

1 **Timing malaria transmission with mosquito fluctuations**

2 Pigeault, R.^{1,2}, Caudron, Q.³, Nicot, A.⁴, Rivero, A.¹, Gandon, S.⁴

3 ¹ MIVEGEC (UMR CNRS 5290), Montpellier, France

4 ² DEE, UNIL, Lausanne, Switzerland

5 ³ Princeton University, USA

6 ⁴ CEFÉ (UMR CNRS 5175), Montpellier, France

7

8 **ABSTRACT**

9 Temporal variations in the activity of arthropod vectors can dramatically affect the
10 epidemiology and evolution of vector-borne pathogens. Here we explore the “Hawking
11 hypothesis” stating that these pathogens may evolve the ability to time investment in
12 transmission to match the activity of their vectors. First, we use a theoretical model to identify
13 the conditions promoting the evolution of time-varying transmission strategies in pathogens.
14 Second, we experimentally test the “Hawking hypothesis” by monitoring the within-host
15 dynamics of *Plasmodium relictum* throughout the acute and the chronic phases of the bird
16 infection. To explore the periodicity in the host parasite density, we develop a new
17 methodology to correct for non-stationarities in the host parasitaemia. We detect a periodic
18 increase of parasitaemia and mosquito infection in the late afternoon that coincides with an
19 increase in the biting activity of its natural vector. We also detect a positive effect of mosquito
20 bites on *Plasmodium* replication in the birds both in the acute and in the chronic phases of the
21 infection. This study highlights that *Plasmodium* parasites use two different strategies to
22 increase the match between transmission potential and vector availability. We discuss the
23 adaptive nature of these unconditional and plastic transmission strategies with respect to the
24 time-scale and the predictability of the fluctuations in the activity of the vector.

25

26 **Key words:** Cinderella hypothesis, circadian rhythm, Hawking hypothesis, periodicity

27

28 Impact Summary

29 Seasonal and daily fluctuations in the environment affect the abundance and the activity of
30 vectors and may therefore have profound consequences on the transmission of infectious
31 diseases. Here we show that, in accord with evolutionary theory, malaria parasites have
32 evolved two different and complementary strategies to cope with fluctuations in mosquito
33 availability. First, *Plasmodium relictum* adopts an unconditional strategy whereby within-host
34 parasitaemia and mosquito infection increases in the afternoon and in the evening, when its
35 vector, the *Culex pipiens* mosquito, is most active. Second, we find evidence for a plastic
36 strategy allowing the parasitaemia to rapidly increase after exposure to mosquito bites.

37 INTRODUCTION

38 All organisms face periodic changes in their environment. These environmental
39 fluctuations, which can happen at time scales ranging from daily to annual, affect the
40 physiological, immunological and behavioural activities of all species (Smaaland *et al.* 2002;
41 Corder *et al.* 2016; Duboscq *et al.* 2016) including parasites (Martinez-Bakker & Helm 2015;
42 Thaiss *et al.* 2015; Rijo-Ferreira *et al.* 2017a). Both short term (circadian) and long term
43 (seasonal) fluctuations in the environment may trigger dramatic perturbations of the
44 physiology of the hosts that can affect the within host dynamics of the parasite and,
45 ultimately, its epidemiology. One potential explanation for these parasite fluctuations is that
46 they are a by-product of the biological rhythms imposed by the host. There is, for example,
47 abundant evidence of the existence of short-term (circadian) rhythms in the expression of
48 physiological and immune host genes that may potentially impact the development of the
49 parasites within (Edgar *et al.* 2016). Longer-term (seasonal) fluctuations may also trigger
50 dramatic perturbations of the physiology and immunology of the host, which may affect the
51 within-host dynamics of some parasites (see Martinez-Bakker & Helm 2015).

52 Alternatively, and arguably more interestingly, these periodic fluctuations may be
53 viewed as pathogen adaptations aimed at maximizing transmission by taking advantage of a
54 transient favourable environment (Hawking 1975; Martinez-Bakker & Helm 2015). For
55 instance, in the coccidian parasite *Isospora* *sp*, the highly synchronized production of
56 transmissible stages in the faeces of infected animals takes place in the late afternoon to
57 minimize mortality through desiccation and UV radiation (Martinaud *et al.* 2009). Crucially,
58 Hawking (Hawking 1970, 1975) argued that similar processes may be acting in vector-borne
59 diseases. He postulated that the timing and the rhythm of many vector-borne pathogens may
60 have evolved to match the daily fluctuations in vector abundance. This so-called "Hawking

61 hypothesis" (Garnham & Powers 1974; Gautret & Motard 1999) has received considerable
62 empirical support from both within and cross-species comparisons of microfilarial parasites,
63 where parasite and mosquito daily rhythms seem to be well matched. For example, the
64 parasite *Wuchereria bancrofti*, which is transmitted by night-biting *Culex* sp mosquitoes,
65 shows a marked nocturnal periodicity where the transmissible microfilaria are sequestered in
66 the lungs during daytime and released into the peripheral blood at night (Hawking 1975).
67 However, in the Pacific islands, where the parasite is transmitted by day-biting *Aedes*
68 *polinesiensis* mosquitoes, *Wuchereria bancrofti* microfilaria are significantly more abundant
69 during the day (Moulia-Pelat *et al.* 1993).

70 Many malaria parasites exhibit striking periodic and synchronized cell cycles leading to
71 the simultaneous burst of infected red blood cells at regular points in time. In spite of
72 numerous studies exploring the adaptive nature of malaria periodicity in relation to vector
73 activity (Hawking 1970, 1975; Gautret & Motard 1999) whether these patterns fit the
74 "Hawking hypothesis" remains a controversial issue. Mideo *et al.* (2013) put forward three
75 main arguments against the validity of the Hawking hypothesis in malaria. First, they argued
76 that an accurate timing of gametocyte *production* requires a very finely-tuned synchronization
77 of the whole parasite life cycle. We agree, but contend that gametocyte *maturity* (the
78 process under which gametocytes become infective to mosquitoes, Alano 2007) may be
79 decoupled from the rest of the parasite's life cycle. Although the process of gametocyte
80 maturation is still not well understood, potential inducers of gametocyte maturation have
81 been described (Sinden 2015), some of which may be under circadian control (Rijo-Ferreira *et*
82 *al.* 2017b). Second, Mideo *et al.* (2013) also argued that even if the timing of the production
83 of infectious gametocytes is perfectly controlled, the long life expectancy of mature
84 gametocytes would erase any daily rhythm imposed on their production. The gametocytes of

85 most *Plasmodium* species seem, however, to have very short lifespans, surviving for a few
86 hours after their production (see Gautret & Motard 1999, Alano 2007). The one exception is
87 *P. falciparum* whose gametocytes seem indeed to live an inordinate amount of time (6 days,
88 Bousema & Drakeley 2011). Whether these mature gametocytes remain infective throughout
89 their lifespan is, however, not entirely clear. Indeed, the expected positive correlation
90 between gametocyte density and mosquito infection is often not very strong (Bousema &
91 Drakeley 2011). Hawking (1966), for instance, observed that “the cycle of infectivity is not due
92 to the cycle of the *number* of gametocytes in the blood but must be due to variation in their
93 *physiological* state – i.e., their suitability to develop in mosquitoes”. This suggests that malaria
94 infectivity is not driven solely by gametocyte abundance. The last of Mideo *et al.*’s (2013)
95 objections is the lack of evidence for a match between the parasite’s cycles in infectivity and
96 the biting activity of mosquitoes. This is a point we agree with, as the large majority of studies
97 aiming to test the Hawking hypothesis in malaria have indeed focused on the within-host
98 dynamics of the parasite, without testing whether this translates into higher mosquito
99 infection.

100 Here, we first present a theoretical model that studies evolution of time-varying
101 transmission strategies of *Plasmodium* in a periodically fluctuating environment. This model
102 identifies the conditions under which a periodic investment in transmission is expected to
103 evolve. Then, we carry out an experiment to explore empirically the validity of the “Hawking
104 hypothesis”. For this purpose, we study the periodicity of the avian malaria parasite,
105 *Plasmodium relictum*, in relation with the timing of the activity of its natural vector in the field,
106 the mosquito *Culex pipiens*. In contrast with human malaria, *P. relictum* does not exhibit
107 synchronous development in its vertebrate host (all erythrocytic stages are present in the
108 blood at all times) but several earlier studies report daily fluctuations in within-host parasite

109 abundance (see Gambrell 1937; Hewitt 1940). Yet, the potential link between these
110 fluctuations and the activity of the mosquito vectors remains to be investigated. To explore
111 the validity of the “Hawking hypothesis” we monitored both blood parasitaemia (Pigeault *et*
112 *al.* 2015) and mosquito activity throughout the day. We use overall parasitaemia as a proxy
113 for transmissible stage (gametocyte) production because in avian malaria the development of
114 gametocytes follows quite closely the development of asexual forms (Hewitt 1940) and our
115 previous work (Pigeault *et al* 2015) has shown that there’s a very good correlation between
116 sexual (gametocyte) and asexual parasitaemia. As pointed out by our theoretical analysis, the
117 adaptive scenario underlying the “Hawking hypothesis” should yield a positive covariance
118 between bird parasitaemia and mosquito activity.

