (v. 4.0.2). Data were analyzed separately for each experiment. The different statistical models built to analyse the data are described in the supplementary material (**Table S1**). Briefly, analyses where a same individual bird was sampled repeatedly, such as corticosterone quantification or fluctuation of blood parasitaemia throughout time, were analysed fitting bird as a random factor into the models (to account for the temporal pseudoreplication), using a mixed model procedure (*lmer*, package: lme4*).* When experimental days were split into two blocks, bird random factor was nested in experimental blocks. Bird treatment and day post-infection (experiment 1&2) or the time of blood sampling (experiment 3) were used as fixed factors. When only one parasitaemia measurement was taken per bird and experimental days were not split into two blocks, the analysis was performed using Mann-Whitney-Wilcoxon Test (i.e. comparison of bird chronic pre-experimental parasitaemia according to bird treatment in the handling experiment). Mosquito-centred traits, such as blood meal size (haematin quantity, ng), infection prevalence (proportion of mosquitoes containing at least one oocyst) and oocyst burden (taking account of mosquitoes with one or more oocysts in the midgut), which may depend on which bird mosquitoes fed on, were analysed fitting bird as a random factor into the models, using *lmer*, *glmer.nb*

or *glmer* (package: lme4) according to whether the errors were normally (haematin quantity), binomially (infection prevalence) or negative binomially distributed (oocyst burden). Bird treatment and, when necessary, blood meal size (haematin) were used as fixed factors.

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

3- RESULTS

3-1 Impact of artificial increase of corticosterone concentration in vertebrate blood on *Plasmodium* replication rate and transmission

Bird parasitaemia decreased drastically between the acute infection stage (10 dpi) and the first blood sample of the chronic infection stage (35 dpi, model 1: $\chi^2 = 61.09$ $p < 0.0001$). Then, a significant increase in host parasitaemia over time was observed during the chronic infection period (from 35 to 75 dpi, model 2: $\chi^2 = 42.13$ $p < 0.0001$, **Figure 1A**).

We further divided the chronic stage of infection into three different stages: the chronic pre-experimental stage (35-47 dpi), the chronic experimental stage (52-62 dpi) and the chronic post-experimental stage (65-75 dpi). During the chronic pre-experimental stage, parasitaemia did not vary between the different blood sampling days (model 3, 35 and 47 dpi, $\chi^2 = 1.29$, $p = 0.255$, **Figure 1A**) and was not different between control individuals and birds that would later ingest corticosterone

(model 3, $\chi^2 = 0.65$, $p = 0.422$). During the chronic experimental stage, an interaction between dpi and bird treatment was observed (model 4, $\chi^2 = 3.95$, $p = 0.046$, **Figure 1A**). While the parasitemia of the control individuals did not vary significantly over time (model 5, $\chi^2 = 1.98$, $p = 0.158$), the parasite burden of CORT birds increased (model 6, $\chi^2 = 16.40$, $p < 0.0001$). Then, during the post-experimental stage, parasitemia did not vary between the different blood sampling days (65 and 75 dpi, model 7, $\chi^2 = 0.96$, $p = 0.326$, **Figure 1A**) and was not different between control and CORT birds (model 7, $\chi^2 = 1.70$, $p = 0.191$).

On the last day of the chronic experimental stage (61/62 dpi), we evaluated the impact of the oral hormone treatment on *Plasmodium* transmission rate. No effect of bird treatment was observed on mosquito blood meal size (mean ± s.e., CORT: 23.15 ng ± 8.26, control: 23.94 ng ± 8.07, model 8, $\chi^2_1 = 0.029$ $p = 0.865$), mosquito infection prevalence (CORT: 0.66, 95% CI: 0.06, control: 0.53, 95%CI: 0.06, model 9, $\chi^2_1 = 1.140$ $p = 0.286$) and oocyst burden (geometric mean ± s.d., CORT: 2.74 ± 2.40, control: 4.07 ± 3.11, model 10, $\chi^2_1 = 0.033$ $p = 0.857$, **Figure 2A**).

3-2 Experiment 2: impact of hosts' stress induces by handling on *Plasmodium* replication rate and transmission

Bird parasitaemia decreased drastically between the acute infection stage (10 dpi) and the first blood sample of the chronic infection stage (35 dpi, model 11: $\chi^2 = 54.39$ $p < 0.0001$). Then, a significant interaction between bird treatment and dpi was observed during the chronic infection period (model 12: $\chi^2 = 4.92$ $p = 0.026$, **Figure 1B**). While the parasiteamia of control birds did not vary, the parasite burden of handling individuals increased over time (**Figure 1B**).

