Can malaria parasites manipulate the odour-mediated host preference of their mosquito vectors?

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Abstract

Malaria parasites can manipulate mosquito feeding behaviours such as motivation and avidity to feed on vertebrate hosts in ways that increase parasite transmission. However, in natural conditions, not all vertebrate blood-sources are suitable hosts for the parasite. Whether malaria parasites can manipulate mosquito host choice in ways that enhance parasite transmission toward suitable hosts and/or reduce mosquito attraction to unsuitable hosts (i.e. specific manipulation) is unknown. To address this question, we experimentally infected three species of mosquito vectors (*Anopheles coluzzii*, *Anopheles gambiae*, and *Anopheles arabiensis*) with wild isolates of the human malaria parasite *Plasmodium falciparum*, and examined the effects of immature (oocyst) and mature (sporozoite) infections on mosquito behavioural responses (activation rate and odour choice) to combinations of calf odour, human odour and outdoor air using a dual-port olfactometer. Regardless of parasite developmental stage and mosquito species, *P. falciparum* infection did not alter mosquito activation rate or their choice for human odours. The overall expression pattern of host choice of all three mosquito species was consistent with a high degree of anthropophily, with both infected and uninfected individuals showing higher attraction toward human odour over calf odour, human odour over outdoor air, and outdoor air over calf odour. Our results suggests that, in this system, the parasite may not be able to manipulate the early long-range behavioural steps involved in the mosquito host-feeding process, including initiation of host-seeking and host orientation. Future studies examining mosquito host-feeding behaviours at a shorter range (i.e. the "at-host" foraging activities) are required to test whether malaria parasites can modify their mosquito host choice to enhance transmission toward suitable hosts and/or reduce biting on unsuitable hosts.

Keywords: *Anopheles*, experimental infection, host manipulation by parasites, host-seeking behaviours, olfactometer, *Plasmodium*.

1. Introduction

Host behavioural manipulation by parasites is a widespread transmission strategy (Moore, 2002; Lefèvre et al., 2009a; Poulin, 2010; Hughes et al. 2012). Trophically-transmitted parasites, for example, can alter the behaviour of their intermediate hosts in ways that increase predation rate by definitive hosts, hence favouring transmission (Lafferty and Morris, 1996; Lagrue et al., 2007; Lagrue et al., 2013). However, altering the behaviour of intermediate hosts can also increase predation rates by unsuitable hosts (Mouritsen and Poulin, 2003; Kaldonski et al., 2008; Seppälä et al., 2008). This higher probability of being killed by dead-end predators can incur significant costs to manipulative parasites, especially when initial predation risk is high (Seppälä and Jokela, 2008). In response, some parasites have evolved specific manipulation, i.e. the ability to enhance transmission toward appropriate hosts and/or reduce predation by unsuitable hosts (Lagrue et al., 2007; Médoc and Beisel, 2009; Médoc et al. , 2009; Seppälä et al., 2012; Lagrue et al., 2013; Jacquin et al., 2014).

In addition to its ecological and evolutionary relevance, host manipulation may also have profound implications for human health. Many manipulative parasites are responsible for devastating vector-borne diseases such as dengue fever, malaria, leishmaniasis, or sleeping sickness. Vector-borne parasites can indeed manipulate phenotypic traits of their vectors and hosts in ways that increase contacts between them, hence favouring parasite transmission (Hurd, 2003; Lacroix et al., 2005; Lefèvre and Thomas, 2008; Cator et al., 2012; Cornet et al., 2013a; De Moraes et al., 2014; Caljon et al., 2016). A frequently reported change induced by vector-borne parasites is alteration of vector motivation and avidity to feed. For example in malaria mosquitoes, individuals infected with *Plasmodium* sporozoites (the mosquito to human transmission stages) can display increased response to host odours (Rossignol et al., 1986; Cator et al., 2013), increased landing and biting activity (Rossignol et al., 1984, 1986;