119 We worked on both the acute and chronic stages of the infection. From the point of
120 view of the parasite these two stages are fundamentally different in terms of transmission
121 opportunities. While the acute phase is very short lived and results in high rates of mosquito
122 infection, the chronic phase can last several months, and even years, but does not yield high
123 transmission rates (Cornet *et al.* 2014; Pigeault *et al.* 2015). We thus compare these two
124 phases of the infections to establish: (i) the existence of fluctuations of blood parasitaemia
125 throughout the day and (ii) whether these fluctuations translate into higher pathogen
126 transmission to mosquitoes. To explore the periodicity in host parasite density, we developed
127 a new methodology to correct for non-stationarities in the host parasitaemia caused by the
128 large-scale changes in within-host dynamics during the acute phase of the infection. In
129 addition, given that mosquito bites may themselves affect within-host dynamics of the
130 parasite (Lawaly *et al.* 2012; Cornet *et al.* 2014; Reece & Mideo 2014) we compared the within-
131 host dynamics of malaria in birds exposed (or not) to mosquitoes. Mosquito bites may be yet
132 another way for the parasite to respond to the variability of the environment, albeit at a

133 different (shorter) temporal scale. We have previously argued that such a strategy may be an
134 adaptation to a fluctuating seasonal environment where mosquitoes are very abundant during
135 certain seasons and absent during others (Cornet *et al.* 2014; Reece & Mideo 2014). The
136 present paper is an attempt to explore another dimension of malaria adaptation to
137 fluctuations in mosquito availability. In the following we show that *Plasmodium* parasites can
138 use both constitutive and plastic variations in within-host investment in transmission to match
139 short-term and long-term fluctuations in vector availability.

140

141 MATERIAL & METHODS

142 Malaria parasites and mosquitoes

143 *Plasmodium relictum* (lineage SGS1) is the aetiological agent of the most prevalent
144 form of avian malaria which is commonly found infecting passeriform birds in Europe (Pigeault
145 *et al.* 2015). Our parasite lineage (SGS1) was isolated from an infected house sparrow caught
146 in the region of Saintes Maries-de-la-Mer (France) in May 2015 and transferred to naïve
147 canaries (*Serinus canaria*, Passeriforms).

148 Mosquito experiments were conducted with a laboratory isogenic strain of *Cx. pipiens*
149 mosquitoes. The susceptibility to infection by *P. relictum* and the behavioural activity of our
150 mosquito strain are similar to what is observed in wild *Cx. pipiens* mosquitoes (Vézilier *et al.*
151 2010, Pigeault *pers. obs.*). Mosquitoes were reared as described by Vézilier *et al.* (2010). We
152 used females 7 ± 2 days after emergence that had no prior access to blood and which were
153 starved for 6h before the experiment. Mosquitoes and canaries were maintained under a
154 12:12-h LD cycle (6h light on, 18h light off).

155

156

157 **Experimental design**

158 Experiments were carried out using (1- year old) domestic canaries (*Serinus canaria*).
159 Prior to the experiments, a small amount of blood (3-5 μ L) was collected from the medial
160 metatarsal vein of each of the birds and used to verify that they were free from any previous
161 haemosporidian infections. Eight canaries were experimentally inoculated by means of an
162 intraperitoneal injection of *ca.* 80 μ L of an infected blood pool (day 0, **Fig. 1**, Pigeault *et al.*
163 2015). The blood pool was constituted of a mixture of blood from 3 infected canaries
164 inoculated with the parasite isolated from the field three weeks before the experiment. The
165 eight infected birds were assigned to two treatments: “exposed” (n=3) or “unexposed” (n=5)
166 to mosquito bites. One “unexposed” bird lost the malaria infection very quickly (10 dpi) and
167 was removed from the analyses. From day 8 to day 70 post-infection parasitaemia of each bird
168 was monitored regularly at noon (12h, **Fig. 1**) except during the experimental sessions when
169 sampling was increased to 4 times per day (see below for details). All blood samples were
170 carried out by collecting 5-10 μ L of blood from the medial metatarsal vein. A drop of this blood
171 sample was smeared onto a slide for the visual quantification of the parasitaemia (Valkiunas
172 2004), and the rest was frozen for the molecular quantification of the parasitaemia (see
173 below). In *Plasmodium relictum* infections parasitaemia and gametocytaemia are strongly
174 positively correlated (see Figure 2 in Pigeault *et al.* 2015). For practical reasons, parasitaemia,
175 which is more rapidly quantified, was therefore used as a proxy of parasite investment in the
176 production of transmissible stage.

177 *Daily fluctuations of Plasmodium infection –*

178 In order to investigate the daily fluctuation of the blood parasitaemia, two experimental
179 sessions were carried out: the first one during the acute stage of infection (Session 1: between
180 day 12 and 14 dpi, **Fig. 1**) and the second one during the chronic stage of infection (Session 2:
181 between day 61 and 64 dpi, **Fig. 1**). During these two experimental sessions blood sampling
182 was carried out every 6 hours (at 6h, 12h, 18h and 00h, **Fig. 1B**). In the acute stage of the
183 infection the existence of a daily fluctuation in the blood parasitaemia was investigated by
184 counting the number of parasites in blood smears (Valkiunas 2004) while in the chronic stage,
185 when parasites in the blood are so scarce that blood smear counts are highly inaccurate,
186 parasite intensities were calculated using molecular tools (see below). In the acute stage of
187 the infection, when the daily fluctuations of parasitaemia may be masked by the large-scale
188 changes in within-host dynamics, the periodicity of the fluctuations in bird parasitaemia was
189 analysed using a new statistical approach that takes into account the overall within-host
190 dynamics of *Plasmodium* infection (see Supplementary Materials, **S1 Text**).

191 *Daily fluctuations of Plasmodium transmission –*

192 In order to estimate whether fluctuations in blood parasitaemia translate into fluctuations in
193 transmission to mosquitoes we: 1) obtained estimates of mosquito activity throughout the
194 day, 2) estimated the number of parasites ingested by the mosquitoes at different times
195 during the day and 3) estimated the success of the infection at the oocyst (midgut) stage. For
196 this purpose, on day 13 (Session 1) and day 62 dpi (Session 2), and straight after each of the
197 blood sampling events (at 6h, 12h, 18h and 00h), the birds from the “exposed” treatment were
198 placed inside a cage (L40 x W30 x H30cm) with a batch of 70 uninfected female mosquitoes
199 for 135 minutes. The remaining (“unexposed”) birds were kept under identical conditions but
200 without the mosquitoes. The cages were visited every 45 minutes and all blood fed females
201 were removed and counted. The number of mosquitoes fed at each time step was recorded

202 and was used as an estimate mosquito activity throughout the day (see below). Thereafter,
203 these recently blood-fed mosquitoes were divided in two groups. One half was frozen
204 individually in order to quantify the parasites ingested in the blood meal (see below). The
205 other half was kept alive to obtain an estimate of the blood meal size and of the success of
206 the infection (number of oocysts in the midgut). This was done by placing these mosquitoes
207 in numbered plastic tubes (30 ml) covered with a mesh with a cotton pad soaked in a 10%
208 glucose solution. Seven days later (day 7 post blood meal) the females were taken out of the
209 tubes and the amount of haematin excreted at the bottom of each tube was quantified as an
210 estimate of the blood meal size (Vézilier *et al.* 2010). Females were then dissected and the
211 number of *Plasmodium* oocysts in their midguts counted with the aid of a binocular
212 microscope (Vézilier *et al.* 2010).

213 At the end of the mosquito exposure session, the parasitaemia of the birds was
214 monitored on a daily basis for a total of 57 days in acute and 8 days in chronic stage of
215 infection. This allowed us to contrast the within-host dynamics of the malaria parasites in birds
216 exposed or not to mosquitoes.