During the chronic pre-experimental stage, parasitemia was not different between control individuals and birds in the "handling" group (model 13, $W = 40$, $p = 0.99$, **Figure 1B**). After the four days of bird manipulation (i.e. chronic experimental stage), the parasitaemia of handled individuals,

measured day 61 or 62 post-infection, was significantly higher than the parasitaemia of control birds (mean RQ10 ± s.e., control = 12.65 ± 4.62, handling = 39.54 ± 14.95, model 14, $\chi^2 = 5.32$ p = 0.021, **Figure 1B**). This significant difference was maintained throughout the chronic post-experiment stage (control = 8.4 ± 2.55, handling = 32.32 ± 9.82, model 15, $\chi^2 = 4.91$ p = 0.027, **Figure 1B**), but a slight decrease of host parasitemia was observed according to blood sampling days (65 versus 75 dpi, model 15, $\chi^2 = 6.206$, p = 0.013, **Figure 1B**).

As in the first experiment, on the last day of the chronic experimental stage (61/62 dpi), no effect of bird treatment was observed on mosquito blood meal size (mean ± s.e., Control: 31.83 ng ± 9.04, Handling: 28.86 ng ± 9.98, model 16, $\chi^2_1 = 1.97$ p = 0.160), infection prevalence (Control: 0.53, CI: 0.06, Handling: 0.55, CI: 0.07, model 17, $\chi^2_1 = 0.08$ p = 0.772) and oocyst burden (geometric mean ± s.d., control: 3.87 ± 2.70, handling: 5.38 ± 3.24, model 18, $\chi^2_1 = 0.69$ p = 0.407, **Figure 2B)**.

3-3 Experiment 3: Effect of mosquito bites on host corticosterone concentration

The mean baseline corticosterone of birds was 2.50 ± 0.66 $ng.mL^{-1}$ (mean ± s.e.). Corticosterone level increased significantly after the handling and isolation period in both “control” and “exposed” groups (model 19, time of blood sampling: $\chi^2 = 19.21$, $p < 0.0001$, contrast analysis: unexposed $\chi^2 = 4.61$, p = 0.032, exposed $\chi^2 = 21.41$, $p < 0.0001$, **Figure 3**). The increase was however significantly higher in birds exposed to mosquito bites (significant interaction between bird treatment and time of blood sampling, model 19, $\chi^2 = 4.56$, p = 0.032). On average, the corticosterone level increased by more than 7.5 times in the “exposed” group (first blood sample: 1.79 $ng.mL^{-1}$ ± 0.31, second blood sample: 13.57 $ng.mL^{-1}$ ± 2.57) and by 2.5 times in the “control” group (first blood sample: 3.30 $ng.mL^{-1}$ ± 1.35, second blood sample: 8.28 $ng.mL^{-1}$ ± 1.90).

4. DISCUSSION

Understanding the mechanism(s) underlying the increase of *Plasmodium* replication rate in the host blood compartment during chronic infection stage is essential for targeting and controlling malaria relapses. Here we provide evidence that increased levels of stress-related hormones can trigger malaria relapses. Our study also supports the hypothesis advanced by Dhondt and Dobson (2017) that the increase in parasitemia observed in hosts exposed to mosquitoes (Billingsley et al., 2005; Cornet, Nicot, et al., 2014; Lawaly et al., 2012; Pigeault et al., 2018) could be mediated by mosquito bite-induced stress. However, while stress caused a long-lasting increase in parasite burden in the peripheral blood of chronically infected hosts, no effect on the transmission rate of the parasite was observed.

4.1 Impact of artificial increases in stress hormone levels on parasite number in the blood of vertebrate host

In the first experiment, we tested whether the artificial increase of corticosterone concentration in the plasma of hosts chronically infected by malaria impacted the replication rate of *Plasmodium*. For this purpose, corticosterone levels were manipulated orally using a minimally invasive technique, in which corticosterone solution was injected directly into birds' beak. Increases in corticosterone levels in response to oral ingestion have been reported in many species (e.g. (Breuner et al., 1998; Lõhmus et al., 2006; Spencer & Verhulst, 2008; Tóth et al., 2018)) and our data was consistent with these finding. Artificial manipulation of corticosterone levels has been shown to affect the intensity of parasite infection in various host/parasite system. For instance, Zebra finches infected by West Nils Virus and implanted with silastic tubules filled with corticosterone showed higher viremia than those observed in control birds (Gervasi et al., 2017; Martin et al., 2019). Tree frog tadpoles treated with exogenous corticosterone and infected by trematode (*Alaria* sp) developed higher parasite loads than control tadpoles (Belden & Kiesecker, 2005). Here, the oral hormone treatment led to a significant increase in parasite burden over time compared to infection dynamics

recorded in control individuals (i.e. significant interaction between days post-infection and CORT treatment). This result is consistent with that obtained in a previous study on house sparrows chronically infected by *P. relictum*, where intramuscular injection of corticosterone led to a considerably higher proportion of infected erythrocytes than in the control group (40-200 times higher parasitaemia, see Figure 3 in (Applegate, 1970)). The difference in effect size between this study and our result could be explained either by an interspecific difference associated with host resistance to malaria infection (Palinauskas et al., 2008), by the mode of corticosterone administration (oral versus intramuscular), or by the amount of corticosterone injected. The dose per body mass used by Appelgate (10 µg/g) was indeed 10 time higher than that used here (1 µg/g).

