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

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Abstract

A pervasive characteristic of malaria parasite infection in mosquito vector populations is their tendency to be overdispersed. Understanding the mechanisms underlying the overdispersed distribution of parasites is of key importance as it may drastically impact the transmission dynamics of the pathogen. The small fraction of heavily infected individuals might serve as superspreaders and cause a disproportionate number of subsequent infections. Although multiple factors ranging from environmental stochasticity to inter-individual heterogeneity may explain parasite overdispersion, *Plasmodium* infection has also been observed to be highly overdispersed in inbred mosquito population maintained under standardized laboratory conditions, suggesting that other mechanisms may be at play. Here, we show that the aggregated distribution of *Plasmodium* within mosquito vectors is partially explained by a temporal heterogeneity in parasite infectivity triggered by the bites of blood-feeding mosquitoes. Several experimental blocks carried out with three different *Plasmodium* isolates have consistently shown that the transmission of the parasite increases progressively with the order of mosquito bites. Surprisingly the increase in transmission is not associated with an increase in *Plasmodium* replication rate or higher investment in the production of the transmissible stage (gametocyte). Adjustment of the physiological state of the gametocytes could be, however, an adaptive strategy to respond promptly to mosquito bites. Overall our data show that malaria parasite appears to be able to respond to the bites of mosquitoes to increase its own transmission at a much faster pace than initially thought (hours rather than days). Further work needs to be carried out to elucidate whether these two strategies are complementary and, particularly, what are their respective underlaying mechanisms. Understanding the processes underlying the temporal fluctuations in *Plasmodium* infectivity

throughout vertebrate host-to-mosquito transmission is essential and could lead to the development of new approaches to control malaria transmission.

Author summary

Plasmodium parasites are known for being the etiological agents of malaria and for the devastating effects they cause on human populations. A pervasive characteristic of *Plasmodium* infection is their tendency to be overdispersed in mosquito vector populations: the majority of mosquitoes tend to harbour few or no parasites while a few individuals harbor the vast majority of the parasite population. Understanding the mechanisms underlying *Plasmodium* overdispersed distribution is of key importance as it may drastically impact the transmission dynamics of the pathogen. Here, we show that the aggregated distribution of *Plasmodium* parasites within mosquito vectors is partially explained by a temporal heterogeneity in *Plasmodium* infectivity triggered by the bites of blood-feeding mosquitoes. In other words, mosquitoes that bite at the beginning of a 3h feeding session have significantly fewer parasites than those that bite towards the end. Malaria parasite is therefore capable of responding to the bites of mosquitoes to increase its own transmission at a much faster pace than thought (hours rather than days). Understanding the processes underlying the temporal fluctuations in *Plasmodium* infectivity throughout vertebrate host-to-mosquito transmission is essential and could lead to the development of new approaches to control malaria transmission.

Keywords: *Plasmodium*, mosquito bites, transmission, overdispersion, aggregation, temporal heterogeneity, avian malaria

Introduction

An ubiquitous feature of parasite infections is their tendency to be overdispersed or aggregated [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 ranging 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 strong repercussions for disease dynamics [9,10]. Overdispersion reduces the deleterious effects of parasites on host populations but also 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 infectious disease epidemiology and parasite transmission. The small fraction of heavily infected individuals may serve as super-spreaders and therefore play 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 the vertebrate host and in the vector populations [18–22]. Despite this, studies to date have mainly focused on the epidemiological consequences of parasite overdispersion in vertebrate host rather than vectors [17,23,24]. Yet, for many of these diseases, key traits determining the transmission dynamics of the pathogen such as the lifespan and feeding behaviour of vectors as well as the length of the parasite's extrinsic incubation period may depend on the intensity of parasite infection in the vector [25–29].

Anderson and Gordon (1982) identified environmental stochasticity as the prime cause of overdispersion in parasite populations [30]. This included not only the physical parameters of the environment, but also the differences in host susceptibility to the infection induced by

behavioural differences, genetic factors or varying past experiences of infection. The mechanisms underlying the aggregated distribution of parasites in vector populations remains however rarely explored and little understood.

Plasmodium parasites are known for being the etiological agents of malaria and for the devastating effects they cause on human populations in the African continent. These vector-borne parasites are however also found infecting many other terrestrial vertebrate species, including other mammals, reptiles and birds. The life cycle of the parasite is the same in all hosts, irrespective of their 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 the sexual reproduction of the parasite, the motile zygotes penetrate the wall of the midgut and start developing into oocysts, which in turn produce the transmissible sporozoites in the mosquito's salivary glands. There is abundant evidence showing that the distribution of oocysts, the most commonly quantified parasite stage in mosquitoes, is highly overdispersed [7,31–34]. The most straightforward explanation for this aggregated distribution of oocysts is that it is driven by heterogeneity in vector susceptibility to *Plasmodium* infection associated to their genetic background or to their physiological status [7,35,36]. Polymorphism in mosquito immune genes is strongly associated with natural resistance to *Plasmodium* [35,37] and aging also tends to decrease the susceptibility of vectors to *Plasmodium* infection [36]. Puzzlingly, however, oocyst overdispersion is also extremely common under controlled laboratory conditions in highly inbred, and therefore physiologically and genetically homogeneous, mosquito populations [7,31,33,34]. This suggests that genetic or physiological heterogeneities between mosquitoes may only be part of the explanation, and that the causes of the aggregated distribution of oocysts in vectors may also lie elsewhere.

One possible explanation is the existence of *spatial* aggregation of gametocytes in the vertebrate blood. Recent work has shown that gametocyte densities in humans can vary in as much as 45% in blood collected from different parts of the body (Pigeault et al *.in prep).* Although the direct connection between spatial heterogeneity in blood and overdispersion in mosquitoes has never been made, it has been reported that *Plasmodium* gametocytes show an aggregated distribution within mosquitoes which recently fed on human host [38].

Alternatively, the aggregated distribution of *Plasmodium* parasites within mosquitoes could be due to a within-host *temporal* variation in parasite densities and/or infectivity. Under this scenario, mosquitoes feeding during the high parasite density/infectivity phase would be 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 within relatively short temporal intervals. A recent study found that rodent malaria *P. chabaudi* gametocytes are twice as infective at night despite being less numerous in the blood [39]. A periodic late afternoon increase in parasitemia is also observed in the avian malaria system [40]. Such temporal variations may respond to changes in the physiological, nutritional or immunological condition of the host [41–43]. They may, however, also be an adaptive strategy of the parasite aimed at maximizing its own transmission [40,44]. 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 [40,44]. These results, however, are not able to explain the aggregated distribution of parasites among mosquitoes feeding within a short feeding bout typically lasting a handful of hours.

Here, we test whether the *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-hour) time interval: do the first mosquitoes to bite the host increase the infectivity of the parasite so that mosquitoes biting last end up with significantly increased oocystaemias? To test this hypothesis, we use the avian malaria experimental 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 (the mosquito *Culex pipiens*). Specifically, we carry out a series of experiments designed to answer the following two main questions: 1) 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 2) Is this due to a temporal increase in the parasitaemia/gametocytaemia of birds as a result of mosquito bites?