217

218 **Molecular analyses**

219 The molecular quantification of parasites in the mosquito blood meal was carried out
220 using a quantitative PCR (qPCR) protocol adapted from (Cornet *et al.* 2013). Briefly, DNA from
221 blood-fed females was extracted using standard protocols (Qiagen DNeasy 96 blood and tissue
222 kit). For each individual, we conducted two qPCRs in the same run: one targeting the nuclear
223 18s rDNA gene of *Plasmodium* (Primers: 18sPlasm7 5'-AGCCTGAGAAATAGCTACC- ACATCTA-
224 3', and 18sPlasm8 5'-TGTTATTCTTGTCACTACCTCTC- TTCTTT-3'), and the other targeting the
225 18s rDNA gene of the bird (18sAv7 5' GAAACTCGCAATGGCTCATTAAATC-3', and 18sAv8 5'-

226 TATTAGCTCTAGAATTACCACAGT TATCCA-3'). All samples were run in triplicate (ABI 7900HT
227 real-time PCR system, Applied Biosystems) and their mean was used to calculate the threshold
228 Ct value (the number of PCR cycles at which fluorescence is first detected, which is inversely
229 correlated with the initial amount of DNA in a sample) using the software Light Cycler 480
230 (Roche). Parasite intensities were calculated as relative quantification values (RQ). RQ can be
231 interpreted as the fold-amount of target gene (*Plasmodium* 18s rDNA) with respect to the
232 amount of the reference gene (Bird18s rDNA) and are calculated as $2^{-(Ct_{18s\ Plasmodium} - Ct_{18s\ Bird})}$. For convenience, RQ values were standardised by $\times 10^4$ factor and log-transformed
233 (Cornet *et al.* 2013).
234

235

236 **Statistical analysis**

237 The statistical analyses were run using the R software (V. 3.3.3). The different statistical
238 models built to analyse the data are described in the supplementary material (**Table S1**).
239 Analyses where a same individual bird was sampled repeatedly, such as the daily fluctuation
240 of blood parasitaemia or the impact of mosquito exposure on the parasite replication rate,
241 were analysed fitting bird as a random factor into the models (to account for the temporal
242 pseudoreplication), using a mixed model procedure (*lme*, package: *nlme*). Similarly, mosquito-
243 centred traits (such as infection prevalence or oocyst burden), which may depend on which
244 bird mosquitoes fed on, were also analysed fitting bird as a random factor into the models (to
245 account for the spatial pseudoreplication), using *lme* or *glmer* (package: *lme4*) according to
246 whether the errors were normally (oocyst burden) or binomially (prevalence) distributed.
247 Time of day and, when necessary, blood meal size (haematin) were used as fixed factors.

248 Mosquito activity (*i.e.* time required to take a blood meal) was analyzed using survival
249 analyses (package: *survival*) with time of day (6h, 12h, 18h 00h) fitted as fixed factors in the

250 model and under the assumption of exponential errors. From this model, we estimated the
251 constant hazard rate for each treatment (time of day).

252 Maximal models, including all higher-order interactions, were simplified by
253 sequentially eliminating non-significant terms and interactions to establish a minimal model
254 (Crawley 2012). The significance of the explanatory variables was established using either a
255 likelihood ratio test (which is approximately distributed as a Chi-square distribution (Bolker
256 2008) or an F test. The significant Chi-square or F values given in the text are for the minimal
257 model, whereas non-significant values correspond to those obtained before the deletion of
258 the variable from the model. *A posteriori* contrasts were carried out by aggregating factor
259 levels together and by testing the fit of the simplified model using an LRT (Crawley 2012).

260 To analyse the existence of a circadian rhythm in the parasite dynamics during the
261 acute stage of infection, in addition to the statistical analyses described above, we also
262 developed a new methodology presented in the supplementary materials (**S1 Text**). In
263 addition, we provide a link to a github notebook with a step-by-step description of this
264 procedure and a code that may be used to analyse other within-host time series
265 (https://github.com/QCaudron/timing_malaria_transmission).

266

267 **RESULTS**

268 **Theory: evolution of adaptive rhythmicity**

269 To model the evolution of rhythmic transmission strategies we first need to model the
270 epidemiological dynamics of malaria. For the sake of simplicity, the vertebrate host population
271 is assumed to be constant and equal to $N_H = S(t) + I(t)$, where $S(t)$ and $I(t)$ are the
272 densities of uninfected and infected hosts, respectively. Similarly, the mosquito vector
273 population is also assumed to be constant and equal to $N_V = V(t) + V_I(t)$, where $V(t)$ and

274 $V_I(t)$ are the densities of uninfected and infected vectors, respectively. The activity of the
275 vector $a(t)$ is assumed to fluctuate with a period $T = 1$ day. Low mosquito activity is assumed
276 to decrease biting rate and transmission and, consequently, the epidemiological dynamics also
277 fluctuate periodically. The following set of differential equations describes the temporal
278 dynamics of the different types of hosts (the dot notation indicates differential over time):

$$\begin{aligned} \dot{I}(t) &= (N_H - I(t))V_I(t)a(t)\beta_2 - (d + \alpha(t))I(t) \\ \dot{V}_I(t) &= I(t)(N_V - V_I(t))a(t)\beta_1(t) - m_I V_I(t) \end{aligned} \tag{1}$$

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

283 The pathogen is allowed to have time-varying investment in transmission and virulence
284 in the vertebrate host. As in classical models of virulence evolution, replication allows the
285 parasite to transmit more efficiently (i.e. higher $\beta_1(t)$) but is assumed to be costly because it
286 may induce the death of the vertebrate host (i.e. higher $\alpha(t)$). To study parasite evolution we
287 track the dynamics of a rare mutant parasite M with different transmission and virulence
288 strategies ($\beta_{1M}(t)$ and $\alpha_M(t)$, respectively):

$$\begin{aligned} \dot{I}_M(t) &= (N_H - I(t))V_{IM}(t)a(t)\beta_2 - (d + \alpha_M(t))I_M(t) \\ \dot{V}_{IM}(t) &= I_M(t)(N_V - V_I(t))a(t)\beta_{1M}(t) - m_I V_{IM}(t) \end{aligned} \tag{2}$$

289 Because the frequency of the fluctuation in mosquito activity is much higher than other
290 dynamical variations of the system we may assume that the density of infected hosts remains

291 approximately stable throughout the day. This separation of time scale allows to focus on the
 292 dynamics of the vector compartment which yields:

$$\dot{V}_I(t) \approx \left(\frac{a(t)N_H\beta_2(N_V - V_I(t))}{(d + \alpha(t) + a(t)V_I(t)\beta_2)} a(t)\beta_1(t) - m_I \right) V_I(t) \quad (3)$$

$$\dot{V}_{IM}(t) \approx \left(\frac{a(t)N_H\beta_2(N_V - V_I(t))(d + \alpha(t))}{(d + \alpha(t) + a(t)V_I(t)\beta_2)(d + \alpha_M(t))} a(t)\beta_{1M}(t) - m_I \right) V_{IM}(t)$$

293 The change in frequency of the mutant is thus given by:

$$\dot{p}_M(t) \propto A(t)(B_{1M}(t) - B_1(t)) p_M(t) \quad (4)$$

294 with $(t) = a(t) \frac{a(t)N_H\beta_2(N_V - V_I(t))}{(d + \alpha(t) + a(t)V_I(t)\beta_2)}$, $B_{1M}(t) = \frac{\beta_{1M}(t)}{(d + \alpha_M(t))}$ and $B_1(t) = \frac{\beta_1(t)}{(d + \alpha(t))}$.

295 The ability of the mutant to invade the resident population is determined by s_M , the selection
 296 coefficient on the mutant, which can be evaluated after integrating the change of the mutant
 297 frequency over one day:

$$s_M = \frac{1}{T} \int_0^T (\dot{p}_M(t)/p_M(t)) dt \quad (5a)$$

298 Which yields:

$$s_M \propto \left(\underbrace{\tilde{A}(\tilde{B}_{1M} - \tilde{B}_1)}_{\substack{\text{Classical} \\ \text{transmission-virulence} \\ \text{trade off}}} + \underbrace{\text{cov}_t(A, B_{1M}) - \text{cov}_t(A, B_1)}_{\substack{\text{Match between mosquito} \\ \text{activity and within-host growth}}} \right) \quad (5b)$$

299 where the tilde refers to the average over a period $T = 1$ day of the fluctuation. The first term
 300 in the above equation for s_M is akin to the classical trade-off between transmission β_1 and
 301 virulence α . The second term measures the benefit associated with a closer match between

302 parasite dynamics in the vertebrate host and the rhythmicity in mosquito behavior. For
303 instance, the above expression is particularly useful to examine the invasion of a mutant with
304 a time-varying strategy in a resident pathogen population with a strategy that does not vary
305 with time (i.e. β_1 is constant and $cov_t(A, B_1) = 0$). The mutant will invade only if $s_M > 0$
306 which yields:

$$cov_t(A, B_{1M}) > \frac{\beta_1}{(d + \alpha)} - \frac{1}{T} \tilde{B}_{1M} \approx \frac{\beta_1}{(d + \alpha)} - \frac{\tilde{\beta}_{1M}}{(d + \tilde{\alpha}_M)} \quad (6)$$

307 Time-varying transmission thus evolves when the temporal covariance between $A(t)$, which
308 is a dynamical variable tightly linked with mosquito activity $a(t)$, and investment in
309 transmission is positive and higher than the potential fitness cost (the right-hand side of
310 equation (6)) associated with this time-varying transmission. In other words, this temporal
311 covariance is a measure of the adaptive nature of time-varying transmission.

312 The above derivation focuses on the evolution of a constitutive time-varying
313 investment in transmission to match fast and periodic fluctuations of vector activity. But when
314 the fluctuation of the environment is slower and/or is less predictable it may be more adaptive
315 to monitor environmental changes and to induce phenotypic modifications accordingly (e.g.
316 Kussell & Leibler 2005). In malaria we developed a similar argument to analyze the evolution
317 of inducible investment in transmission after mosquito bites (Cornet *et al.* 2014).