4.2 Impact of handling stress on parasite number in the blood of vertebrate host

During the chronic experiment and post-experiment stages, both CORT and control individuals showed a higher parasite burden compared to that measured during the chronic pre-experimental stage (i.e. 35 and 45 dpi). CORT but also control birds were manipulated during the chronic experimental stage and we demonstrated that handling and restraint induced a significant increase in bird plasma corticosterone levels (see experiment 3). Since previous studies have shown that experimental handling stress can negatively impact the immune system of several bird species (Cīrule et al., 2012; D'Amico et al., 2017; Matson et al., 2006), we hypothesized that handling stress may ultimately underlie the increase of parasite replication rate. Our second experiment confirmed our prediction by demonstrating that, unlike unstressed birds (i.e., unhandled and unrestrained), for which parasitemia remained constant throughout the chronic infection stage, parasitemia of handled individuals increased significantly. At the end of the chronic experimental stage, the parasitemia of the handled birds was on average more than six times higher than that of the control individuals (handling = 39.54 ± 14.95, control = 6.22 ± 6.53). In addition, the stress associated with handling the birds had a long-lasting impact. Thirteen days after the end of the stress period (i.e. chronic

experimental stage), the parasite burden of handling individuals was still more than six times higher than that of control ones. It would have been relevant to continue the monitoring to determine when the parasitemia of the stressed birds would have decreased.

4.3 Relationship between host stress and parasite transmission to mosquito vector

While host stress impacted the replication rate of *Plasmodium,* we did not observe any effect on the transmission rate of the parasite to the mosquitoes. The parasite burden of CORT and control birds was relatively close on the day of exposure to mosquitoes (i.e. 61/62 dpi, CORT: 41.39 ± 14.39, control: 29.02 ± 8.98), which can explain this result. However, in the second experiment, the parasitemia of the handled birds was on average more than six times higher than that of the control individuals. Considering the positive relationship reported between vertebrate host parasitaemia and the transmission rate of *Plasmodium relictum* to *Culex pipiens* mosquitoes (Pigeault et al., 2015), we would expect to observe a higher oocyst burden in mosquitoes fed on stressed hosts. Nonetheless, we cannot exclude that, despite a large difference in parasitemia, the gametocyte (sexual, transmissible stage of *Plasmodium*) burden was similar between the two groups of individuals. But this hypothesis is unlikely because *Plasmodium relictum* infection is characterized by a strong association between parasitaemia and gametocytaemia (Pigeault et al., 2015). On the other hand, the birds might have been exposed to the mosquitoes too soon after the beginning of the stress period, and thus of the increase in *Plasmodium*'s investment in the production of both asexual and sexual stages, to observe a significant effect on transmission (but see (Cornet, Nicot, et al., 2014)). Gametocytes go through several stages (from one to eight depending on *Plasmodium* species, (Gautret & Motard, 1999)) of development before reaching the 'mature' infectious stage, but the time required to reach the mature stage in *P. relictum* is not known.

4.4 Relationship between mosquito bites and stress hormone levels in vertebrate hosts

Contrary to what has been shown or suggested in several studies (Cornet, Nicot, et al., 2014; Lawaly et al., 2012; Pigeault et al., 2018), parasitaemia of chronically infected host did not increase after exposure to mosquito bites. Previous experimental studies that have reported an effect of mosquito bites on the parasitemia of infected individuals have exposed the hosts to several successive batches of 50 to 70 mosquitoes each (Cornet, Nicot, et al., 2014; Pigeault et al., 2018). In our study, individuals were exposed to a single batch of 60 mosquitoes of which only 39 ± 9 on average took a blood meal. Exposure to a minimum number of mosquitoes seems therefore required to trigger a relapse. While the proximal mechanism governing this phenomenon is not known, several hypotheses have been proposed. The stimulation of within-host growth and investment in transmission induced by mosquito bites could be due to a plastic response of the parasite triggered by the detection of cues from the mosquitoes (e.g. proteins present in saliva injected during the blood meal) or from the vertebrate host's response to mosquito bites (e.g. molecules product during the inflammatory response). Alternatively, these relapses could also be a side effect mediated by the anemia caused by blood feeding mosquitoes ((Shutler et al., 2005) but see (Cornet, Nicot, et al., 2014)) or by the stress response of the host induced by exposure to blood sucking insects (Dhondt & Dobson, 2017). Here, we demonstrated that mosquito exposure induces a significant increase in stress hormone levels and we highlighted that stress induction can generate a relapse. Our study does not establish a direct link between mosquito exposure, increased stress hormone levels, and ultimately increased parasite burden, but supports the hypothesis of Dhont and Dobson (2017). The most efficient way to demonstrate unequivocally the role played by stress hormone changes in relapse following exposure to mosquitoes would be to use drugs that block the production and/or activity of corticosterone (e.g. metyrapone, (Aharon-Rotman et al., 2021).