78 Wekesa et al. 1992; Anderson et al. 1999; Koella et al., 2002; Smallegange et al., 2013;),
79 increased number of feeds (Koella et al., 1998) and increased blood volume intake (Koella
80 and Packer, 1996; Koella et al., 1998; Koella et al. 2002). In contrast, mosquitoes infected
81 with oocysts (the immature non-transmissible stage of the parasite), are less persistent and
82 less likely to attempt to feed (Anderson et al., 1999; Koella et al., 2002; Cator et al., 2013).
83 Since biting is risky (e.g., host defensive behaviours can kill the vector and its parasite),
84 reduced feeding attempts seems beneficial to the parasite (Schwartz and Koella, 2001).
85 In natural conditions, these "stage-dependent" behavioural changes presumably
86 increase the rate at which a mosquito will feed on a vertebrate blood-source, not all of which
87 are suitable hosts for the parasite. The very few epidemiological models that have considered
88 mosquito behavioural manipulation by malaria parasites have assumed that suitable hosts for
89 parasite development were the only source of blood (Dobson, 1988; Cator et al., 2014). These
90 models have ignored the possibility that malaria parasites may increase biting rate on
91 vertebrates that do not act as suitable hosts. Similarly, no study has, to our knowledge,
92 investigated whether malaria parasites can manipulate mosquito vertebrate choice in ways that
93 enhance parasite transmission toward suitable hosts and/or reduce mosquito attraction to
94 unsuitable hosts (i.e. specific manipulation).
95 Mosquito choice for vertebrate blood-source is an important key predictor for the
96 transmission intensity of vector-borne diseases. This choice may be influenced by genetic and
97 environmental factors such as the innate preference of the mosquito and the availability of the
98 vertebrate species (Lyimo and Ferguson, 2009). While some malaria vectors can display
99 propensity to feed on different vertebrate species (i.e. generalist or opportunistic feeding
100 behaviour) (Takken and Verhulst, 2013), the parasites they transmit are often highly host-
101 specific, infecting only one or a few vertebrate species (Perkins, 2014). Because of this strong
102 host specificity, it is possible that vector-borne parasites acquired, during the course of

103 evolution, the ability to target appropriate host and/or avoid unsuitable ones (Lefèvre et al.,
104 2006). Accordingly, generalist mosquitoes, once infected, should develop a feeding
105 preference for vertebrates that are suitable for parasite development. Studies exploring this
106 possibility may yield important information about the diversity of transmission strategies used
107 by malaria parasites and show that mosquitoes infected with transmissible parasite stages not
108 only bite "more" but perhaps also "better".

109 Theoretically, there are several ways through which malaria parasites could maximise
110 transmission towards suitable vertebrate hosts. First, the parasite may induce in the mosquito
111 vector a sensory bias for host traits (e.g. specific odours) that are correlated with optimal
112 suitability for the parasite. Second, the parasite may induce alteration of mosquito
113 microhabitat choice, in a way that spatially matches the microhabitat of the suitable host
114 species. Finally, the parasite may induce changes in time activity in a way that temporally
115 matches the resting time of the suitable host.

116 Here, we explored the first possibility using the natural association between
117 *Plasmodium falciparum*, which causes the most severe form of human malaria, the mosquito
118 species *Anopheles coluzzii*, *Anopheles gambiae* and *Anopheles arabiensis*, three major vectors
119 of *P. falciparum* in Africa, and calves and human, two common mosquito vertebrate hosts. *An.*
120 *coluzzii* and *An. gambiae* are considered anthropophilic (they are attracted to human stimuli)
121 throughout its distribution, whereas *An. arabiensis* can display a weak host tropism
122 (plastic/opportunistic) and can display either anthropophilic or zoophilic preference
123 depending on the geographic area and the relative abundance of cattle and human (Costantini
124 et al., 1999; Takken and Verhulst, 2013). *P. falciparum* displays an extreme form of
125 specificity and can develop and reproduce in hominids only (predominantly in human and to a
126 lesser extent in chimpanzee, bonobo, and gorilla) (Prugnolle et al., 2011; Rayner et al., 2011;
127 Ngoubangoye et al., 2016), such that any mosquito bite on another vertebrate species, such as

cattle, would be a dead-end for the parasite. We experimentally challenged local colonies of three mosquito species with sympatric field isolates of *P. falciparum* using direct membrane feeding assays in Burkina Faso, and examined the effects of immature (oocyst) and mature (sporozoite) infections on mosquito choice between human and calf odours using a dual-port olfactometer.