318 **Experiment: “Hawking hypothesis” in avian malaria**

319 Blood parasitaemia initially followed a bell-shape function typical of acute *Plasmodium*
320 infections: peaking at day 12 post-infection (dpi) and decreasing thereafter (**Fig. 1**). The
321 infection subsequently entered a long-lasting chronic state, which was characterized by a low
322 blood parasitaemia over several weeks (**Fig. 1**). During the acute phase of the infection, and

323 before the exposure to the mosquitoes, there was no significant difference in the parasitaemia
324 of the hosts assigned to the “exposed” and “unexposed” treatments (model 1: $\chi^2_1 = 0.01$, $p =$
325 0.941, **Fig. 2A**). However, after they had been exposed to the mosquito bites, the acute-phase
326 parasitaemia of the “exposed” birds was significantly higher than that of their “unexposed”
327 counterparts (model 2: $\chi^2_1 = 8.59$, $p = 0.003$, **Fig. 2A**). This effect was short-lived and only lasted
328 around 48h (peak reached in 24h, **Fig. 2A**). During the chronic phase of the infection, there
329 was a significant difference in the parasitaemia of the birds before the exposure session
330 (model 3: $\chi^2_1 = 10.83$, $p = 0.001$, **Fig. 2B**): “unexposed” birds had a higher parasitaemia than
331 “exposed” hosts. After exposure to mosquito bites, while the parasitaemia of the “unexposed”
332 birds did not vary (model 4: $\chi^2_1 = 0.086$ $p = 0.771$, **Fig. 2B**), the parasitaemia of the “exposed”
333 chronically-infected hosts increased over time (peak reached in 6 days, model 5: $\chi^2_1 = 22.99$ p
334 < 0.0001, **Fig. 2B**).

335

336 Daily fluctuations of blood parasitaemia

337 The periodicity of the fluctuations in bird parasitaemia was explored using a new
338 statistical approach that takes into account the overall within-host dynamics of *Plasmodium*
339 infection during the acute phase of the infection (See **Supporting Information**). In spite of a
340 limited number of samples this analysis suggests that bird parasitaemia fluctuates periodically
341 with a peak in the late afternoon (See **Supporting Information**). We then examined in depth
342 these daily fluctuations in parasitaemia and their consequences on mosquito transmission in
343 both the acute and chronic phases of the infection. To avoid the potential effect of mosquito
344 bites on within-host dynamics, we focused our analyses on “exposed” birds. In the acute phase
345 of the infection we found a significant effect of the time of day on blood parasitaemia (model
346 6: $\chi^2_1 = 11.58$, $p = 0.009$, **Fig. 3A**). The parasitaemia was highest in the evening (18h) and lowest

347 early in the morning (6h, **Fig. 3A**). During the chronic phase of the infection blood parasitaemia
348 was very low in all exposed birds (parasitaemia < 0.001%, **Fig. 1**). Molecular methods,
349 however, allowed us to detect daily variations in parasitaemia. Parasite burden was null at 6h
350 and 12h, or below the detection levels, but increased in the evening (**Fig. 3B**).

351

352 **Daily fluctuations of *Plasmodium* transmission**

353 As mentioned above, the aim of this section was to investigate whether fluctuations in
354 blood parasitaemia translate into fluctuations in transmission to mosquitoes. For this purpose,
355 we first quantify the number of oocysts in mosquitoes fed at different times of the day. We
356 then explore whether these differences can be explained by differences in the amount of
357 parasites ingested by the mosquitoes at different times of the day. Finally, we explore whether
358 the fluctuations in blood parasitaemia and mosquito infectivity match the daily patterns of
359 mosquito activity.

360 In the acute stage, the mosquito infection prevalence was 100% for all feeding times.
361 Blood feeding time, however, had a very significant effect on the oocyst burden of mosquitoes
362 (model 7: $\chi^2_1 = 42.69$, $p < 0.0001$, **Fig. 3A**). Females that fed in the evening (18h and 00h) had
363 more than twice as many oocysts as those feeding at noon (contrast analyses: 12h/18h : $\chi^2_1 =$
364 8.28 , $p = 0.004$, 12h/00h : $\chi^2_1 = 13.92$, $p < 0.0001$, oocysts burden: 12h: mean \pm s.e: 108 ± 25 ,
365 18h: 262 ± 51 and 00h: 314 ± 52) and noon-feeding mosquitoes had significantly more oocysts
366 than those feeding in the early morning (contrast analyses: 6h/12h : $\chi^2_1 = 5.03$, $p = 0.025$,
367 oocysts burden: 6h: 62 ± 14). As expected, haematin, a proxy for blood meal size, has an
368 impact on mosquito oocyst burden (model 7: $\chi^2_1 = 49.17$, $p < 0.0001$). Crucially, however, the
369 time of day has no impact on haematin production (model 8: $\chi^2_1 = 6.54$, $p = 0.091$) implying
370 that the blood meal sizes do not change according to the feeding times. An impact of bird

371 parasitaemia on oocyst burden was observed but only when the feeding time was removed
372 from our statistical model (model 7: with time of day as covariate $\chi^2_1 = 1.70$, $p = 0.192$, model
373 9: without time of day as covariate $\chi^2_1 = 15.09$, $p < 0.001$).

374 The quantification of parasites ingested by mosquitoes showed a significant positive
375 correlation with both haematin and time of day (model 10: $\chi^2_1 = 28.01$, $p < 0.0001$, $\chi^2_1 = 41.71$,
376 $p < 0.0001$ respectively). The quantity of parasite ingested by mosquito was highest at
377 midnight (00h) and lowest early in the morning (6h). Bird parasitaemia also had an impact on
378 the quantity of parasites ingested by females but only when the time of day was removed
379 from the statistical model (model 10: with time of day as covariate $\chi^2_1 = 0.12$, $p = 0.727$, model
380 11: without time of day as covariate $\chi^2_1 = 3.62$, $p = 0.047$).

381 In the chronic stage of the infection, mosquito infection prevalence varied throughout
382 the day (model 12: $\chi^2_1 = 6.98$, $p = 0.030$, **Fig. 3B**). Infection prevalence was 0% at noon, 7%
383 (mean \pm s.e: 7.1 ± 4) at 18h and 22% (22.2 ± 7.1) at 00h (no data available for 6h, contrast
384 analyses: 12h/18h : $\chi^2_1 = 3.89$, $p = 0.049$, 12h/00h : $\chi^2_1 = 6.34$, $p = 0.012$, 18h/00h : $\chi^2_1 = 3.91$,
385 $p = 0.048$). However, oocyst numbers were too low (all infected females had a single oocyst)
386 to detect any effect of time of day on parasite burden. Bird parasitaemia and blood meal size
387 (haematin) had no impact on mosquito infection prevalence (model 12: with time of day as
388 covariate $\chi^2_1 = 0.58$, $p = 0.447$, model 13: without time of day as covariate $\chi^2_1 = 1.64$, $p = 0.201$,
389 model 12: with time of day as covariate $\chi^2_1 = 0.64$, $p = 0.725$, model 13: without time of day as
390 covariate $\chi^2_1 = 0.17$, $p = 0.679$ respectively).

391

392 **Daily fluctuations of mosquito activity**

393 Mosquito activity was significantly impacted by the time of day (model 14: $\chi^2_1 = 204.15$,
394 $p < 0.0001$, **Fig. 3C**). Overall, the activity of vectors was higher in the evening (18h, 00h) than

395 in the morning (6h, 12h). The maximal activity was observed at dusk (contrast analyses:
396 18h/00h: $\chi^2_1 = 28.78$, $p < 0.0001$, 18h/12H: $\chi^2_1 = 148.89$, $p < 0.0001$, 18h/6H: $\chi^2_1 = 166.48$, $p <$
397 0.0001, **Fig. 3C**) and the minimal activity at dawn (contrast analyses: 6h/12h: $\chi^2_1 = 4.09$, $p =$
398 0.026, 6h/00h: $\chi^2_1 = 38.90$, $p < 0.0001$, **Fig. 3C**). Interestingly, these daily variations in mosquito
399 activity were positively correlated with both bird parasitaemia and parasite transmission to
400 mosquito in acute (**Fig. 4A**) but also in chronic stage of infection (**Fig. 4B**).

401

402 **DISCUSSION**

403 Temporal fluctuations of the activity of mosquito vectors have profound consequences
404 on malaria transmission (Barrozo *et al.* 2004; Lalubin *et al.* 2013). Here we argue that
405 *Plasmodium* parasites have evolved two different and complementary transmission strategies
406 to cope with these variations of their environment: a constitutive time-varying strategy that
407 generates a covariance between parasite investment in transmission and vector activity and
408 a plastic, fast acting, strategy that allows the parasite to react rapidly to the presence of
409 mosquitoes.