5. CONCLUSION

To conclude, we identified stress as a trigger for avian malaria relapses. We showed that both artificial and natural increase in host stress-related hormone levels boost *P. relictum* investment in its replication during the chronic stage of the vertebrate infection. Whether this phenomenon is mediated by stress immuno-suppressive effect or by other physiological modifications remains to be investigated. It also remains to be ascertained whether this feature extends to other malaria system and in particular to human malaria. Finally, our study highlights that the stress associated with the handling of infected birds, whether for experimental studies or for epidemiological monitoring, can have consequences on the within-host infection dynamics which may impact results interpretation and ultimately bird's life history traits.

REFERENCES

Aharon-Rotman, Y., Buttemer, W. A., Koren, L., & Wynne-Edwards, K. (2021). Experimental corticosterone manipulation increases mature feather corticosterone content: Implications for inferring avian stress history from feather analyses. *Canadian Journal of Zoology*, *99*(10), 948–952. https://doi.org/10.1139/cjz-2021-0091

Applegate, J. E. (1970). Population changes in latent avian malaria infections associated with season and corticosterone treatment. *The Journal of Parasitology*, *56*(3), 439–443.

Applegate, J. E. (1971). Spring Relapse of Plasmodium relictum Infections in an Experimental Field Population of English Sparrows (Passer domesticus). *Journal of Wildlife Diseases*, *7*(1), 37–42. https://doi.org/10.7589/0090-3558-7.1.37

Applegate, J. E., & Beaudoin, R. L. (1970). Mechanism of Spring Relapse in Avian Malaria: Effect of Gonadotropin and Corticosterone. *Journal of Wildlife Diseases*, *6*(4), 443–447. https://doi.org/10.7589/0090-3558-6.4.443

Arsenopoulos, K., Triantafillou, E., & Papadopoulos, E. (2017). Reduced stress and fatigue indicators (cortisol and creatinine kinase) in dairy cattle due to fly repellency using deltamethrin (Butox®, MSD). *Bulgarian Journal of Veterinary Medicine*, *20*(1), 123–129.

Ashley, E. A., & White, N. J. (2014). The duration of Plasmodium falciparum infections. *Malaria Journal*, *13*(1), 500. https://doi.org/10.1186/1475-2875-13-500

Battle, K. E., Karhunen, M. S., Bhatt, S., Gething, P. W., Howes, R. E., Golding, N., Van Boeckel, T. P., Messina, J. P., Shanks, G. D., Smith, D. L., Baird, J. K., & Hay, S. I. (2014). Geographical variation in Plasmodium vivax relapse. *Malaria Journal*, *13*(1), 144. https://doi.org/10.1186/1475-2875-13-144

Becker, D. J., Singh, D., Pan, Q., Montoure, J. D., Talbott, K. M., Wanamaker, S. M., & Ketterson, E. D. (2020). Artificial light at night amplifies seasonal relapse of haemosporidian parasites in a widespread songbird. *Proceedings of the Royal Society B: Biological Sciences*, *287*(1935), 20201831. https://doi.org/10.1098/rspb.2020.1831

Belden, L. K., & Kiesecker, J. M. (2005). Glucocorticosteroid hormone treatment of larval treefrogs increases infection by Alaria sp. Trematode cercariae. *The Journal of Parasitology*, *91*(3), 686–688. https://doi.org/10.1645/GE-397R

Betuela, I., Rosanas-Urgell, A., Kiniboro, B., Stanisic, D. I., Samol, L., de Lazzari, E., Del Portillo, H. A., Siba, P., Alonso, P. L., Bassat, Q., & Mueller, I. (2012). Relapses contribute significantly to the risk of Plasmodium vivax infection and disease in Papua New Guinean children 1-5 years of age. *The Journal of Infectious Diseases*, *206*(11), 1771–1780. https://doi.org/10.1093/infdis/jis580

Bichet, C., Scheifler, R., Cœurdassier, M., Julliard, R., Sorci, G., & Loiseau, C. (2013). Urbanization, Trace Metal Pollution, and Malaria Prevalence in the House Sparrow. *PLOS ONE*, *8*(1), e53866. https://doi.org/10.1371/journal.pone.0053866

Billingsley, P. F., Snook, L. S., & Johnston, V. J. (2005). Malaria parasite growth is stimulated by mosquito probing. *Biology Letters*, *1*(2), 185–189. https://doi.org/10.1098/rsbl.2004.0260

Bishop, A., Tate, P., & Thorpe, M. V. (1938). The duration of Plasmodium relictum infection in canaries1. *Parasitology*, *30*(3), 388–391. https://doi.org/10.1017/S0031182000025944

Bolker, B. M. (2008). *Ecological Models and Data in R*. Princeton University Press.