Results

To investigate the impact of mosquito bite-driven plasticity on *Plasmodium* transmission we used the avian malaria biological system [45]. Birds (*Serinus canaria*) infected by *Plasmodium relictum*, the causative agent of the most prevalent form of avian malaria in Europe, were exposed to a wild-caught lineage of *Culex pipiens* mosquitoes for 3 hours (6 – 9 p.m.). Mosquitoes were sampled at regular intervals thereafter (different protocols for the three experiments, see below) and dissected one week later to count the number of oocysts in the midgut. To investigate the impact of vector bites on parasite population growth, the parasitaemia (number of parasites in the blood) and gametocytaemia (number of gametocytes in the blood) of vertebrate hosts exposed or not (control) to mosquitoes were measured just before and just after the mosquito exposure period (6 – 9 p.m.).

Experiment 1: Oocyst burden and mosquito biting order: batch experiment

In the first experiment, birds were exposed to four successive batches of 25 ± 3 uninfected mosquitoes. Each mosquito batch was kept in the cage for 45 minutes before being replaced with a new batch (batch 1 ($T0_{min}$), batch 2 ($T45_{min}$), batch 3 ($T90_{min}$) and batch 4 ($T135_{min}$)). At the end of each exposure period, all mosquitoes were removed from the cages and were immediately replaced by a new batch. 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 (model 1: $\chi^2 = 5.90$, $p = 0.116$, model 2: $\chi^2 = 3.55$, $p = 0.314$ respectively, statistical models are described in **Table S1**). Although mosquitoes from batches 3 and 4 tend to have a higher infection prevalence (proportion of mosquitoes containing at least 1 oocyst in the midgut; mean ± SE: batch 3: 64.4% ± 11.9, batch 4: 78.2% ± 8.6) than those from batches 1 and 2 (batch 1: 56.7% ± 15, 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 1A**, mean ± se Variance-to-Mean Ratio = 11.48 ± 3.37). 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 1A**). Females from batch 4 had almost twice as many oocysts as those from batch 3 (contrast analyses: batch4/batch3: $\chi^2 = 11.02$, $p < 0.001$) and three times more than females from batches 1 and 2 (batch4/batch2: $\chi^2 = 17.95$, $p < 0.001$, batch4/batch1: $\chi^2 = 19.31$, $p < 0.0001$, **Figure 1A**). No significant difference was however observed between mosquitoes from batches 1 and 2 (contrast analyses: batch1/batch2: $\chi^2 = 0.15$, $p = 0.697$) or between mosquitoes from batches 2 and 3 (batch2/batch3: $\chi^2 = 2.29$, $p = 0.129$, **Figure 1A**). Haematin quantity had no effect on the oocyst burden (model 4: $\chi^2 = 0.02$, $p = 0.875$).

171 The increase in *Plasmodium* oocyst burden with mosquito batch was not explained by
172 an increase in total parasite or gametocyte burden in the birds' peripheral blood. The
173 parasitaemia and gametocytaemia of exposed birds remained roughly constant between the
174 beginning and the end of the experiment (parasitaemia= model 5: χ^2 = 0.39, p = 0.529,
175 gametocytaemia = model 6: χ^2 = 0.02, p = 0.877 respectively) and were similar between exposed
176 and control (unexposed) birds (parasitaemia = model 5: χ^2 = 0.29, p = 0.5907, gametocytaemia
177 = model 6: χ^2 = 0.60, p = 0.4364).

178 To test the repeatability of our results, a second experimental block, with a new
179 *Plasmodium relictum* strain freshly collected in the field from an infected House sparrow (*Passer*
180 *domesticus*), was performed. The results of block 2 fully confirmed those of the first block. The
181 blood meal rate and the quantity of haematin excreted by mosquitoes was similar for all
182 batches (model 7: χ^2= 1.77 p = 0.621, model 8: χ^2= 1.13, p = 0.770**)**. The difference in infection
183 prevalence between the different batches was not statistically significant (model 9: χ^2 = 5.70, p
184 = 0.127) although mosquitoes from batches 2, 3 and 4 tend to have a higher prevalence (mean
185 ± SE, batch 2: 73.1 % ± 7.0, batch 3: 69.4% ± 7.8 and batch 4: 70.3% ± 7.6 %) than those from
186 batch 1 (mean ± SE, batch 1: 51.1% ± 7.5). The distribution of oocyst burden in mosquitoes was
187 overdispersed (**Figure 1B**, mean ± se VMR = 11.40 ± 5.66) and we observed a significant increase
188 in oocyst burden with mosquito batches order (model 10: χ^2 = 30.73, p < 0.0001, geometric
189 mean: batch 1: 3.47 ± 2.69, batch 2: 3.52 ± 2.95, batch 3: 6.06± 2.85 and batch 4: 10.38 ± 2.76,
190 all contrast analyses were significant, **Figure 1B**). A significant positive correlation between
191 haematin and oocyst burden was found (model 10: χ^2 = 4.46, p = 0.03). As in the previous
192 experimental block, the vertebrate host parasitaemia and gametocytaemia remained constant
193 between the beginning and the end of the experiment (parasitaemia = model 11: χ^2 = 1.29, p =
194 0.256, gametocytaemia = model 12: χ^2 = 0.88, p = 0.349 respectively) and were similar between

exposed and control (unexposed) birds (parasitaemia= model 11: $\chi^2 = 2.44$, $p = 0.118$, gametocytaemia = model 12: $\chi^2 = 2.45$, $p = 0.117$ respectively).

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 batches. Each mosquito batch was left for 45 minutes in contact with birds (batch 1 ($T0_{min}$), batch 2 ($T45_{min}$), batch 3 ($T90_{min}$) and batch 4 ($T135_{min}$)). Birds were either infected by a *Plasmodium relictum* lab strain (experimental block 1, panel **A**) or by a *Plasmodium relictum* strain freshly collected in the field (experimental block 2, panel **B**). Black horizontal lines represent medians and black diamond represent geometric means. Levels not connected by 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 colors represent the mosquito batches (from 1 to 4).

Experiment 2: Oocyst burden and mosquito biting order: individual monitoring

A second experiment, with another *Plasmodium relictum* strain freshly collected in the field from an infected Great tit (*Parus major*), was carried out to obtain a finer measurement of the impact of mosquito biting order on their oocyst burden. Here, infected individuals were exposed to 100 mosquitoes for 3h (6.00 pm – 9.00 pm). Cages were continuously observed and mosquitoes were individually removed from the cages immediately after their blood feeding bout. The order of biting of each individual female was recorded.