410 First, our theoretical model indicates that fast and predictable oscillations in mosquito
411 activity can select for a constitutive time-varying strategy in the parasite, provided this
412 strategy generates a positive covariance between the activity of the vector and the parasite's
413 investment in transmission (see equation (6)). Our experimental results show both that the
414 activity of *Culex* mosquitoes oscillates throughout the day in a predictable way (**Fig. 3C**) but
415 also, that these daily fluctuations of mosquito activity are matched with periodic fluctuations
416 in malaria transmission during both phases of the infection (*i.e.* acute and chronic, **Fig. 4**). This
417 positive covariance supports the "Hawking hypothesis" and the idea that this time-varying
418 transmission may result from an adaptation of the pathogen.

419 Second, our experiment demonstrates the existence of plastic transmission strategies
420 enabling avian malaria parasites to respond to mosquito bites. In a previous study, we showed
421 that mosquito bites stimulate within-host growth and investment in transmission during the
422 chronic phase of *Plasmodium relictum* infections (Cornet *et al.* 2014). In the present study, we
423 obtain a similar effect in the chronic but also in the acute phase of the infection. This plastic
424 transmission strategy is expected to evolve when variations in the abundance of their
425 mosquito vectors are less predictable (Cornet *et al.* 2014; Reece & Mideo 2014). During the
426 chronic phase of the infection, such plastic transmission strategies may allow the parasite to
427 react to the seasonal variations in mosquito abundance and to reactivate its transmission
428 when mosquitoes are around (Cornet *et al.* 2014; Reece & Mideo 2014). During the acute
429 phase of the infection, this strategy may also allow the parasite to respond to unexpected
430 variations in the abundance of mosquitoes driven by stochastic processes such as variations
431 in temperature and humidity (Yamana & Eltahir 2013).

432 In spite of the match between these theoretical predictions and our experimental
433 results, our adaptive hypothesis is challenged by alternative explanations for the existence of
434 periodic variations in parasitaemia and mosquito infection. Several studies suggest that the
435 dynamics of the infectivity of *Plasmodium* might not be underpinned by the feeding activity
436 cycle of its vector but induced by the vertebrate immunity (see Mideo *et al.* 2013), whose
437 activity is known to vary during the day (Scheiermann *et al.* 2013; Curtis *et al.* 2014). This
438 variation may alter the number and/or the infectiousness of gametocytes and explain (at least
439 partly) the increase of transmissibility during the evening. It would be interesting to monitor
440 whether the efficacy of the birds' immune system to fight against a *Plasmodium* infection
441 fluctuates throughout the day, and to evaluate its potential effect on the transmissibility of
442 avian malaria.

443 In addition, the increase in mosquito infection may also be explained by physiological
444 cycles in the vector. Daily cycles in the production of immune compounds (Rund *et al.* 2016;
445 Tsoumtsa *et al.* 2016) or molecules (e.g. nutrients) used by *Plasmodium* (Carter *et al.* 2007;
446 Dinglasan *et al.* 2007) may impact the viability of ookinetes or their ability to invade the midgut
447 epithelia. One way to quantify this effect would be to perform similar experiments with
448 vectors with the circadian rhythm experimentally inverted (jet-lagged). Reversed patterns of
449 time-varying infectivity in jet-lagged and control mosquitoes would indicate a strong effect of
450 the circadian rhythm of the insect vector. In contrast, if both jet-lagged and control
451 mosquitoes exhibit similar patterns of infection this would indicate that the infectivity is under
452 the parasite's control and would support the "Hawking hypothesis".

453 The most efficient way to demonstrate unequivocally the adaptive nature of these
454 time-varying transmission strategies would be to perform experimental evolution (Johnson
455 2005). For instance, does the parasite lose its ability to react to mosquito bites if the parasite
456 is always transmitted from bird to bird by intraperitoneal injection (Pigeault *et al.* 2015)? Could
457 the parasite be made to evolve other patterns of daily investment in transmission if the
458 mosquitoes are allowed to feed on birds at very specific time of the day? Avian malaria
459 provides a perfect experimental system to carry out such experiments. Earlier studies have
460 observed a great degree of variation in the period and in the phase of the fluctuations of
461 within-bird dynamics. For instance, *P. circumflexum* has a periodicity of 48h peaking in the late
462 afternoon, while *P. elongatum*'s periodicity is 24h and peaks in the early morning (see Hewitt
463 1940 for a review). Besides, the amplitude of the fluctuations of parasitaemia reported in
464 some of these earlier experimental studies is orders of magnitude higher than the one we
465 observed in the present study (Taliaferro 1925, Huff & Bloom 1935, Hewitt 1940). What
466 factors explain the maintenance of such a large amount of natural variation? Additional

467 experimental studies using different avian *Plasmodium* lineages would yield unique
468 perspectives on the adaptive nature of the rhythmicity of malaria within-host dynamics. The
469 genomic analysis of evolved lines would also yield new candidate genes governing these key
470 adaptations. This deeper understanding of malaria transmission may thus yield practical
471 implications for the control of human malaria parasites.

472 **Data archiving:** Data for this study will be made available once the manuscript accepted for
473 publication (Dryad website)

474 **Authors' contributions:** Conceived and designed the experiments: RP AR SG. Performed the
475 experiments: RP AN. Analysed the data: RP. Developed and analysed the theoretical model:
476 SG. Developed the new statistical methodology to study daily fluctuations of parasitaemia:
477 QC. Wrote the paper: RP AR SG. All authors read and commented the paper and approved the
478 final version of the manuscript.

479 **LITERATURE CITED**

480 Alano, P. (2007) *Plasmodium falciparum* gametocytes: still many secrets of a hidden life. *Mol.*
481 *Microbiol.* 66 : 291–302.

482 Barrozo, R.B., Schilman, P.E., Minoli, S.A. & Lazzari, C.R. (2004). Daily rhythms in disease-vector
483 insects. *Biol. Rhythm Res.* 35:79–92.

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

485 Bousema, T. & Drakeley, C. (2011). Epidemiology and infectivity of *Plasmodium falciparum*
486 and *Plasmodium vivax* gametocytes in relation to malaria control and elimination. *Clin.*
487 *Microbiol. Rev.* 24:377–410.

488 Carter, V., Nacer, A.M.L., Underhill, A., Sinden, R.E. & Hurd, H. (2007). Minimum requirements
489 for ookinete to oocyst transformation in *Plasmodium*. *Int. J. Parasitol.* 37:1221–1232.

490 Corder, K.R., DeMoranville, K.J., Russell, D.E., Huss, J.M. & Schaeffer, P.J. (2016). Annual life-
491 stage regulation of lipid metabolism and storage and association with PPARs in a
492 migrant species: the gray catbird (*Dumetella carolinensis*). *J. Exp. Biol.* 219:3391–3398.

493 Cornet, S., Nicot, A., Rivero, A. & Gandon, S. (2013). Malaria infection increases bird
494 attractiveness to uninfected mosquitoes. *Ecol. Lett.* 16:323–329.

495 Cornet, S., Nicot, A., Rivero, A. & Gandon, S. (2014). Evolution of plastic transmission Strategies
496 in avian malaria. *PLoS Pathog.* 10:e1004308.

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

498 Curtis, A.M., Bellet, M.M., Sassone-Corsi, P. & O'Neill, L.A.J. (2014). Circadian clock proteins
499 and immunity. *Immunity* 40:178–186.

500 Dinglasan, R.R., Alagangan, A., Ghosh, A.K., Saito, A., Kuppevelt, T.H. van & Jacobs-Lorena, M.
501 (2007). *Plasmodium falciparum* ookinetes require mosquito midgut chondroitin
502 sulfate proteoglycans for cell invasion. *Proc. Natl. Acad. Sci.* 104:15882–15887.

503 Duboscq, J., Romano, V., Sueur, C. & MacIntosh, A.J.J. (2016). Network centrality and
504 seasonality interact to predict lice load in a social primate. *Sci. Rep.*, 6.

505 Edgar, R.S., Stangerlin, A., Nagy, A.D., Nicoll, M.P., Efstathiou, S., O'Neill, J.S., et al. (2016).
506 Cell autonomous regulation of herpes and influenza virus infection by the circadian
507 clock. *Proc. Natl. Acad. Sci.* 113:10085–10090.

508 Gambrell, W.E. (1937). Variations in gametocyte production in avian malaria. *Am. J. Trop. Med.*
509 *Hyg.* s1-17:689–727.

510 Garnham, P.C. & Powers, K.G. (1974). Periodicity of infectivity of plasmodial gametocytes: the
511 “Hawking phenomenon.” *Int. J. Parasitol.* 4:103–106.

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

514 Hawking, F. (1970). The clock of the malaria parasite. *Sci. Am.* 222:123–131.

515 Hawking, F. (1975). Circadian and other rhythms of parasites. *Adv. Parasitol.* 13:123–182.

516 Hawking, F., Worms, M.J., Gammie, K. & Goddard, P.A. (1966). The biological purpose of the
517 blood-cycle of the malaria parasite *Plasmodium cynomolgi*. *The Lancet*, Originally
518 published as Volume 2, Issue 7460, 288:422–424.