Bourgeon, S., & Raclot, T. (2006). Corticosterone selectively decreases humoral immunity in female eiders during incubation. *Journal of Experimental Biology*, *209*(24), 4957–4965. https://doi.org/10.1242/jeb.02610

Breuner, C. W., Greenberg, A. L., & Wingfield, J. C. (1998). Noninvasive Corticosterone Treatment Rapidly Increases Activity in Gambel's White-Crowned Sparrows (Zonotrichia leucophrys gambelii). *General and Comparative Endocrinology*, *111*(3), 386–394. https://doi.org/10.1006/gcen.1998.7128

Cain, D. W., & Cidlowski, J. A. (2017). Immune regulation by glucocorticoids. *Nature Reviews Immunology*, *17*(4), 233–247. https://doi.org/10.1038/nri.2017.1

Cīrule, D., Krama, T., Vrublevska, J., Rantala, M. J., & Krams, I. (2012). A rapid effect of handling on counts of white blood cells in a wintering passerine bird: A more practical measure of stress? *Journal of Ornithology*, *153*(1), 161–166. https://doi.org/10.1007/s10336-011-0719-9

Cogswell, F. B. (1992). The hypnozoite and relapse in primate malaria. *Clinical Microbiology Reviews*, *5*(1), 26–35. https://doi.org/10.1128/CMR.5.1.26

Commons, R. J., Simpson, J. A., Watson, J., White, N. J., & Price, R. N. (2020). Estimating the Proportion of Plasmodium vivax Recurrences Caused by Relapse: A Systematic Review and Meta-Analysis. *The American Journal of Tropical Medicine and Hygiene*, *103*(3), 1094–1099. https://doi.org/10.4269/ajtmh.20-0186

Cornelius, J. M., Zylberberg, M., Breuner, C. W., Gleiss, A. C., & Hahn, T. P. (2014). Assessing the role of reproduction and stress in the spring emergence of haematozoan parasites in birds. *The Journal of Experimental Biology*, *217*(Pt 6), 841–849. https://doi.org/10.1242/jeb.080697

Cornet, S., Bichet, C., Larcombe, S., Faivre, B., & Sorci, G. (2014). Impact of host nutritional status on infection dynamics and parasite virulence in a bird-malaria system. *The Journal of Animal Ecology*, *83*(1), 256–265. https://doi.org/10.1111/1365-2656.12113

Cornet, S., Nicot, A., Rivero, A., & Gandon, S. (2013). Malaria infection increases bird attractiveness to uninfected mosquitoes. *Ecology Letters*, *16*(3), 323–329. https://doi.org/10.1111/ele.12041

Cornet, S., Nicot, A., Rivero, A., & Gandon, S. (2014). Evolution of Plastic Transmission Strategies in Avian Malaria. *PLoS Pathog*, *10*(9), e1004308. https://doi.org/10.1371/journal.ppat.1004308

Cosgrove, C. L., Wood, M. J., Day, K. P., & Sheldon, B. C. (2008). Seasonal variation in Plasmodium prevalence in a population of blue tits Cyanistes caeruleus. *The Journal of Animal Ecology*, *77*(3), 540–548. https://doi.org/10.1111/j.1365-2656.2008.01370.x

Crawley, M. J. (2012). *The R Book*. John Wiley & Sons.

D'Amico, V. L., Palacios, M. G., Baker, A. J., González, P. M., Madrid, E., & Bertellotti, M. (2017). PHYSIOLOGIC PARAMETERS AND THEIR RESPONSE TO HANDLING STRESS IN A NEOTROPICAL MIGRATORY SHOREBIRD DURING THE NONBREEDING SEASON. *Journal of Wildlife Diseases*, *53*(3), 437–446. https://doi.org/10.7589/2016-02-039

Dhondt, A. A., & Dobson, A. P. (2017). Stress Hormones Bring Birds, Pathogens and Mosquitoes Together. *Trends in Parasitology*, *33*(5), 339–341. https://doi.org/10.1016/j.pt.2017.01.001

Eggermann, J., Theuerkauf, J., Pirga, B., Milanowski, A., & Gula, R. (2013). Stress-Hormone Levels of Wolves in Relation to Breeding Season, Pack Size, Human Activity, and Prey Density. *Annales Zoologici Fennici*, *50*(3), 170–175. https://doi.org/10.5735/086.050.0304

Eisen, R. J. (2000). Variation in life-history traits of Plasmodium mexicanum, a malaria parasite infecting western fence lizards: A longitudinal study. *Canadian Journal of Zoology*, *78*(7), 1230–1237. https://doi.org/10.1139/z00-057

Gao, S., Sanchez, C., & Deviche, P. J. (2017). Corticosterone rapidly suppresses innate immune activity in the house sparrow (Passer domesticus). *Journal of Experimental Biology*, *220*(2), 322–327. https://doi.org/10.1242/jeb.144378

Gautret, P., & Motard, A. (1999). Periodic infectivity of Plasmodium gametocytes to the vector. A review. *Parasite (Paris, France)*, *6*(2), 103–111.