Haematin quantity and infection prevalence were independent of the mosquito biting order (model 13: $\chi^2 = 2.44$, $p = 0.118$, model 14: $\chi^2 = 0.83$, $p = 0.363$, respectively). The distribution of oocyst burdens across all mosquitoes was highly overdispersed (mean ± SE. VMR = 90.26 ± 41.53, **Figure 2A**). Biting order was a significant explanatory factor of oocyst burden:

mosquitoes that bit later showed higher oocyst burden than mosquitoes that bit first (model 15: $\chi^2 = 8.28$ p = 0.004, **Figure 2A**). A significant positive correlation between haematin quantity and oocyst burden was found (model 15: $\chi^2 = 19.151$, p <0.001). As for the first experiment, vertebrate host parasitaemia and gametocytaemia remained constant between the beginning and the end of the experiment (parasitaemia = model 16: $\chi^2 = 2.03$, p = 0.154, gametocytaemia = model 17: $\chi^2 = 0.13$, p = 0.718 respectively) and were similar between exposed and unexposed (control) birds (parasitaemia = model 16: $\chi^2 = 0.98$, p = 0.321, gametocytaemia = model 17: $\chi^2 = 0.12$, p = 0.731 respectively).

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 (Log(RQ+1), in red), or the oocyst burden (in black), 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. Histogram in each panel show the distribution of oocyst burden in mosquitoes in the experiment 2 (**A**) and 3 (**B**).

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 without, however, showing an increase of the parasite density in the peripheral blood of vertebrate hosts (measured from blood samples). We carried out a third experiment to determine whether the total number of parasites in the blood meal, immediately after the blood feeding, fluctuated during the feeding bout. As for the experiment 2, birds were exposed to 100 mosquitoes for 3h (6.00 pm – 9.00 pm) and mosquitoes were individually removed from the cages immediately after blood feeding. Every second mosquito collected was either

immersed immediately in liquid nitrogen or stored in plastic tubes and dissected one week later to count the number of oocysts in the midgut. Frozen blood-fed mosquitoes were used to quantify the number of parasites ingested by qPCR.

The amount of parasite ingested by the mosquitoes remained roughly constant throughout the exposure period (model 18: $\chi^2 = 1.54$, $p = 0.215$ **Figure 2B**). The hematin quantity and the infection prevalence (oocyst stage) were also independent of the mosquito biting order (model 19: $\chi^2 = 1.89$, $p = 0.169$, and model 20: $\chi^2 = 0.37$, $p = 0.545$ respectively). In contrast, the distribution of oocyst burden across all mosquitoes was still overdispersed (mean ± SE. VMR = 15.03 ± 1.86, **Figure 2B)** and was significantly explained by the mosquito biting order (model 21: $\chi^2 = 6.45$, $p = 0.011$, **Figure 2B**). As in the experiment 2, mosquitoes that bit later showed higher oocyst burden than mosquitoes that bit first (**Figure 2B**).

Discussion

Overdispersed distribution of vector-borne parasite within vertebrate and invertebrate host populations has profound consequences on parasite transmission and disease control strategies [16,28,46]. Parasite overdispersion is driven by multiple factors ranging from population processes to inter-individual heterogeneity in susceptibility and parasite exposure [11,47–49]. Here, using three different isolates of *Plasmodium relictum*, we provide evidence that the aggregated distribution of malaria parasites within mosquito vectors may also be explained by the mosquito biting order: mosquitoes that bite first have a lower intensity of infection than those that bite later on. This fluctuation in *Plasmodium* infectivity may reflect an adaptive strategy of parasites selected to optimize transmission.

The abundance of invertebrate vectors fluctuates at time scales ranging from daily to annual [40,50–52]. 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 increases daily when its vector is most active [39,40] but also a plastic strategy allowing parasite growth to increase after exposure to mosquito bites [40,44,53]. This plastic strategy allows the parasite to react to daily and seasonal fluctuations in mosquito abundance [40,44].

In this study we demonstrate that *Plasmodium* plastic response is much faster than initially thought [40,44]. When vertebrate hosts were exposed to mosquito bites during a short period of time (3 hours), parasite transmission increased gradually with the biting order of mosquitoes. *Plasmodium* transmission was tripled between the first and the last blood fed mosquito. Although the biting order of the mosquito cannot be decoupled from the biting time (these two parameters are obviously highly correlated), the increase in transmission 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 [40,50] but stochastic environmental factors such as variations in temperature, wind or humidity impact drastically the abundance of mosquitoes from one day to another [54–56]. 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 [40,44] 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 gametocythemia. Our study suggests that malaria parasite have evolved an alternative strategy acting at a shorter term. This strategy could be to adjust the physiological state of the gametocytes to respond promptly to mosquito bites. It has been suggested as early as 1966 [57] 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 on rodent malaria parasite: *P. chabaudi* gametocytes were twice as infective at night despite being less numerous in the blood [39]. Mechanisms underlying gametogenesis remains poorly understood. Although we know that gametocytes go through several stages of development before reaching the so-called "mature" stage, from 1 to 8 stages depending on the species of *Plasmodium* [58], we do not know whether mature stage is systematically infectious. The ability of malaria parasites to accelerate the rate of maturation and/or infectivity of gametocytes in response to mosquito bites should be explored.

Alternatively, 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 experiences strong fluctuations during its journey within the mosquito, which are partly intertwined with the kinetics of blood digestion [32]. Within seconds of ingestion into the mosquito blood meal, the drop in temperature and the rise in pH, associated to the presence of xanthurenic acid, triggers gametocyte activation and differentiation into gametes [59–61]. Studies on ookinete production have revealed that not only mosquito-derived xanthurenic acid but also undefined blood-derived factors ingested by mosquito are significant sources of gametocyte activating

313 factor [62,63]. Indeed, numerous host blood-derived compounds remain or become active
314 through the mosquito blood digestion, especially since the parasite is no longer protected by
315 the red blood cell membrane. For instance, complement components, vertebrate antibodies or
316 regulator factor H, may impact gametocytes-to-zygote and zygote-to-ookinetes stages
317 transition and survival [64–66]. Several studies also showed that ingested vertebrate-derived
318 factors negatively impact mosquito microbiota (e.g. complement cascade [67,68]) and their
319 peritrophic matrix (e.g. chitinase [66,69]) both known to play a key role in the mosquito
320 refractoriness to *Plasmodium* infection [70,71].The concentration of these vertebrate-derived
321 compounds in the ingested blood and, ultimately, their impact on parasite infectivity and/or
322 vector susceptibility, might vary progressively as the number of bites increases and thus explain
323 the increase in oocyst density with mosquito biting order.

324 In summary, we provide evidence that the overdispersion of parasite burden observed
325 in mosquitoes fed on a same infected host is partly explained by a temporal heterogeneity in
326 *Plasmodium* infectivity resulting from the biting order of mosquitoes. These results show that
327 the parasite is either directly or indirectly capable of responding to the bites of mosquitoes to
328 increase its own transmission at a much shorter time scales than initially thought (hours rather
329 than days [40,44]). Further work needs to be carried out to elucidate whether these two
330 strategies are complementary and, particularly, what are their respective underlaying
331 mechanisms. According to estimates by the World Health Organization, 228 million cases of
332 human malaria occurred in 2018, with 405 000 resulting in death. Despite recent progress
333 towards disease control, the number of malaria cases has increased in several countries. The
334 efficacy of control strategies is continually challenged and threatened by the evolution of
335 insecticide [72] and drug [73] resistances. To overcome these issues, the development of
336 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.