2. Material and methods

2.1. Mosquitoes

Anopheles gambiae and An. coluzzii mosquitoes originated from outbred colonies established in 2008 and repeatedly replenished with F1 from wild-caught mosquito females collected in Soumousso (*An. gambiae*) (11°01'00"N, 4°02'59"W) and Kou Valley (*An. coluzzii*) (11°23'14"N, 4°24'42"W), south-western Burkina Faso (West Africa) and identified by PCR-RFLP (Fanello et al., 2002; Santolamazza et al., 2008). Mosquitoes were maintained under standard insectary conditions (27 ± 2°C, 70 ± 10% relative humidity, 12:12 LD). Larvae were bred in the laboratory with *ad libitum* Tetramin® and adult mosquitoes were provided with a solution of 5% glucose.

Anopheles arabiensis mosquitoes originated from wild caught larvae in Dioulassoba (11°10'42"N, 4°18'26"W), a district of Bobo Dioulasso, where previous surveys ensured that *An. arabiensis* population was dominant (Dabiré et al., 2012, 2014). Collection of field larvae was conducted three times (twice in October 2014 and once in November 2014). Larvae were reared under the same standard insectary conditions as the mosquito colonies and F0 females were used for the experiments. Species identification of 35 individual from each wild caught batch (a total of 105 mosquitoes) was performed to confirm that *An. arabiensis* was the dominant species (Fanello et al., 2002). Samples from the 1st and 2nd batches in October

151 contained 60% and 90.91% of *An. arabiensis* respectively; sample from the 3rd batch in
152 November contained 100% *An. arabiensis*.

153 **2.2. Experimental infections**

154 Experimental infections of mosquitoes were performed by membrane feeding of infectious
155 blood (DMFA for Direct Membrane feeding Assay) as described previously (Ouédraogo et al.,
156 2013; Vantaux et al., 2014; Vantaux et al., 2015; Roux et al., 2015; Hien et al., 2016). Briefly,
157 three- to five-days old females were fed through membranes on *P. falciparum* gametocyte
158 (the human to mosquito transmission stage) -infected blood from malaria patients in Burkina
159 Faso. Mosquitoes were starved of glucose solution for 12 h (*An. gambiae* and *An. arabiensis*)
160 or 24 h (*An. coluzzii*) prior to the infection. Gametocyte carriers were selected by examining
161 thick blood smears from children aged between 5 and 11 from two villages in southwestern
162 Burkina Faso (Dande and Soumousso, located 60km north and 40km southeast of Bobo
163 Dioulasso, respectively) and blood drawing was carried out at laboratory. For *An. gambiae*
164 and *An. arabiensis* mosquitoes, when gametocytemia was below 160 gametocytes/µl, blood
165 serum was replaced with European naive AB serum to limit potential effect of human
166 transmission blocking immunity and hence maximize the number of successfully infected
167 mosquitoes (Gouagna et al., 2004). Serum was not changed in *An. coluzzii* experiments. As a
168 negative control (uninfected mosquitoes), females were fed on the same blood in which
169 gametocytes were heat-inactivated. Parasite inactivation was performed by placing the
170 infectious blood in a thermo-mixer and heating at 43°C for 15 min and 900 rpm. This heat-
171 inactivation prevents from infectiousness of gametocytes and does not affect the blood
172 nutritive quality (Sangare et al., 2013). Mosquitoes exposed/unexposed to infection were
173 therefore fed with blood from the same individual, avoiding the potential confounding effects
174 of different blood origins on mosquito behaviours. Mosquito blood feeding was performed by

distributing three hundred µl of blood in membrane feeders maintained at 37°C by water jackets; cups containing 60-80 mosquitoes were placed under the feeders to allow blood engorgement through Parafilm® membranes for 2 hours. Fully blood-fed females were sorted out and placed in new cages (30x30x30 cm) where they had constant access to 5% glucose solution on cotton wool pads until the behavioural assays. A total of eight experimental replicates using 9 different gametocyte carriers were performed (see Table A1 for details).