Gervasi, S. S., Burgan, S. C., Hofmeister, E., Unnasch, T. R., & Martin, L. B. (2017). Stress hormones predict a host superspreader phenotype in the West Nile virus system. *Proceedings. Biological Sciences*, *284*(1859), 20171090. https://doi.org/10.1098/rspb.2017.1090

Glaizot, O., Fumagalli, L., Iritano, K., Lalubin, F., Rooyen, J. V., & Christe, P. (2012). High Prevalence and Lineage Diversity of Avian Malaria in Wild Populations of Great Tits (Parus major) and Mosquitoes (Culex pipiens). *PLOS ONE*, *7*(4), e34964. https://doi.org/10.1371/journal.pone.0034964

Gross, S. J. (1947). Male hormones for the prevention of relapses in malaria. *The Indian Medical Gazette*, *82*(2), 65.

Hellgren, O., Waldenström, J., & Bensch, S. (2004). A new pcr assay for simultaneous studies of leucocytozoon, plasmodium, and haemoproteus from avian blood. *Journal of Parasitology*, *90*(4), 797–802. https://doi.org/10.1645/GE-184R1

Huldén, L., & Huldén, L. (2011). Activation of the hypnozoite: A part of Plasmodium vivax life cycle and survival. *Malaria Journal*, *10*, 90. https://doi.org/10.1186/1475-2875-10-90

Huldén, L., Huldén, L., & Heliövaara, K. (2008). Natural relapses in vivax malaria induced by Anopheles mosquitoes. *Malaria Journal*, *7*, 64. https://doi.org/10.1186/1475-2875-7-64

Ishtiaq, F. (2021). Ecology and Evolution of Avian Malaria: Implications of Land Use Changes and Climate Change on Disease Dynamics. *Journal of the Indian Institute of Science*, *101*(2), 213–225. https://doi.org/10.1007/s41745-021-00235-3

Jenkins, T., Delhaye, J., & Christe, P. (2015). Testing Local Adaptation in a Natural Great Tit-Malaria System: An Experimental Approach. *PLOS ONE*, *10*(11), e0141391. https://doi.org/10.1371/journal.pone.0141391

Kavaliers, M., & Colwell, D. D. (1996). Synergism Between Stress Responses Induced by Biting Flies and Predator Odours. *Ethology*, *102*(1), 89–98. https://doi.org/10.1111/j.1439-0310.1996.tb01106.x

Lalubin, F., Delédevant, A., Glaizot, O., & Christe, P. (2013). Temporal changes in mosquito abundance (Culex pipiens), avian malaria prevalence and lineage composition. *Parasites & Vectors*, *6*, 307. https://doi.org/10.1186/1756-3305-6-307

Lawaly, R., Konate, L., Marrama, L., Dia, I., Diallo, D., Sarr, F. D., Schneider, B. S., Casademont, I., Diallo, M., Brey, P. T., Sakuntabhai, A., Mecheri, S., & Paul, R. (2012). Impact of Mosquito Bites on Asexual Parasite Density and Gametocyte Prevalence in Asymptomatic Chronic Plasmodium falciparum Infections and Correlation with IgE and IgG Titers. *Infection and Immunity*, *80*(6), 2240–2246. https://doi.org/10.1128/IAI.06414-11

Lõhmus, M., Sundström, L. F., & Moore, F. R. (2006). Non-invasive corticosterone treatment changes foraging intensity in red-eyed vireos Vireo olivaceus. *Journal of Avian Biology*, *37*(5), 523–526. https://doi.org/10.1111/j.0908-8857.2006.03733.x

Lormée, H., Jouventin, P., Trouve, C., & Chastel, O. (2003). Sex-specific patterns in baseline corticosterone and body condition changes in breeding Red-footed Boobies Sula sula. *Ibis*, *145*(2), 212–219. https://doi.org/10.1046/j.1474-919X.2003.00106.x

Markus, M. B. (2018). Biological concepts in recurrent Plasmodium vivax malaria. *Parasitology*, *145*(13), 1765–1771. https://doi.org/10.1017/S003118201800032X

Martin, L. B., Kernbach, M. E., & Unnasch, T. R. (2019). Distinct effects of acute versus chronic corticosterone exposure on Zebra finch responses to West Nile virus. *Conservation Physiology*, *7*(1), coz094. https://doi.org/10.1093/conphys/coz094

Martínez-de la Puente, J., Merino, S., Tomás, G., Moreno, J., Morales, J., Lobato, E., & Martínez, J. (2011). Nest ectoparasites increase physiological stress in breeding birds: An experiment. *Naturwissenschaften*, *98*(2), 99–106. https://doi.org/10.1007/s00114-010-0746-z