Materials and Methods

Malaria parasites and mosquito vector

Plasmodium relictum (lineage SGS1) is the most prevalent form of avian malaria in Europe [74]. The parasite strain used in the first block of the first experiment was isolated from an infected Great tit (*Parus major*) in 2015. The parasite used in the second experiment was isolated from an infected Great tit (*Parus major*) in April 2018. The parasite strain used in the second block of the first experiment and in the third experiment was isolated in January 2019 from an infected House sparrow (*Passer domesticus*). All strains were maintained by carrying out regular passages across our stock canaries (*Serinus canaria*) through intraperitoneal injections (i.p) until the beginning of the experiment.

All the experiments were conducted with a lineage of *Culex pipiens* mosquitoes, the main vector of *Plasmodium relictum* in Europe, collected in Lausanne (Switzerland) and maintained in insectary since August 2017. Mosquitoes were reared using standard protocols [75]. We used females 7-13 days after emergence that had no 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 24h before the experiment.

Experimental design

Prior to the experiments, a small amount (ca.3-5 µL) of blood was collected from the medial metatarsal vein of each canary to ensure that they were free from any previous haemosporidian infections [76]. Birds were inoculated by intraperitoneal injection of 100µL of

359 an infected blood pool (day 0). The blood pool was made with a 1:1 mixture of PBS and blood
360 sampled from 2-4 canaries infected with the parasite three weeks before the experiment.

361 ***Experiment 1***

362 The two experimental blocks of the first experiment were carried out with 14 and 5
363 infected birds respectively. Day 11-13 post-infection, corresponding to the acute phase of
364 infection, blood was sampled from each bird at 5:45 p.m. Straight afterwards blood sampling,
365 birds were placed individually in an experimental cage (L40 x W40 x H40 cm). At 6:00 p.m., 8
366 and 3 haphazardly chosen birds, for block 1 and 2 respectively, were exposed to mosquito bites
367 following the protocols described below.

368 Birds from the exposed treatment were exposed to four successive batches of 25 ± 3
369 uninfected females' mosquitoes. Each mosquito batch was left in the cage for 45 minutes
370 before being taken out and replaced by a new batch (i.e. batch 1 ($T0_{min}$), batch 2 ($T45_{min}$), batch
371 3 ($T90_{min}$) and batch 4 ($T135_{min}$)). Blood fed mosquitoes in each batch were counted and
372 individually placed in numbered plastic tubes (30 ml) covered with a mesh with a cotton pad
373 soaked in a 10% glucose solution. At the end of the last mosquito exposure session (9:00 p. m.)
374 a second blood sample was taken from each bird. A red lamp was used to capture blood fed
375 mosquitoes without disturbing the birds and the mosquitoes. Unexposed birds (control) were
376 placed in the same experimental condition but without mosquitoes.

377 Tubes containing the blood fed mosquitoes were kept in standard insectary conditions
378 to obtain an estimate of the blood meal size and the success of the infection (infection
379 prevalence and oocyst burden). For this purpose, 7-8 days post blood meal, the females were
380 taken out of the tubes and the amount of haematin excreted at the bottom of each tube was
381 quantified as an estimate of the blood meal size [75]. Females were then dissected and the

number of *Plasmodium* oocysts in their midgut counted with the aid of a binocular microscope [75].

Experiment 2

The same protocol as described above was used for the second experiment, except that birds exposed to mosquitoes (4 of the 8 infected birds) were exposed to a single batch of 100 uninfected mosquitoes for 3h (6:00-9:00 p.m.). 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.

Experiment 3

Two infected birds were exposed to a single batch of 100 mosquitoes for 3h (6.00 pm – 9.00 pm) and 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 qPCR or stored in plastic tubes and dissected one week later to count the number of oocysts in the midgut.

Vertebrate host infection

The parasitaemia (total proportion of red blood cells infected) and gametocytaemia (proportion of red blood cells infected by mature gametocytes, the sexual stages of the parasite) of vertebrate hosts exposed or not (control) to mosquitoes were measured just before and just after the mosquito exposure period (6 – 9 p.m.) using blood smears [74].

Molecular analyses

The quantification of parasites contained within the blood meal was carried out using a quantitative PCR (qPCR) with a protocol adapted from Cornet et al. (2013). Briefly, DNA was extracted from blood-fed females using standard protocols (Qiagen DNeasy 96 blood and tissue

406 kit). For each individual, we conducted two qPCRs: one targeting the nuclear 18s rDNA gene
407 of *Plasmodium* (Primers 18sPlasm7 5'-AGCCTGAGAAATAGCTACCACATCTA-3', 18sPlasm8
408 5'-TGTTATTTCTTGTCACTACCTCTCTTCTTT-3') and the other targeting the 18s rDNA gene of the
409 bird (Primers 18sAv7 5'-GAAACTCGCAATGGCTCATTAAATC-3', 18sAv8
410 5'-TATTAGCTCTAGAATTACCACAGTTATCCA-3'). All samples were run in triplicate (Bio-Rad
411 CFX96^{TM} Real-Time System) and the mean of the two closet samples was used to calculate the
412 threshold Ct value using the Bio-Rad CFX Maestro v1.1 software. Samples with a threshold Ct
413 value higher than 35 were considered uninfected. The number of parasites were calculated as
414 relative quantification values (RQ). RQ can be interpreted as the fold-amount of target gene
415 (*Plasmodium* 18s rDNA) with respect to the amount of the reference gene (Bird18s rDNA) and
416 are calculated as $2^{-(Ct18s\ Plasmodium\ -\ Ct18s\ Bird)}$. For convenience, RQ values were standardized by
417 $\times 10^4$ factor and log-transformed.

418 **Statistical analyses**

419 Analyses were carried out using the R statistical package (v. 3.4.1). Data were analysed
420 separately for each experiment and each experimental block.

421 Blood meal rate, blood meal size, infection prevalence, oocyst burden (where only
422 individuals that developed ≥ 1 oocyst were included), quantity of parasites contained within the
423 blood meal, which may depend on which bird mosquitoes fed on, were analysed fitting bird as
424 a random factor into the models using *lmer, glmer* or *glmer.nb* (package: lme4, [77]) according
425 to whether the errors were normally (haematin quantity, oocyst burden, quantity of parasites
426 contained within the blood meal), binomially (blood meal rate, infection prevalence) or
427 negative binomially distributed (oocyst burden). Mosquito batches (experience 1), mosquito
428 biting order (experiment 2 and 3) and blood meal size (when it was not a response variable)
429 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 (5:45 and 9:00 p.m.) and bird group (exposed to mosquito bites or control) were used as fixed factors.