2.3. Behavioural assays

A dual-choice olfactometer was used to study odour-mediated host choice by infected and uninfected mosquitoes (Lefèvre et al., 2009b; Lefèvre et al. 2010; Vantaux et al., 2015). *An. coluzzii* behavioural assays were carried out as in Vantaux et al. (2015). There was one slight modification in the set-up when used for *An. gambiae* and *An. arabiensis* assays for which the two tents were placed next to each other (Figure 1a). The olfactometer consisted of a source of two odours connected to two collecting boxes (30 x 30 x 40cm) linked by two glass tubes (L = 52cm, □ = 10cm) to a downwind box (L x l x h=60 x 40 x 40cm) (Figure 1a). Odour stimuli (from human, calf or outdoor air) came from two tents connected to the two collecting boxes of the olfactometer by air vent hoses (Scanpart®, D x L = 10 x 300 cm). Gauze was placed at the junction between the air vent hose and the collecting boxes to prevent mosquitoes entering the tent. A fan was set up at the junction of the air vent hose and the tent to draw air from the tent to the collecting box and the downwind box. The wind speed in the two downwind arms was regulated at 15cm/s (±2cm/s) using a 435-4 Testo multi-functional meter (Testor, Forbach, France) equipped with a hot wire probe (range: 0 to 20m/s, accuracy: ± (0.03m/s +5% of mv)). The two tents were left outdoor while the collecting boxes and the downwind box were located inside an experimental room (Figure 1a).

198 Mosquitoes were coloured with either red or yellow powders (Luminous Powder Kit,
199 BioQuip) corresponding to their exposure status (received either an infectious blood-meal or a
200 heat-inactivated uninfectious blood-meal) (Verhulst et al., 2013; Vantaux et al., 2015). The
201 matching between exposure status and colours was switched between each run within a test
202 day (Figure 1b). To increase mosquito response to host odours in the olfactometer set-up, the
203 three mosquito species were deprived from glucose solution for 10 hours prior to the
204 behavioural tests. During this period, mosquitoes had access to water only. For *An. gambiae*
205 and *An. arabiensis* assays, in a single test day, a maximum of 6 runs each lasting 30 minutes
206 were conducted using one mosquito species, and a total of 3 odour combinations: human *vs.*
207 calf odour (H-C), human *vs.* outdoor air (H-O) and calf *vs.* outdoor air (C-O) (Figure 1b). For
208 *An. coluzzii* experiments, only the human *vs.* calf odour (H-C) and human *vs.* outdoor air (H-
209 O) combinations were carried out. In a single test day, 4 runs each lasting 30 minutes were
210 carried out.

211 For each run, 20 uninfected controls and 20 gametocyte-exposed mosquitoes of
212 similar age from the same experimental infection were simultaneously released in the
213 downwind box of the apparatus. At the end of a run, the mosquitoes inside each of the two
214 collecting boxes and the downwind box were removed with an aspirator, counted and kept in
215 paper cups for subsequent analyzes (dissection of mosquito midgut and head/thorax, see
216 below). Each batch of mosquitoes was tested once, so that a fresh batch of naive mosquitoes
217 was used for each run. After each run, the olfactometer was washed with 70% alcohol to
218 remove odour contaminants left from previous tests. Latex gloves were worn by the
219 experimenter to avoid contamination of the equipment. The chronological order of the odour
220 combinations was changed to eliminate possible confounding effect of odour combination and
221 test time. The side of the tent connected to the collecting boxes was also switched to avoid
222 positional effect. Different combinations of calves and humans were used as odour sources on

223 each testing day to obviate any individual effect (a total of 21 volunteers and 18 calves). All
224 volunteers who served as the human odour source were male Burkinabe around 20-30 years
225 old who lived in Bobo Dioulasso. Calves of about similar size and weight as human
226 volunteers were used to equalize quantity of emitted odours.

227 Mosquito host choice was tested at different time points corresponding to two distinct
228 phases of the parasite development: (i) Test period 1 (5-8 days post-infection (dpi))
229 corresponding to the period of immature parasite development in the midgut (i.e. oocyst
230 stage), (ii) Test period 2 (13-19 dpi) corresponding to the period of parasite transmission
231 potential (when the sporozoites have invaded the mosquito salivary glands). The total number
232 of run performed for each mosquito species, test period and odour combination is indicated in
233 Table A1.