519 Hewitt, R.J. (1940) *Bird Malaria. The American journal of hygiene*. Baltimore: The Johns
520 Hopkins Press.

521 Huff, C.G. & Bloom, W. (1935) A malarial parasite infecting all blood and blood-forming cells
522 of birds. *J. Infect. Dis.* 57: 315–336.

523 Johnson, C.H. (2005). Testing the adaptive value of circadian systems. In: *Methods in*
524 *Enzymology, Circadian Rhythms* (ed. Young, M.W.). Academic Press, pp. 818–837.

525 Kussell, E. & Leibler, S. (2005). Phenotypic diversity, population growth, and information in
526 fluctuating environments. *Science* 309:2075–2078.

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

530 Lawaly, R., Konate, L., Marrama, L., Dia, I., Diallo, D., Sarr, F.D., *et al.* (2012). Impact of
531 mosquito bites on asexual parasite density and gametocyte prevalence in
532 asymptomatic chronic *Plasmodium falciparum* infections and correlation with IgE and
533 IgG Titers. *Infect. Immun.* 80:2240–2246.

534 Martinaud, G., Billaudelle, M. & Moreau, J. (2009). Circadian variation in shedding of the
535 oocysts of *Isospora turdi* (*Apicomplexa*) in blackbirds (*Turdus merula*): An adaptative
536 trait against desiccation and ultraviolet radiation. *Int. J. Parasitol.* 39:735–739.

537 Martinez-Bakker, M. & Helm, B. (2015). The influence of biological rhythms on host–parasite
538 interactions. *Trends Ecol. Evol.* 30:314–326.

539 Mideo, N., Reece, S.E., Smith, A.L. & Metcalf, C.J.E. (2013). The Cinderella syndrome: why do
540 malaria-infected cells burst at midnight? *Trends Parasitol.* 29:10–16.

541 Moulia-Pelat, J.P., Glaziou, P., Chanteau, S., Nguyen-Ngoc, L., Marcet, Y., Gardines, R., *et al.*
542 (1993). Periodicity of *Wuchereria bancrofti* var. *pacifica filariasis* in French Polynesia.
543 *Trop. Med. Parasitol. Off. Organ Dtsch. Tropenmedizinische Ges. Dtsch. Ges. Tech.*
544 *Zusammenarbeit GTZ* 44:83–85.

545 Pigeault, R., Vézilier, J., Cornet, S., Zélé, F., Nicot, A., Perret, P., *et al.* (2015). Avian malaria: a
546 new lease of life for an old experimental model to study the evolutionary ecology of
547 *Plasmodium*. *Phil Trans R Soc B* 370: 20140300.

548 Reece, S.E. & Mideo, N. (2014). Malaria parasites prepare for flight. *Trends Parasitol.* 30:551–
549 553.

550 Rijo-Ferreira, F., Pinto-Neves, D., Barbosa-Morais, N.L., Takahashi, J.S. & Figueiredo, L.M.
551 (2017a). *Trypanosoma brucei* metabolism is under circadian control. *Nat. Microbiol.*
552 2:17032.

553 Rijo-Ferreira, F., Takahashi, J.S. & Figueiredo, L.M. (2017b). Circadian rhythms in parasites.
554 *PLOS Pathog.*, 13, e1006590.

555 Rund, S.S.C., O'Donnell, A.J., Gentile, J.E. & Reece, S.E. (2016). Daily rhythms in mosquitoes
556 and their consequences for malaria transmission. *Insects* 7:14.

557 Scheiermann, C., Kunisaki, Y. & Frenette, P.S. (2013). Circadian control of the immune system.
558 *Nat. Rev. Immunol.* 13:190–198.

559 Sinden, R.E. (2015). The cell biology of malaria infection of mosquito: advances and
560 opportunities. *Cell. Microbiol.* 17:451–466.

561 Smaaland, R., Sothern, R.B., Laerum, O.D. & Abrahamsen, J.F. (2002). Rhythms in human bone
562 marrow and blood cells. *Chronobiol. Int.* 19:101–127.

563 Taliaferro, L.G. (1925) Periodicity of reproduction, infection and resistance in bird malaria.
564 *Proc. Natl. Acad. Sci.* 11:348–352.

565 Thaiss, C.A., Levy, M. & Elinav, E. (2015). Chronobiomics: the biological clock as a new principle
566 in host–microbial interactions. *PLOS Pathog.* 11:e1005113.

567 Tsoumpta, L.L., Torre, C. & Ghigo, E. (2016). Circadian control of antibacterial immunity:
568 findings from animal models. *Front. Cell. Infect. Microbiol.* 6.

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

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

573 Yamana, T.K. & Eltahir, E.A.B. (2013). Incorporating the effects of humidity in a mechanistic
574 model of *Anopheles gambiae* mosquito population dynamics in the Sahel region of
575 Africa. *Parasit. Vectors* 6:235.

576

577 **FIGURE LEGENDS**

578 **Figure 1: Overview of the experiment with the 2 experimental sessions (grey areas).** (A)
579 Mean parasitaemia ($\text{Log}(1 + \text{parasitaemia})$), measured at noon, across time post-infection for
580 “unexposed” birds (control). The variation of the parasitaemia among birds is indicated with
581 the shaded envelope (standard error). The dashed boxes represent the two experimental
582 sessions performed in acute (12–14 day post-infection) and in chronic stage (61–64 day post-
583 infection) of infection. In each session, the grey areas correspond to the day at which birds
584 (“exposed”) were exposed to mosquito bites (day 13 post-infection in acute and day 62 post-

585 infection in chronic stage of infection). (B) Zoom on the days where the birds were exposed to
586 mosquito bites. The grey shaded area on x-axis represents the night period. Arrows indicate
587 the time of day at which birds were exposed to mosquito bites. Mosquito exposure were
588 carried out straight after each of the blood sampling events (at 6h, 12h, 18h and 00h).

589

590 **Figure 2: Within-host dynamics of blood parasitaemia (mean \pm se) of *Plasmodium relictum***
591 **in birds.** The dynamics in birds exposed or unexposed to mosquito bites is represented in red
592 and blue, respectively. Mosquito exposure took place (A) day 13 (at 6AM, 12AM, 6PM and
593 12PM) and (B) day 62 (at 6AM, 12AM, 6PM and 12PM) post-infection.

594

595 **Figure 3: Timing of malaria within-host dynamics and mosquito activity in avian malaria.** (A)
596 Daily fluctuations of *Plasmodium* transmission in acute phase of infection (session 1: day 13
597 post-infection, see **Fig. 1**). Boxplot represent the blood parasitaemia ($\text{Log}(1 + \text{parasitaemia})$)
598 of the exposed birds measured at 6h, 12h, 18h and 00h, 13 days after the infection by
599 *Plasmodium*. The red points represent the distribution of the number of oocysts in the midgut
600 of *Plasmodium*-infected females 7 days after the blood meal. Blood meals were taken on the
601 birds whose parasitaemia is described by the boxplots. (B) Daily fluctuations of *Plasmodium*
602 transmission in chronic phase of infection (session 2: day 62 post-infection, see **Fig. 1**). Boxplot
603 represent the blood parasitaemia ($\text{Log}(1 + \text{Relative Quantification values})$) of the exposed
604 birds measured at 6h, 12h, 18h and 00h, 62 days after the infection by *Plasmodium*. The red
605 points represent the prevalence of *Plasmodium* infection in females 7 days after the blood
606 meal. Blood meals were taken on the birds whose parasitaemia is described by the boxplots.
607 (C) Daily fluctuations of mosquito activity. From the survival analyses (see Materials and

608 Methods), the constant hazard rate for each treatment (time of day) and the standard errors
609 were calculated. Levels not connected by same letter are significantly different.

610

611 **Figure 4: Testing the “Hawking hypothesis” in avian malaria.** (A) Correlation between
612 mosquito activity (constant hazard rate estimated from model 14, **TableS1**) and bird
613 parasitaemia ($\log(1+\text{parasitaemia})$, in grey) and parasite transmission to mosquito (infection
614 intensity: oocyst burden, in red) in acute stage of infection. (B) Correlation between
615 mosquito activity (constant hazard rate estimated from model 14, **TableS1**) and bird
616 parasitaemia ($\log(1 + \text{Relative Quantification values})$, in grey) and parasite transmission to
617 mosquito (infection prevalence (%), in red) in chronic stage of infection.