The different statistical models (maximal and minimal models) built to analyse 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 [78]. The significance of the explanatory variables was established using a likelihood ratio test [79]. 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. *A posteriori* contrasts were carried out by aggregating factor levels together and by testing the fit of the simplified model using an LRT [78].

Ethics statements

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 will be available on the Dryad website after acceptance.

References

1. Shaw DJ, Dobson AP. Patterns of macroparasite abundance and aggregation in wildlife populations: a quantitative review. Parasitology. 1995;111: S111–S133. doi:10.1017/S0031182000075855

2. Shaw DJ, Grenfell BT, Dobson AP. Patterns of macroparasite aggregation in wildlife host populations. Parasitology. 1998;117: 597–610. doi:10.1017/S0031182098003448

3. Lord CC, Barnard B, Day K, Hargrove JW, McNamara JJ, Paul RE, et al. Aggregation and distribution of strains in microparasites. Philos Trans R Soc B Biol Sci. 1999;354: 799–807.

4. Poulin R. Explaining variability in parasite aggregation levels among host samples. Parasitology. 2013;140: 541–546. doi:10.1017/S0031182012002053

5. Ruiz L, Janssen D, Martín G, Velasco L, Segundo E, Cuadrado IM. 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. 2006;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, et al. Endemicity of chytridiomycosis features pathogen overdispersion. J Anim Ecol. 2016;85: 806–816. doi:10.1111/1365-2656.12500

7. Medley GF, Sinden RE, Fleck S, Billingsley PF, Tirawanchai N, Rodriguez MH. Heterogeneity in patterns of malarial oocyst infections in the mosquito vector. Parasitology. 1993;106 (Pt 5): 441–449.

8. Bundy D a. P, Anderson RM, Thresh JM. Population ecology of intestinal helminth infections in human communities. Philos Trans R Soc Lond B Biol Sci. 1988;321: 405–420. doi:10.1098/rstb.1988.0100

9. Anderson RM, May RM. Regulation and stability of host-parasite population interactions: I. regulatory processes. J Anim Ecol. 1978;47: 219–247. doi:10.2307/3933

10. May RM, Anderson RM. Regulation and stability of host-parasite population interactions: II. destabilizing processes. J Anim Ecol. 1978;47: 249–267. doi:10.2307/3934

11. Anderson RM. The regulation of host population growth by parasitic species. Parasitology. 1978;76: 119–157. doi:10.1017/S0031182000047739

12. Jaenike J. Population-level consequences of parasite aggregation. Oikos. 1996;76: 155–160. doi:10.2307/3545757

13. Lloyd-Smith JO, Schreiber SJ, Kopp PE, Getz WM. Superspreading and the effect of individual variation on disease emergence. Nature. 2005;438: 355–359. doi:10.1038/nature04153

14. Martin LB, Addison B, Bean AGD, Buchanan KL, Crino OL, Eastwood JR, et al. Extreme competence: Keystone hosts of infections. Trends Ecol Evol. 2019;34: 303–314. doi:10.1016/j.tree.2018.12.009

15. Poulin R. The disparity between observed and uniform distributions: A new look at parasite aggregation. Int J Parasitol. 1993;23: 937–944. doi:10.1016/0020-7519(93)90060-C

16. Woolhouse MEJ, Dye C, Etard J-F, Smith T, Charlwood JD, Garnett GP, et al. Heterogeneities in the transmission of infectious agents: Implications for the design of control programs. Proc Natl Acad Sci. 1997;94: 338–342. doi:10.1073/pnas.94.1.338

17. Cooper L, Kang SY, Bisanzio D, Maxwell K, Rodriguez-Barraquer I, Greenhouse B, et al. Pareto rules for malaria super-spreaders and super-spreading. Nat Commun. 2019;10: 1–9. doi:10.1038/s41467-019-11861-y

18. Das PK, Subramanian S, Manoharan A, Ramaiah KD, Vanamail P, Grenfell BT, et al. Frequency distribution of *Wuchereria bancrofti* infection in the vector host in relation to human host: evidence for density dependence. Acta Trop. 1995;60: 159–165.

19. Churcher TS, Ferguson NM, Basáñez M-G. Density dependence and overdispersion in the transmission of helminth parasites. Parasitology. 2005;131: 121–132. doi:10.1017/S0031182005007341

20. Wetten S, Collins RC, Vieira JC, Marshall C, Shelley AJ, Basáñez M-G. Vector competence for *Onchocerca volvulus* in the *Simulium* (*Notolepria*) *exiguum* complex: Cytoforms or density-dependence? Acta Trop. 2007;103: 58–68. doi:10.1016/j.actatropica.2007.05.009

21. Paul REL, Bonnet S, Boudin C, Tchuinkam T, Robert V. Aggregation in malaria parasites places limits on mosquito infection rates. Infect Genet Evol. 2007;7: 577–586. doi:10.1016/j.meegid.2007.04.004

22. Churcher TS, Bousema T, Walker M, Drakeley C, Schneider P, Ouédraogo AL, et al. Predicting mosquito infection from *Plasmodium falciparum* gametocyte density and estimating the reservoir of infection. eLife. 2013;2: e00626. doi:10.7554/eLife.00626

23. Bousema T, Griffin JT, Sauerwein RW, Smith DL, Churcher TS, Takken W, et al. Hitting hotspots: Spatial targeting of malaria for control and elimination. PLOS Med. 2012;9: e1001165. doi:10.1371/journal.pmed.1001165

24. Paull SH, Song S, McClure KM, Sackett LC, Kilpatrick AM, Johnson PT. From superspreaders to disease hotspots: linking transmission across hosts and space. Front Ecol Environ. 2012;10: 75–82. doi:10.1890/110111

25. Basáñez MG, Townson H, Williams JR, Frontado H, Villamizar NJ, Anderson RM. Density-dependent processes in the transmission of human onchocerciasis: relationship between microfilarial intake and mortality of the simuliid vector. Parasitology. 1996;113: 331–355. doi:10.1017/S003118200006649X

26. Dawes EJ, Zhuang S, Sinden RE, Basáñez M-G. The temporal dynamics of *Plasmodium* density through the sporogonic cycle within *Anopheles* mosquitoes. Trans R Soc Trop Med Hyg. 2009;103: 1197–1198. doi:10.1016/j.trstmh.2009.03.009

27. Dawes EJ, Churcher TS, Zhuang S, Sinden RE, Basáñez M-G. Anopheles mortality is both age- and *Plasmodium*-density dependent: implications for malaria transmission. Malar J. 2009;8: 228. doi:10.1186/1475-2875-8-228

28. Courtenay O, Peters NC, Rogers ME, Bern C. Combining epidemiology with basic biology of sand flies, parasites, and hosts to inform leishmaniasis transmission dynamics and control. PLOS Pathog. 2017;13: e1006571. doi:10.1371/journal.ppat.1006571