234 The day following behavioural testing, oocyst prevalence (proportion of *P.*
235 *falciparum*-infected females) and intensity (number of oocysts in the midgut of infected
236 females) were assessed by dissecting the midguts of mosquitoes that received an infectious
237 blood-meal. Midguts were stained in a 1% mercurochrome solution and examined under a
238 microscope (Vantaux et al., 2015). Heads and thoraces were used to determine sporozoite
239 prevalence (proportion of infected females) by PCR assays for *An. coluzzii* females (Morassin
240 et al., 2002) and by qPCR assays for the two other species (Boissière et al., 2013). Three
241 groups of mosquitoes were thus obtained: (i) females that received a gametocyte-positive
242 blood and became successfully infected; (ii) females that received a gametocyte-positive
243 blood and remained uninfected; and (iii) females that received a heat-treated gametocytic
244 blood (uninfected control).

2.4. Statistical analysis

We performed two sets of analyzes. In the first set, all data, including exposed-uninfected mosquitoes, were analyzed. In the second set, we excluded exposed-uninfected individuals to focus on the difference between infected and uninfected control mosquitoes. The two sets of analyzes yielded the same results, and, for the sake of clarity, only the second is reported in the main text (see Tables A2-A5 for the detailed output of the first set of analyzes).

All analyzes were performed in R (R Development Core Team, 2008). Binomial generalized linear mixed models (GLMMs) were fitted to investigate mosquito activation rate (proportion of mosquitoes caught in both collecting boxes out of the total number released) and odour choice (calculated separately for each odour combination, for example, human odour choice in the H-C combination was given as the proportion of mosquitoes entering human trap over the total mosquitoes entering both human and calf traps). In these models, infection treatment (two levels: infected and uninfected control), test period (two levels: test period 1 (oocyst stage) and test period 2 (sporozoite stage)), odour combination (three levels: H-C, H-O, and C-O for *An. gambiae* and *An. arabiensis*, and two levels: H-C, H-O for *An. coluzzii*) and relevant interactions were coded as fixed factors. Human volunteer and calf individual, and replicate were coded as random factors. Details of the fixed-effect and random-effect variables are shown in Tables A6 – A15. Because the *An. arabiensis* data were unbalanced (i.e. no data for carriers D and E during test period 1, see Table A1), activation rate and odour choice we analyzed separately for each test period. As there was complete separation of the data for one pair of human volunteer-calf individual in the H-C combination of test period 1 in the *An. arabiensis* data, volunteer was coded as a fixed factor in this host choice model only. We also verified for each combination, infection status and test period whether odour choice significantly differed from a random distribution between the two

collecting boxes or whether mosquitoes displayed a statistically significant attraction to one odour.

For model selection, we used the stepwise removal of terms, followed by likelihood ratio tests (LRT). Term removals that significantly reduced explanatory power ($P < 0.05$) were retained in the minimal adequate model (Crawley, 2007). Post-hoc tests were carried out using the *testFactor* function in *phia* R package (Rosario-Martinez et al., 2015).

2.5. Ethical statement

Ethical approval was obtained from the Centre Muraz Institutional Ethics Committee (A003-2012/CE-CM) and National Ethics Committee of Burkina Faso (2014-0040). The protocol conforms to the declaration of Helsinki on ethical principles for medical research involving human subjects (version 2002) and informed written consent were obtained from all volunteers. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by both the Office of Laboratory Animal Welfare of US Public Health Service (Assurance Number: A5928-01) and national committee of Burkina Faso (IRB registration #00004738 and FWA 00007038). Animals were cared for by trained personnel and veterinarians.