Matson, K. D., Tieleman, B. I., & Klasing, K. C. (2006). Capture Stress and the Bactericidal Competence of Blood and Plasma in Five Species of Tropical Birds. *Physiological and Biochemical Zoology*, *79*(3), 556–564. https://doi.org/10.1086/501057

Mayor, A., Serra-Casas, E., Bardají, A., Sanz, S., Puyol, L., Cisteró, P., Sigauque, B., Mandomando, I., Aponte, J. J., Alonso, P. L., & Menéndez, C. (2009). Sub-microscopic infections and long-term recrudescence of Plasmodium falciparum in Mozambican pregnant women. *Malaria Journal*, *8*(1), 9. https://doi.org/10.1186/1475-2875-8-9

Names, G. R., Schultz, E. M., Krause, J. S., Hahn, T. P., Wingfield, J. C., Heal, M., Cornelius, J. M., Klasing, K. C., & Hunt, K. E. (2021). Stress in paradise: Effects of elevated corticosterone on immunity and avian malaria resilience in a Hawaiian passerine. *Journal of Experimental Biology*, *224*(20), jeb242951. https://doi.org/10.1242/jeb.242951

Palinauskas, V., Valkiūnas, G., Bolshakov, C. V., & Bensch, S. (2008). Plasmodium relictum (lineage P-SGS1): Effects on experimentally infected passerine birds. *Experimental Parasitology*, *120*(4), 372–380. https://doi.org/10.1016/j.exppara.2008.09.001

Pearson, R. D. (2002). Is prolactin responsible for avian, saurian, and mammalian relapse and periodicity of fever in malarial infections? *Canadian Journal of Zoology*, *80*(7), 1313–1315. https://doi.org/10.1139/z02-121

Pigeault, R., Caudron, Q., Nicot, A., Rivero, A., & Gandon, S. (2018). Timing malaria transmission with mosquito fluctuations. *Evolution Letters*. https://doi.org/10.1002/evl3.61

Pigeault, R., Vézilier, J., Cornet, S., Zélé, F., Nicot, A., Perret, P., Gandon, S., & Rivero, A. (2015). Avian malaria: A new lease of life for an old experimental model to study the evolutionary ecology of

Plasmodium. *Phil. Trans. R. Soc. B*, *370*(1675), 20140300. https://doi.org/10.1098/rstb.2014.0300

Popovici, J., Pierce-Friedrich, L., Kim, S., Bin, S., Run, V., Lek, D., Hee, K. H. D., Lee Soon-U, L., Cannon, M. V., Serre, D., & Menard, D. (2019). Recrudescence, Reinfection, or Relapse? A More Rigorous Framework to Assess Chloroquine Efficacy for Plasmodium vivax Malaria. *The Journal of Infectious Diseases*, *219*(2), 315–322. https://doi.org/10.1093/infdis/jiy484

Reedy, A. M., Edwards, A., Pendlebury, C., Murdaugh, L., Avery, R., Seidenberg, J., Aspbury, A. S., & Gabor, C. R. (2014). An acute increase in the stress hormone corticosterone is associated with mating behavior in both male and female red-spotted newts, Notophthalmus viridescens. *General and Comparative Endocrinology*, *208*, 57–63. https://doi.org/10.1016/j.ygcen.2014.08.008

Rivero, A., & Gandon, S. (2018). Evolutionary Ecology of Avian Malaria: Past to Present. *Trends in Parasitology*. https://doi.org/10.1016/j.pt.2018.06.002

Schall, J. J. (1996). Malarial parasites of lizards: Diversity and ecology. *Advances in Parasitology*, *37*, 255–333. https://doi.org/10.1016/s0065-308x(08)60222-5

Schoenle, L. A., Moore, I. T., Dudek, A. M., Garcia, E. B., Mays, M., Haussmann, M. F., Cimini, D., & Bonier, F. (2019). Exogenous glucocorticoids amplify the costs of infection by reducing resistance and tolerance, but effects are mitigated by co-infection. *Proceedings of the Royal Society B: Biological Sciences*, *286*(1900), 20182913. https://doi.org/10.1098/rspb.2018.2913

Shutler, D., Reece, S. E., Mullie, A., Billingsley, P. F., & Read, A. F. (2005). Rodent malaria parasites Plasmodium chabaudi and P. vinckei do not increase their rates of gametocytogenesis in response to mosquito probing. *Proceedings of the Royal Society of London B: Biological Sciences*, *272*(1579), 2397–2402. https://doi.org/10.1098/rspb.2005.3232

Slowinski, S. P., Geissler, A. J., Gerlach, N., Heidinger, B. J., & Ketterson, E. D. (2022). The probability of being infected with haemosporidian parasites increases with host age but is not affected by experimental testosterone elevation in a wild songbird. *Journal of Avian Biology*, *2022*(1). https://doi.org/10.1111/jav.02819