528 29. Gutiérrez-López R, Martínez-de la Puente J, Gangoso L, Yan J, Soriguer R, Figuerola J.
529 Experimental reduction of host *Plasmodium* infection load affects mosquito survival. Sci
530 Rep. 2019;9. doi:10.1038/s41598-019-45143-w

531 30. Anderson RM, Gordon DM. Processes influencing the distribution of parasite numbers
532 within host populations with special emphasis on parasite-induced host mortalities.
533 Parasitology. 1982;85: 373–398. doi:10.1017/S0031182000055347

534 31. Sinden RE, Dawes EJ, Alavi Y, Waldock J, Finney O, Mendoza J, et al. Progression of
535 *Plasmodium berghei* through *Anopheles stephensi* is density-dependent. PLoS Pathog.
536 2007;3. doi:10.1371/journal.ppat.0030195

537 32. Vaughan JA. Population dynamics of *Plasmodium* sporogony. Trends Parasitol. 2007;23:
538 63–70. doi:10.1016/j.pt.2006.12.009

539 33. Zélé F, Nicot A, Berthomieu A, Weill M, Duron O, Rivero A. *Wolbachia* increases
540 susceptibility to *Plasmodium* infection in a natural system. Proc R Soc Lond B Biol Sci.
541 2014;281: 20132837. doi:10.1098/rspb.2013.2837

542 34. Pigeault R, Vézilier J, Nicot A, Gandon S, Rivero A. Transgenerational effect of infection in
543 *Plasmodium*-infected mosquitoes. Biol Lett. 2015;11: 20141025.
544 doi:10.1098/rsbl.2014.1025

545 35. Harris C, Lambrechts L, Rousset F, Abate L, Nsango SE, Fontenille D, et al. Polymorphisms
546 in *Anopheles gambiae* immune genes associated with natural resistance to *Plasmodium*
547 *falciparum*. PLoS Pathog. 2010;6: e1001112. doi:10.1371/journal.ppat.1001112

548 36. Pigeault R, Nicot A, Gandon S, Rivero A. Mosquito age and avian malaria infection. Malar
549 J. 2015;14: 383. doi:10.1186/s12936-015-0912-z

550 37. Riehle MM, Markianos K, Niaré O, Xu J, Li J, Touré AM, et al. Natural malaria infection in
551 *Anopheles gambiae* is regulated by a Ssngle genomic control region. Science. 2006;312:
552 577–579. doi:10.1126/science.1124153

553 38. Pichon G, Awono-Ambene HP, Robert V. High heterogeneity in the number of
554 *Plasmodium falciparum* gametocytes in the bloodmeal of mosquitoes fed on the same
555 host. Parasitology. 2000;121 (Pt 2): 115–120.

556 39. Schneider P, Rund SSC, Smith NL, Prior KF, O'Donnell AJ, Reece SE. Adaptive periodicity in
557 the infectivity of malaria gametocytes to mosquitoes. Proc Biol Sci. 2018;285.
558 doi:10.1098/rspb.2018.1876

559 40. Pigeault R, Caudron Q, Nicot A, Rivero A, Gandon S. Timing malaria transmission with
560 mosquito fluctuations. Evol Lett. 2018. doi:10.1002/evl3.61

561 41. Mideo N, Reece SE, Smith AL, Metcalf CJE. The Cinderella syndrome: why do malaria-
562 infected cells burst at midnight? Trends Parasitol. 2013;29: 10–16.
563 doi:10.1016/j.pt.2012.10.006

564 42. Prior KF, Veen DR van der, O'Donnell AJ, Cumnock K, Schneider D, Pain A, et al. Timing of
565 host feeding drives rhythms in parasite replication. PLOS Pathog. 2018;14: e1006900.
566 doi:10.1371/journal.ppat.1006900

567 43. Prior KF, Rijo-Ferreira F, Assis PA, Hirako IC, Weaver DR, Gazzinelli RT, et al. Periodic
568 parasites and Ddily host rhythms. Cell Host Microbe. 2020;27: 176–187.
569 doi:10.1016/j.chom.2020.01.005

570 44. Cornet S, Nicot A, Rivero A, Gandon S. Evolution of plastic transmission strategies in
571 avian malaria. PLoS Pathog. 2014;10: e1004308. doi:10.1371/journal.ppat.1004308

572 45. Rivero A, Gandon S. Evolutionary ecology of avian malaria: Past to present. Trends
573 Parasitol. 2018. doi:10.1016/j.pt.2018.06.002

574 46. Churcher TS, Sinden RE, Edwards NJ, Poulton ID, Rampling TW, Brock PM, et al.
575 Probability of transmission of malaria from mosquito to human is regulated by mosquito
576 parasite density in naïve and vaccinated hosts. PLOS Pathog. 2017;13: e1006108.
577 doi:10.1371/journal.ppat.1006108

578 47. Wassom DL, Dick TA, Arnason N, Strickland D, Grundmann AW. Host genetics: A key
579 factor in regulating the distribution of parasites in natural host populations. J Parasitol.
580 1986;72: 334–337. doi:10.2307/3281615

581 48. Hansen F, Jeltsch F, Tackmann K, Staubach C, Thulke H-H. Processes leading to a spatial
582 aggregation of *Echinococcus multilocularis* in its natural intermediate host *Microtus*
583 *arvalis*. Int J Parasitol. 2004;34: 37–44.

584 49. Rosà R, Pugliese A. Aggregation, stability, and oscillations in different models for host-
585 macroparasite interactions. Theor Popul Biol. 2002;61: 319–334.
586 doi:10.1006/tpbi.2002.1575

587 50. Barrozo RB, Schilman PE, Minoli SA, Lazzari CR. Daily rhythms in disease-vector insects.
588 Biol Rhythm Res. 2004;35: 79–92. doi:10.1080/09291010412331313250

589 51. Lalubin F, Delédevant A, Glaizot O, Christe P. Temporal changes in mosquito abundance
590 (*Culex pipiens*), avian malaria prevalence and lineage composition. Parasit Vectors.
591 2013;6: 307. doi:10.1186/1756-3305-6-307

592 52. Duffield GE, Acri DJ, George GF, Sheppard AD, Beebe NW, Ritchie SA, et al. Diel flight
593 activity of wild-caught *Anopheles farauti* (*s.s.*) and *An. hinesorum* malaria mosquitoes
594 from northern Queensland, Australia. Parasit Vectors. 2019;12: 48. doi:10.1186/s13071-
595 018-3271-0

596 53. Billingsley PF, Snook LS, Johnston VJ. Malaria parasite growth is stimulated by mosquito
597 probing. Biol Lett. 2005;1: 185–189. doi:10.1098/rsbl.2004.0260