3. Results

3.1. Infection

Of the 1448 *An. coluzzii* mosquitoes exposed to an infectious blood meal, 622 became infected (percentage ± 95% confidence interval: 42.96 ± 2.55 %, Figure A1), with a mean (± se) parasite intensity of 14.67 ± 1.41 (Figure A2). A total of 2161 *An. coluzzii* females (622 infected + 1539 uninfected control mosquitoes) were used for the analyzes. Of the 744

292 exposed *An. gambiae*, 570 were infected (76.61 ± 3.07 %, Figure A1), with a mean parasite
293 intensity of 18.45 ± 1.71 (Figure A2). A total of 1260 *An. gambiae* females (570 infected +
294 670 control) were used for the analyzes. Finally, of the 447 exposed *An. arabiensis*, 243 were
295 infected (54.36 ± 4.62%, Figure A1), with a mean parasite intensity of 8.77 ± 0.61 (Figure
296 A2). A total of 694 *An. arabiensis* females (243 infected + 451 uninfected controls) were used
297 for the analyzes. The differences in parasite prevalence and intensity observed across the
298 different mosquito species can be explained by the use of different wild parasite isolates
299 containing varying densities of gametocytes.

300 **3.2. Activation rate**

301 *Anopheles coluzzii*. Overall, 228 of 622 infected mosquitoes and 548 of 1539 control
302 mosquitoes left the downwind box of the olfactometer to fly upwind into one of the two
303 collection boxes (activation rate of 36.66 ± 3.79% and 35.61 ± 2.39%, respectively). *P.*
304 *falciparum* infection did not affect *An. coluzzii* activation rate (χ^2_1 = 0.5, *P* = 0.48; Figure 2a).
305 *An. coluzzii* displayed similar activation rate between the two test periods (χ^2_1 = 0.08, *P* = 0.78,
306 Figure 2a) and the three odour combinations (χ^2_1= 3.21, *P* = 0.07, Figure 2a). Finally, there
307 was no statistically significant interactions (test period x odour combination: χ^2_1= 1.06, *P* =
308 0.3; infection x odour combination: χ^2_1= 0.22, *P* = 0.64; infection x test period: χ^2_1= 1.13, *P* =
309 0.29; Table A6, Figure 2a).
310
311 *Anopheles gambiae*. The overall activation rates of infected and uninfected control
312 mosquitoes were 18.42 ± 3.18% (105/570) and 19.25 ± 2.99% (129/670) respectively.
313 Infection did not significantly affect *An. gambiae* activation rate (χ^2_1 = 0.22, *P* = 0.64, Figure
314 2b). There was a marginally significant effect of test period (χ^2_1 = 3.94, *P* = 0.047; Figure 2b).
315 Although there was no significant main effect of odour combination on *An. gambiae*

316 activation rate (χ^2_2 = 3.26, *P* = 0.2), there was a significant test period by odour combination
317 interaction (χ^2_2= 8.44, *P* = 0.01, Table A7) such that the H-O odour combination induced the
318 highest mosquito activation rate during test period 1 and the lowest during test period 2
319 (Figure 2b; Table A8). Finally, there was no infection by test period interaction (χ^2_1 = 0.23, *P*
320 = 0.63), and no infection by combination interaction (χ^2_2 = 2.21, *P* = 0.33; Table A7).
321
322 *Anopheles arabiensis*. The activation rates of infected and uninfected control mosquitoes
323 during test period 1 were 37.62 ± 9.45% (38/101) and 41.59 ± 6.6% (89/214) respectively.
324 There was no influence of infection on *An. arabiensis* activation rate (χ^2_1 = 0.58, *P* = 0.45;
325 Figure 2c; Table A9). There was a significant effect of odour combination on activation rate
326 (χ^2_2 = 10.34, *P* = 0.005). In particular, mosquitoes were more activated in the C-O
327 combination than in the H-O combination (post hoc tests: C-O *vs*. H-O: χ^2_1 = 9.96, *P* = 0.002;
328 H-C *vs*. C-O: χ^2_1 = 1.79, *P* = 0. 18; H-O *vs*. H-C: χ^2_1 = 3.43, *P* = 0.06; Table A10). There was
329 no significant interaction between infection and odour combination (χ^2_2= 4.95, *P* = 0.08;
330 Table A9). The activation rate of infected *An. arabiensis* during test period 2 (16.90 ± 6.16%,
331 n=142) was lower than that of uninfected individuals (29.11 ± 5.78%, n=237) (χ^2_1 = 6.69, *P* =
332 0.01; Figure c). We also found a significant effect of odour combination (χ^2_2 = 6.61, *P* = 0.04),
333 such that mosquitoes were more activated in the H-O combination compared to the two other
334 combinations (post hoc tests: H-O *vs*. H-C: χ^2_1 = 4.97, *P* = 0.03; H-O *vs*. C-O: χ^2_1 = 4.68, *P* =
335 0. 03; H-C *vs*. C-O: χ^2_1 = 0.01, *P* = 0.9; Table A11). We found no significant effect of
336 infection by odour combination interaction (χ^2_2 = 0.18, *P* = 0.92 Figure 2c; Table A9).
337
338 **3.3. Odour choice**
339