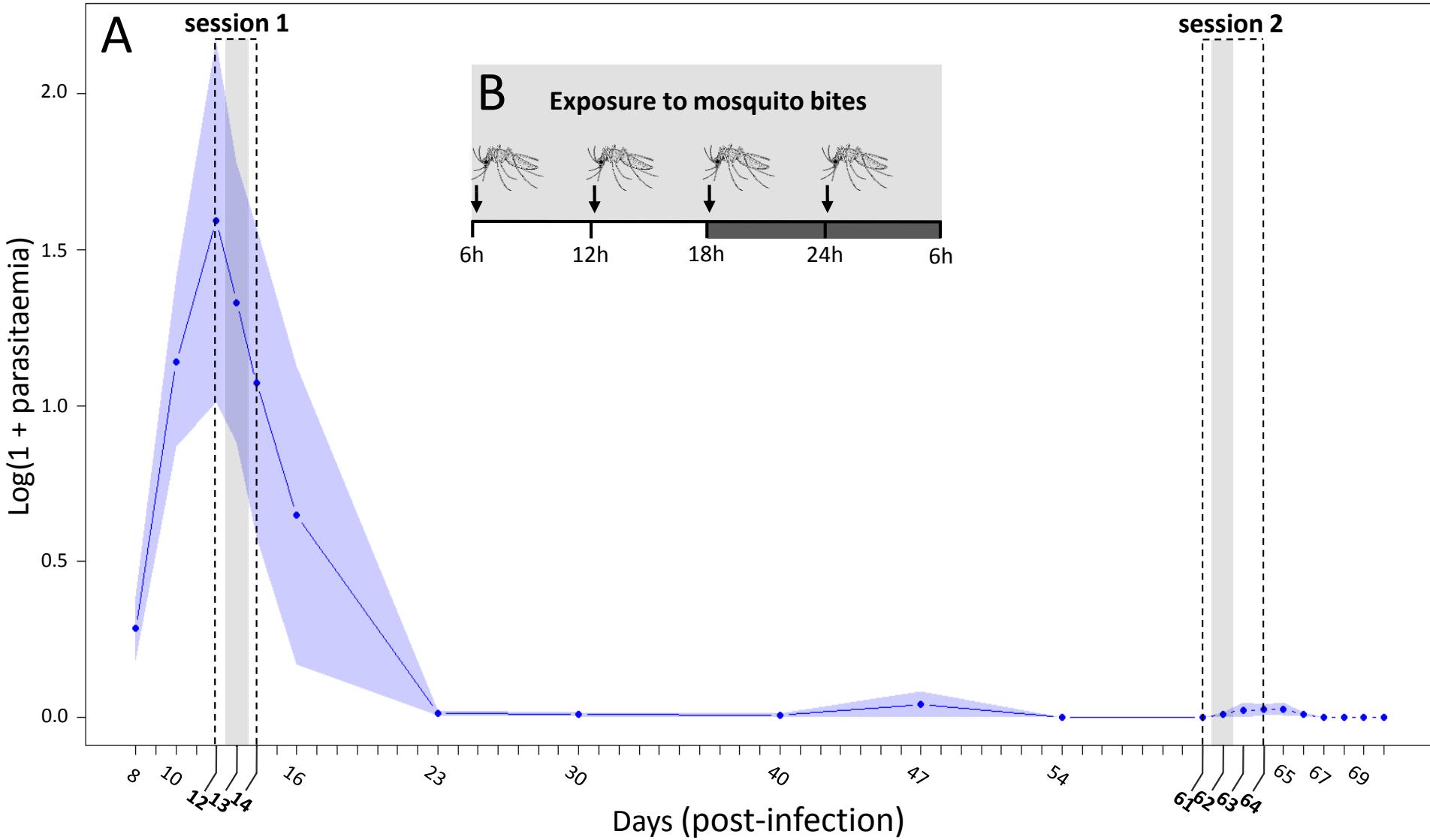


Figure 1: Overview of the experiment with the 2 experimental sessions (grey areas). (A) Mean parasitaemia ($\text{Log}(1 + \text{parasitaemia})$), measured at noon, across time post-infection for “unexposed” birds (control). The variation of the parasitaemia among birds is indicated with the shaded envelope (standard error). The dashed boxes represent the two experimental sessions performed in acute (12-14 day post-infection) and in chronic stage (61-64 day post-infection) of infection. In each session, the grey areas correspond to the day at which birds (“exposed”) were exposed to mosquito bites (day 13 post-infection in acute and day 62 post-infection in chronic stage of infection). (B) Zoom on the days where the birds were exposed to mosquito bites. The grey shaded area on x-axis represents the night period. Arrows indicate the time of day at which birds were exposed to mosquito bites. Mosquito exposure were carried out straight after each of the blood sampling events (at 6h, 12h, 18h and 00h).

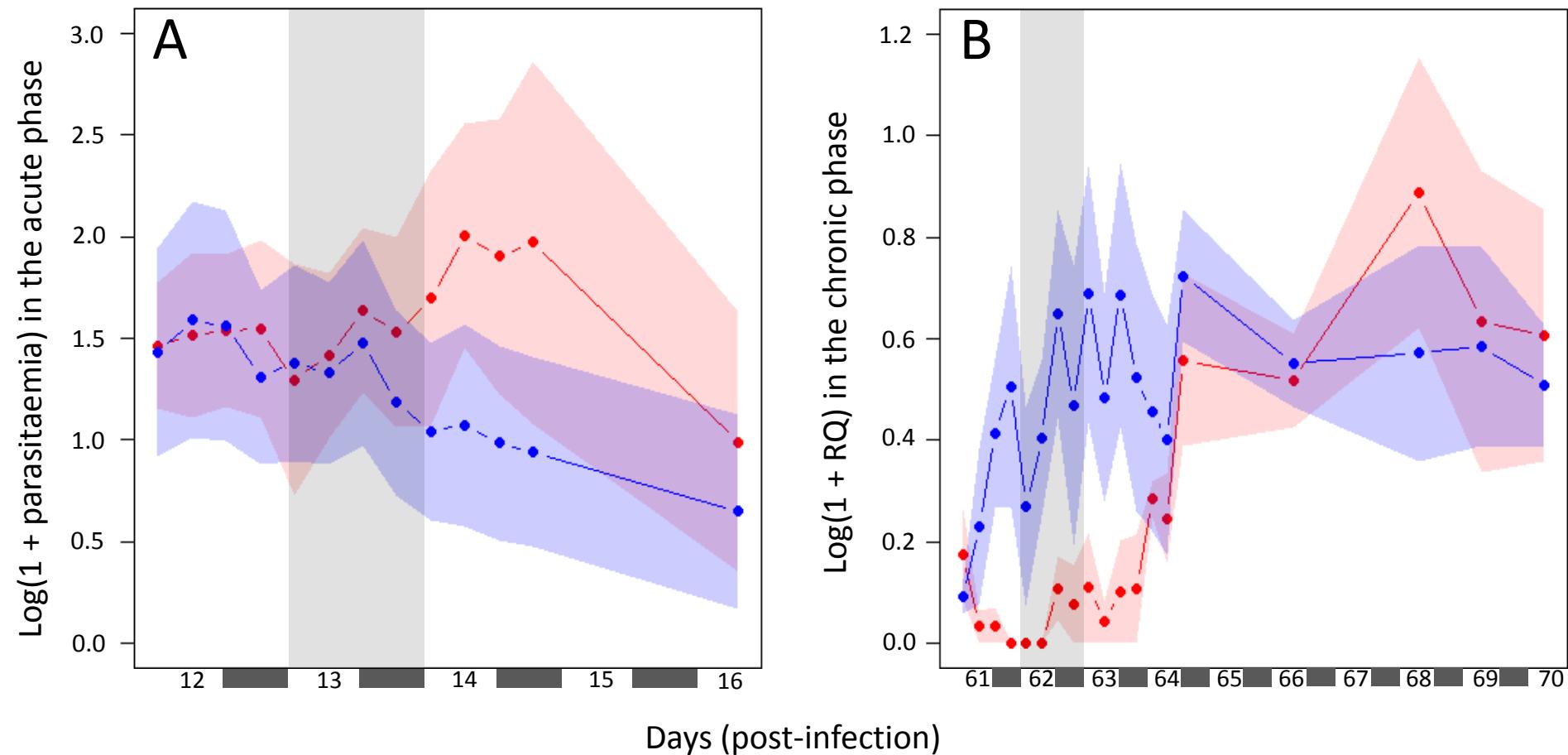


Figure 2: Within-host dynamics of blood parasitaemia (mean \pm se) of *Plasmodium relictum* in birds. The dynamics in birds exposed or unexposed to mosquito bites is represented in red and blue, respectively. Mosquito exposure took place (A) day 13 (at 6AM, 12AM, 6PM and 12PM) and (B) day 62 (at 6AM, 12AM, 6PM and 12PM) post-infection.