598 54. Chao S, Zhu D, Dixon D, Khater E, Xue R-D. Diel activity patterns of adult female
599 mosquitoes (*Diptera*: *Culicidae*) determined by a novel rotated trap in northeastern
600 Florida, U.S.A. J Vector Ecol. 2019;44: 149–153. doi:10.1111/jvec.12339

601 55. Ngowo HS, Kaindoa EW, Matthiopoulos J, Ferguson HM, Okumu FO. Variations in
602 household microclimate affect outdoor-biting behaviour of malaria vectors. Wellcome
603 Open Res. 2017;2. doi:10.12688/wellcomeopenres.12928.1

604 56. Sallam MF, Pereira RM, Batich C, Koehler P. Factors affecting short-range host-seeking
605 for the yellow fever mosquito (*Diptera*: *Culicidae*). J Med Entomol. 2019;56: 609–616.
606 doi:10.1093/jme/tjy230

607 57. Hawking F, Worms MJ, Gammage K, Goddard PA. The biological purpose of the blood-
608 cycle of the malaria parasite *Plasmodium cynomolgi*. The Lancet. 1966;288: 422–424.
609 doi:10.1016/S0140-6736(66)92722-X

610 58. Gautret P, Motard A. Periodic infectivity of *Plasmodium* gametocytes to the vector. A
611 review. Parasite Paris Fr. 1999;6: 103–111.

612 59. Billker O, Lindo V, Panico M, Etienne AE, Paxton T, Dell A, et al. Identification of
613 xanthurenic acid as the putative inducer of malaria development in the mosquito.
614 Nature. 1998;392: 289–292. doi:10.1038/32667

615 60. Billker O, Miller AJ, Sinden RE. Determination of mosquito bloodmeal pH in situ by ion-
616 selective microelectrode measurement: implications for the regulation of malarial
617 gametogenesis. Parasitology. 2000;120 (Pt 6): 547–551.
618 doi:10.1017/s0031182099005946

619 61. Angrisano F, Tan Y-H, Sturm A, McFadden GI, Baum J. Malaria parasite colonisation of the
620 mosquito midgut – Placing the *Plasmodium* ookinete centre stage. Int J Parasitol.
621 2012;42: 519–527. doi:10.1016/j.ijpara.2012.02.004

622 62. Sinden RE, Butcher GA, Billker O, Fleck SL. Regulation of Infectivity of *Plasmodium* to the
623 mosquito vector. Adv Parasitol. 1996;38: 53–117. doi:10.1016/S0065-308X(08)60033-0

624 63. Arai M, Billker O, Morris HR, Panico M, Delcroix M, Dixon D, et al. Both mosquito-derived
625 xanthurenic acid and a host blood-derived factor regulate gametogenesis of *Plasmodium*
626 in the midgut of the mosquito. Mol Biochem Parasitol. 2001;116: 17–24.
627 doi:10.1016/S0166-6851(01)00299-7

628 64. Margos G, Navarette S, Butcher G, Davies A, Willers C, Sinden RE, et al. Interaction
629 between host complement and mosquito-midgut-stage *Plasmodium berghei*. Infect
630 Immun. 2001;69: 5064–5071. doi:10.1128/IAI.69.8.5064-5071.2001

631 65. Simon N, Lasonder E, Scheuermayer M, Kuehn A, Tews S, Fischer R, et al. Malaria
632 parasites co-opt human factor H to prevent complement-mediated lysis in the mosquito
633 midgut. Cell Host Microbe. 2013;13: 29–41. doi:10.1016/j.chom.2012.11.013

634 66. Pakpour N, Riehle MA, Luckhart S. Effects of ingested vertebrate-derived factors on
635 insect immune responses. Curr Opin Insect Sci. 2014;3: 1–5.
636 doi:10.1016/j.cois.2014.07.001

637 67. Wang Y, Gilbreath TM, Kukutla P, Yan G, Xu J. Dynamic gut microbiome across life history
638 of the malaria mosquito *Anopheles gambiae* in Kenya. PloS One. 2011;6: e24767.
639 doi:10.1371/journal.pone.0024767

640 68. Khattab A, Barroso M, Miettinen T, Meri S. Anopheles midgut epithelium evades human
641 complement activity by capturing factor H from the blood meal. PLoS Negl Trop Dis.
642 2015;9. doi:10.1371/journal.pntd.0003513

643 69. Di Luca M, Romi R, Severini F, Toma L, Musumeci M, Fausto AM, et al. High levels of
644 human chitotriosidase hinder the formation of peritrophic membrane in anopheline
645 vectors. Parasitol Res. 2007;100: 1033–1039. doi:10.1007/s00436-006-0372-z

646 70. Cirimotich CM, Dong Y, Clayton AM, Sandiford SL, Souza-Neto JA, Mulenga M, et al.
647 Natural microbe-mediated refractoriness to *Plasmodium* infection in *Anopheles gambiae*.
648 Science. 2011;332: 855–858. doi:10.1126/science.1201618

649 71. Boissière A, Tchioffo MT, Bachar D, Abate L, Marie A, Nsango SE, et al. Midgut microbiota
650 of the malaria mosquito vector *Anopheles gambiae* and interactions with *Plasmodium*
651 *falciparum* infection. PLoS Pathog. 2012;8: e1002742. doi:10.1371/journal.ppat.1002742

652 72. Ranson H, Lissenden N. Insecticide resistance in african *Anopheles* mosquitoes: A
653 worsening stuation that needs urgent action to maintain malaria control. Trends
654 Parasitol. 2016;32: 187–196. doi:10.1016/j.pt.2015.11.010

655 73. Ariey F, Witkowski B, Amaratunga C, Beghain J, Langlois A-C, Khim N, et al. A molecular
656 marker of artemisinin-resistant *Plasmodium falciparum* malaria. Nature. 2014;505: 50–
657 55. doi:10.1038/nature12876

74. Valkiunas G. Avian malaria parasites and other Haemosporidia. CRC Press; 2004.

75. 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. 2010;9: 379. doi:10.1186/1475-2875-9-379

76. Waldenström J, Bensch S, Hasselquist D, Ostman O. A new nested polymerase chain reaction method very efficient in detecting *Plasmodium* and *Haemoproteus* infections from avian blood. J Parasitol. 2004;90: 191–194. doi:10.1645/GE-3221RN

77. Bates D, Mächler M, Bolker B, Walker S. Fitting Linear Mixed-Effects Models Using lme4. J Stat Softw. 2015;067. Available: https://ideas.repec.org/a/jss/jstsof/v067i01.html

78. Crawley MJ. The R Book. John Wiley & Sons; 2012.

79. Bolker BM. Ecological Models and Data in R. Princeton University Press; 2008.

Supporting information captions

Table S1: Description of statistical models presented in the main text. "N" gives the number of mosquitoes or birds included in each analysis. "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, nb: negative binomial errors).