Spencer, K. A., & Verhulst, S. (2008). Post-natal exposure to corticosterone affects standard metabolic rate in the zebra finch (Taeniopygia guttata). *General and Comparative Endocrinology*, *159*(2), 250–256. https://doi.org/10.1016/j.ygcen.2008.09.007

Taylor, A. R., Watson, J. A., Chu, C. S., Puaprasert, K., Duanguppama, J., Day, N. P. J., Nosten, F., Neafsey, D. E., Buckee, C. O., Imwong, M., & White, N. J. (2019). Resolving the cause of recurrent Plasmodium vivax malaria probabilistically. *Nature Communications*, *10*(1), 5595. https://doi.org/10.1038/s41467-019-13412-x

Telford, S. R. (1989). Discovery of the pre-erythrocytic stages of a saurian malaria parasite, hypnozoites, and a possible mechanism for the maintenance of chronic infections throughout the life of the host. *International Journal for Parasitology*, *19*(6), 597–616. https://doi.org/10.1016/0020-7519(89)90038-6

Tóth, Z., Ouyang, J. Q., & Lendvai, Á. Z. (2018). Exploring the mechanistic link between corticosterone and insulin-like growth factor-1 in a wild passerine bird. *PeerJ*, *6*, e5936. https://doi.org/10.7717/peerj.5936

Valkiunas, G. (2004). *Avian Malaria Parasites and other Haemosporidia*. CRC Press.

Vézilier, J., Nicot, A., Gandon, S., & Rivero, A. (2010). Insecticide resistance and malaria transmission: Infection rate and oocyst burden in Culex pipiens mosquitoes infected with Plasmodium relictum. *Malaria Journal*, *9*, 379. https://doi.org/10.1186/1475-2875-9-379

Warren, M., Powers, K. G., Garnham, P. C. C., & Shiroishi, T. (1974). Plasmodium cynomolgi: Influence of X-irradiation and sporozoite dilution on relapse patterns in infected rhesus monkeys. *Experimental Parasitology*, *35*(2), 266–271. https://doi.org/10.1016/0014-4894(74)90032-0

White, M. T., Shirreff, G., Karl, S., Ghani, A. C., & Mueller, I. (2016). Variation in relapse frequency and the transmission potential of Plasmodium vivax malaria. *Proceedings. Biological Sciences*, *283*(1827), 20160048. https://doi.org/10.1098/rspb.2016.0048

White, N. J. (2011). Determinants of relapse periodicity in Plasmodium vivax malaria. *Malaria Journal*, *10*, 297. https://doi.org/10.1186/1475-2875-10-297

Wingfield, J. C., & Romero, L. M. (2011). Adrenocortical Responses to Stress and Their Modulation in Free-Living Vertebrates. In *Comprehensive Physiology* (pp. 211–234). American Cancer Society. https://doi.org/10.1002/cphy.cp070411

Zanghi, G., & Vaughan, A. M. (2021). Plasmodium vivax pre-erythrocytic stages and the latent hypnozoite. *Parasitology International*, 102447. https://doi.org/10.1016/j.parint.2021.102447

FIGURE CAPTIONS

Figure 1: Dynamics of blood parasitaemia (Log(RQ+1)) of *Plasmodium relictum* in vertebrate hosts. **(A)** Dynamics of parasitaemia in control individuals (blue) or in individuals that ingested corticosterone (green) during the chronic experiment stage (light blue areas, refer to Materials & Methods for details). **(B)** Dynamics of parasitaemia in unstressed individuals (i.e. control, blue) or in individuals that were stressed by handling (green) during the chronic experiment stage (light blue area, refer to Materials & Methods for details). The impact of bird treatment on the transmission rate of *Plasmodium* from vertebrate hosts to mosquitoes was explored on day 61/62 post-infection (dark blue area, refer to Materials & Methods for details). The dashed red line indicates the average parasitemia measured during the chronic pre-experimentation stage. The colored dots correspond to the raw data and the black points represent the means.

Figure 2: Number of oocyst per mosquito midgut fed either on control or artificially/naturally stressed vertebrate host. (A) Oocyst burden in mosquitoes blood fed either on control birds (blue) or on birds that ingested corticosterone (green). **(B)** Oocyst burden in mosquitoes blood fed either on

unstressed birds (i.e. control, blue) or on birds that have been stressed by handling (green). Violin plots were constructed to show the spread and density of the raw data. White points represent the means.

Figure 3: Baseline and stress induced corticosterone (ng/ml) in birds exposed or not to mosquito bites. Baseline corticosterone was measured from blood collected within 3 min of disturbance. Immediately after the blood sampling, all birds were immobilized and placed in individual cages. After one hour of acclimatization, mosquitoes were released in each cage containing an “exposed” bird (green boxplot). One hour later, a new blood samples were taken from the brachial vein of all birds to quantify corticosterone concentrations (refer to Materials & Methods for details). The grey dots correspond to the raw data.