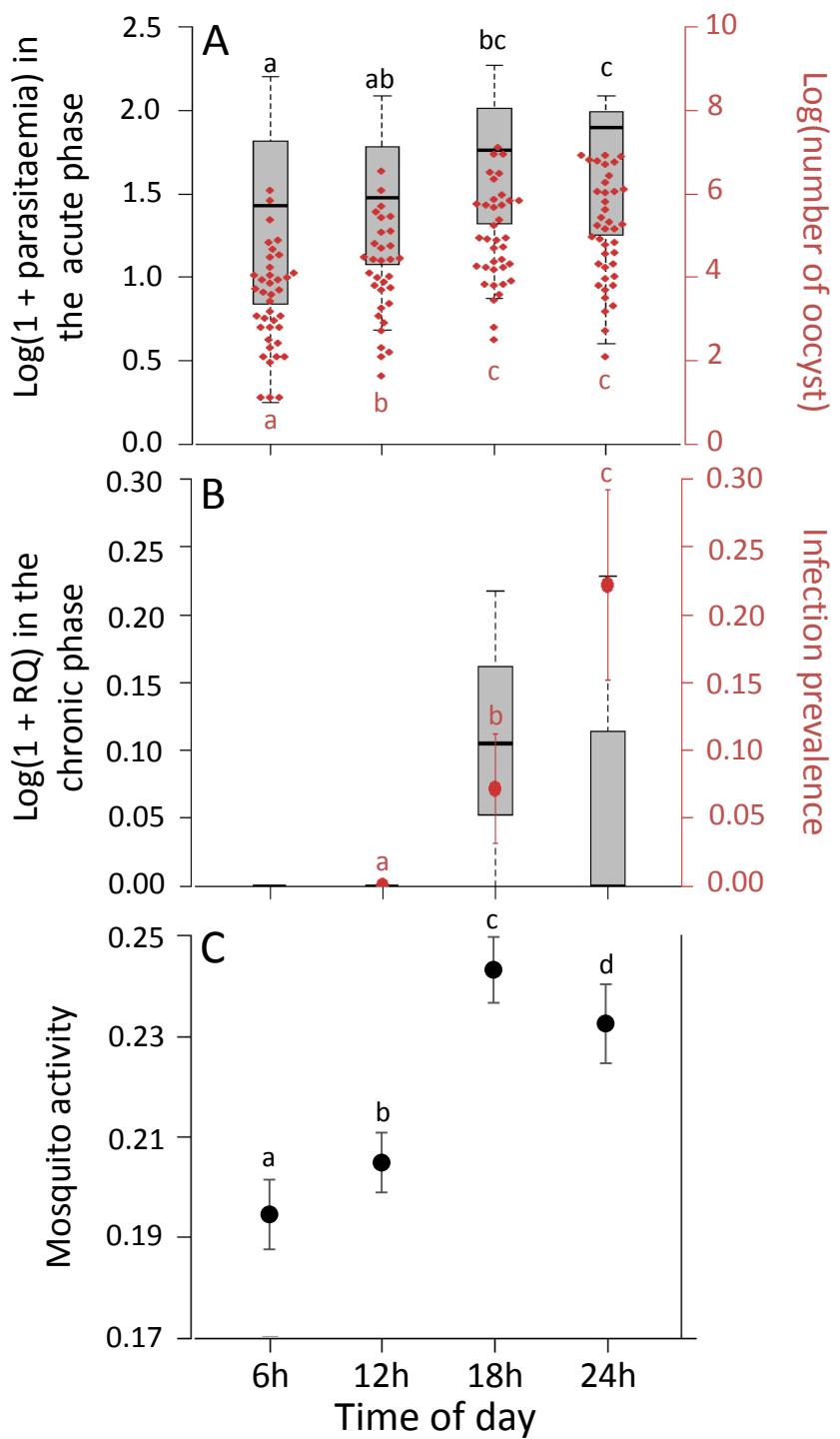


Figure 3: Timing of malaria within-host dynamics and mosquito activity in avian malaria. (A) Daily fluctuations of *Plasmodium* transmission in acute phase of infection (session 1: day 13 post-infection, see **Fig. 1**). Boxplot represent the blood parasitaemia (Log(1 + parasitaemia)) of the exposed birds measured at 6h, 12h, 18h and 00h, 13 days after the infection by *Plasmodium*. The red points represent the distribution of the number of oocysts in the midgut of *Plasmodium*-infected females 7 days after the blood meal. Blood meals were taken on the birds whose parasitaemia is described by the boxplots. (B) Daily fluctuations of *Plasmodium* transmission in chronic phase of infection (session 2: day 62 post-infection, see **Fig. 1**). Boxplot represent the blood parasitaemia (Log(1 + Relative Quantification values)) of the exposed birds measured at 6h, 12h, 18h and 00h, 62 days after the infection by *Plasmodium*. The red points represent the prevalence of *Plasmodium* infection in females 7 days after the blood meal. Blood meals were taken on the birds whose parasitaemia is described by the boxplots. (C) Daily fluctuations of mosquito activity. From the survival analyses (see Materials and Methods), the constant hazard rate for each treatment (time of day) and the standard errors were calculated. Levels not connected by same letter are significantly different.

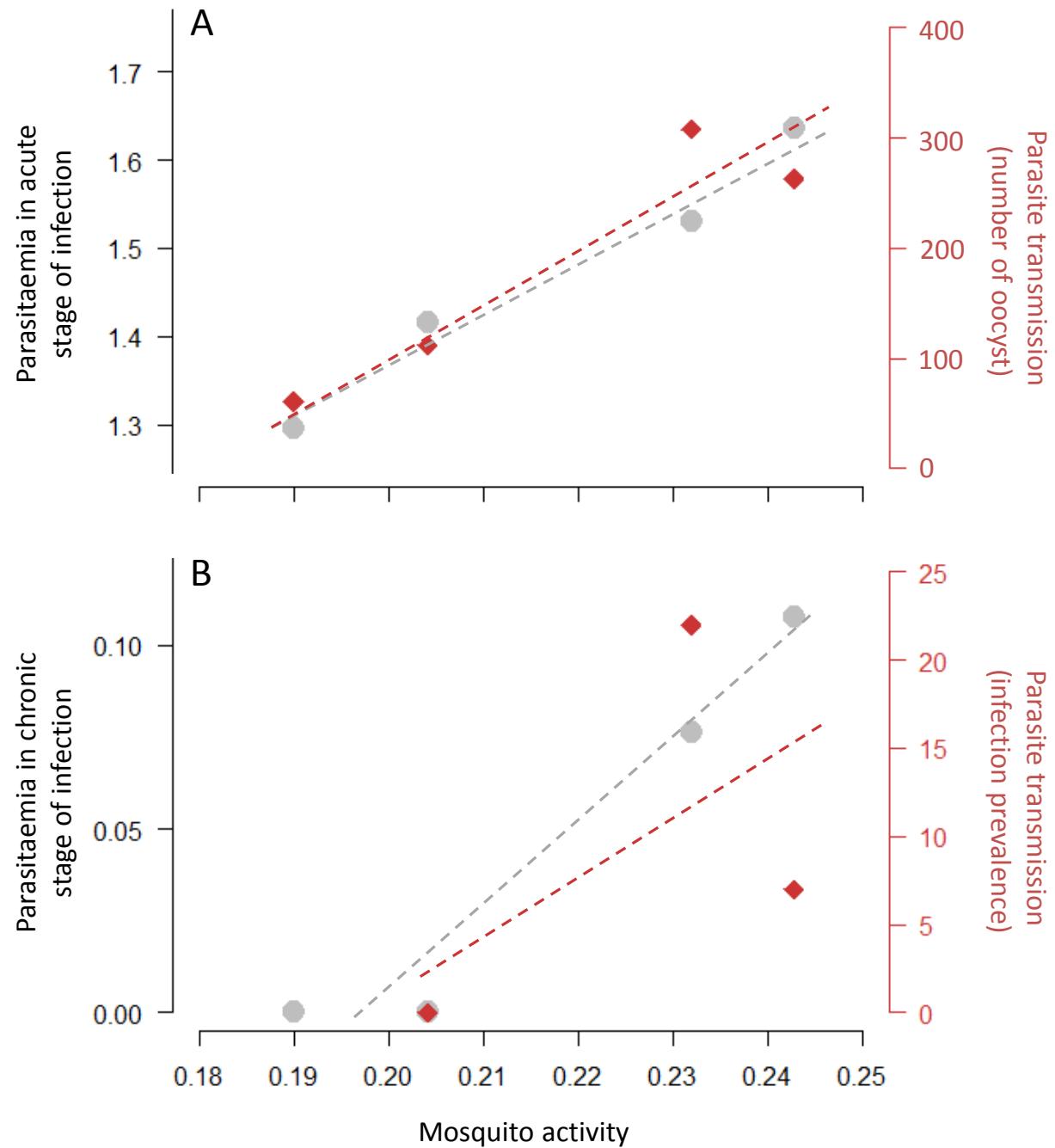


Figure 4: Testing the “Hawking hypothesis” in avian malaria. (A) Correlation between mosquito activity (constant hazard rate estimated from model 14, **TableS1**) and bird parasitaemia ($\log(1+\text{parasitaemia})$, in grey) and parasite transmission to mosquito (infection intensity: oocyst burden, in red) in acute stage of infection. (B) Correlation between mosquito activity (constant hazard rate estimated from model 14, **TableS1**) and bird parasitaemia ($\log(1 + \text{Relative Quantification values})$, in grey) and parasite transmission to mosquito (infection prevalence (%), in red) in chronic stage of infection.