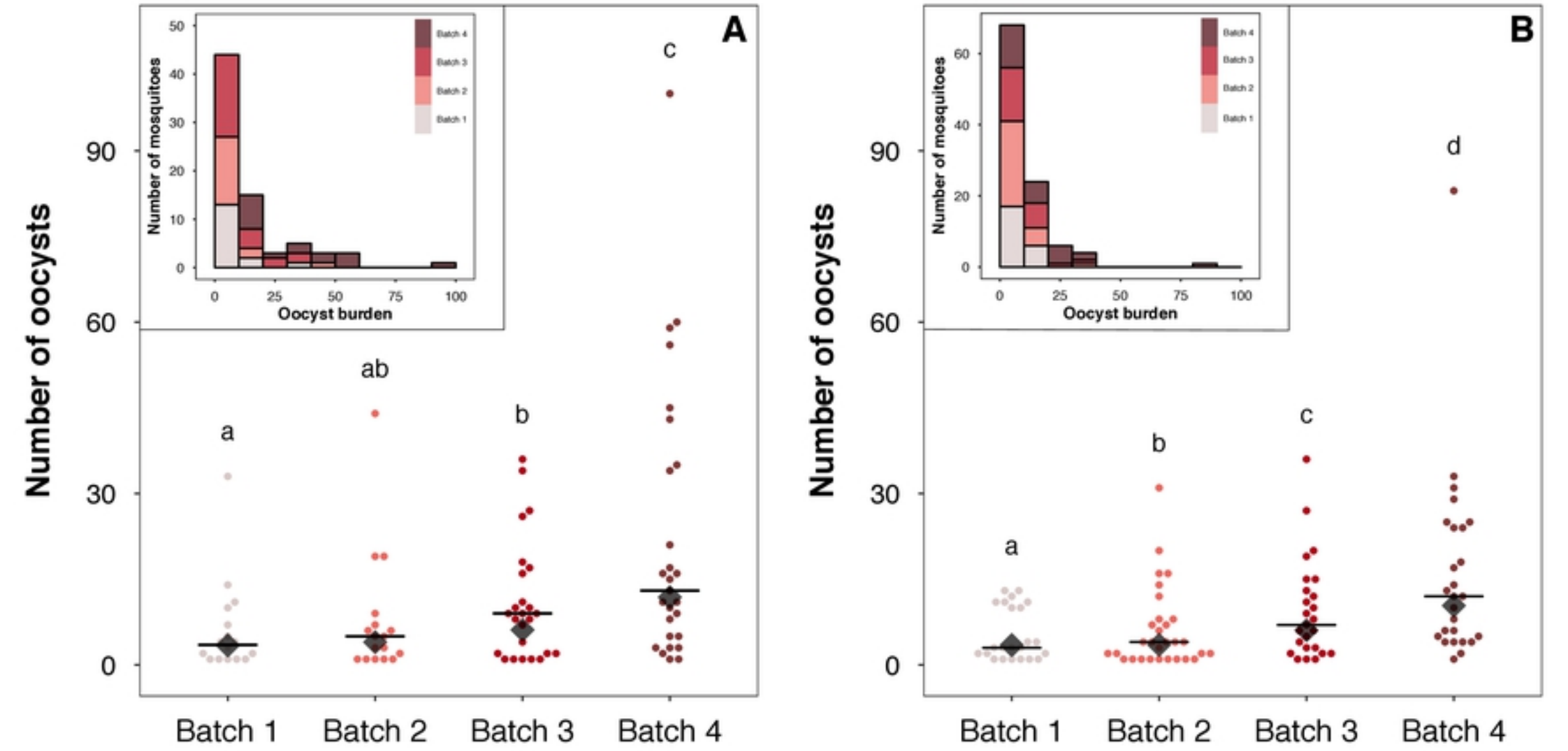
A
B
Number of oocysts
Number of mosquitoes
Oocyst burden
Batch 1
Batch 2
Batch 3
Batch 4
a
ab
b
c
d

Figure_1

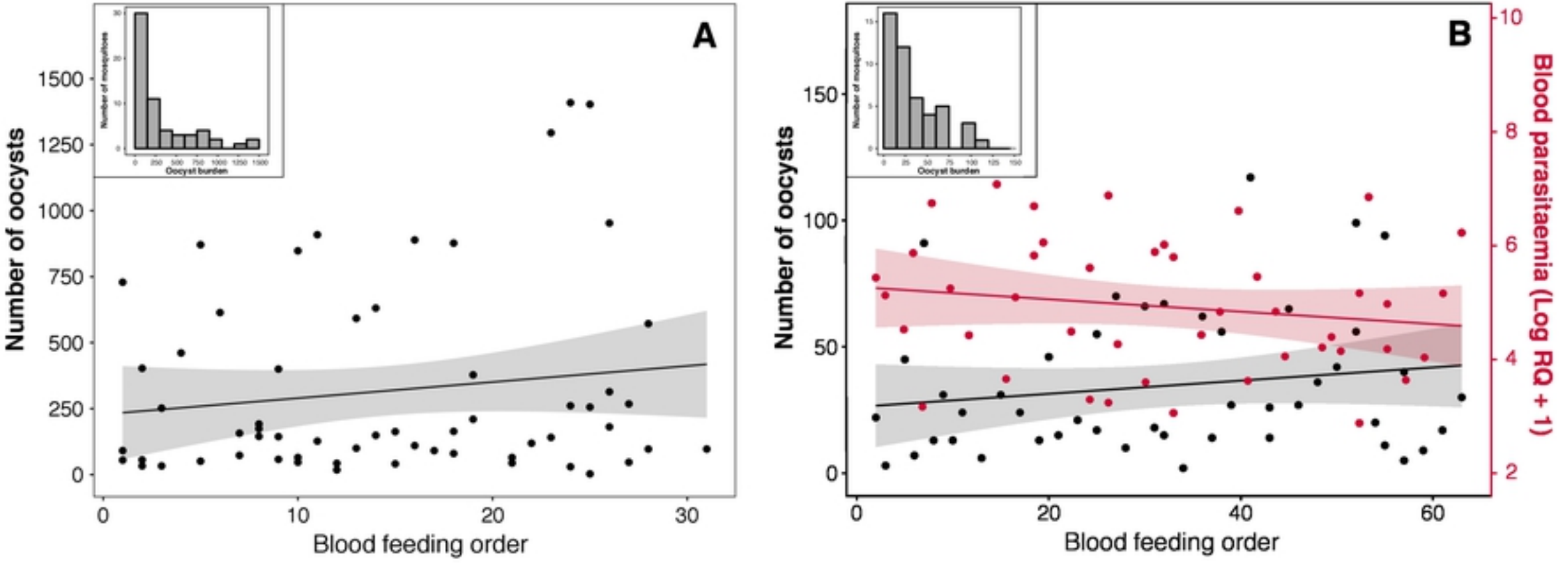
A
Number of oocysts
Blood feeding order
Number of mosquitoes
Oocyst burden
B
Number of oocysts
Blood parasitaemia (Log RQ + 1)
Blood feeding order
Number of mosquitoes
Oocyst burden

Figure_2