340 *Anopheles coluzzii*. Infected and uninfected mosquitoes showed similar odour choice (H-C: $\chi^2_1 = 0.73$, $P = 0.4$; H-O: $\chi^2_1 = 1.24$, $P = 0.43$) with an overall attraction toward human odours
342 of 71.70 ± 4.32% in the H-C and of 87.47 ± 3.42% in the H-O combinations (Figure 3a).
343 There was no significant difference in odour choice between test period 1 and 2 in both the H-
344 C ($\chi^2_1 = 0.03$, $P = 0.87$) and the H-O odour combinations ($\chi^2_1 = 0.02$, $P = 0.89$). Finally, there
345 was no interaction between test period and infection in both the H-C ($\chi^2_1 = 0.31$, $P = 0.58$)
346 and H-O combinations ($\chi^2_1 = 0.62$, $P = 0.43$; Figure 3a, Table A12).
347
348 *Anopheles gambiae*. In all three odour combinations, infected and uninfected mosquitoes
349 displayed similar odour choice (H-C: $\chi^2_1 = 0.12$, $P = 0.73$; H-O: $\chi^2_1 = 2.92$, $P = 0.09$; C-O: χ^2_1
350 $= 0.75$, $P = 0.75$) with an overall attraction to human odours of 76.12 ± 10.21% in the H-C
351 and 69.14 ± 10.06% in the H-O combinations, and a repulsion by calf odours of 62.79 ±
352 10.22% in the C-O combination (Figure 3b). There was no significant effect of test period (H-
353 C combination: $\chi^2_1 = 1.96$, $P = 0.16$; H-O combination: $\chi^2_1 = 1.66$, $P = 0.2$; C-O combination:
354 $\chi^2_1 = 0.4$, $P = 0.53$) on *An. gambiae* odour choice. We found a significant infection by test
355 period interaction for the C-O combination ($\chi^2_3 = 4.67$, $P = 0.03$) with calf odours inducing a
356 stronger repellence to infected mosquitoes than to uninfected mosquitoes during test period 1
357 and inversely during test period 2; Figure 3b, Table A13). Finally, there was no significant
358 interaction between infection and test period in the H-C and H-O combinations ($\chi^2_1 = 0.47$, P
359 $= 0.49$ and $\chi^2_1 = 0.82$, $P = 0.37$, respectively).
360
361 *Anopheles arabiensis*. For both test periods and all odour combinations, infected and
362 uninfected mosquitoes displayed similar host choice (test period 1, H-C: $\chi^2_1 = 0.87$, $P = 0.35$;
363 H-O: $\chi^2_1 = 0.16$, $P = 0.69$; C-O: $\chi^2_1 = 1.2$, $P = 0.27$; test period 2, H-C: $\chi^2_1 = 0.36$, $P = 0.55$, H-
364 O: $\chi^2_1 = 1.39$, $P = 0.24$, C-O: $\chi^2_1 = 0.06$, $P = 0.81$; Figure 3c, Tables A14 and A15) with an

365 attraction to human odours of 88.23 ± 7.66% in the H-C and 60.56 ± 11.37% in the H-O
366 combinations and a repulsion by calf odour of 64.19 ± 10.44% in the C-O odour combination.
367 There was no influence of volunteer/calf individual on odour choice (test period 1, H-C: χ^2_1 =
368 0.69, P = 0.41; Table A14).
369
370 **4. Discussion**
371 Several studies have demonstrated that malaria parasites can alter important behavioural
372 features of their mosquito vectors in a stage dependant manner: oocyst-infected mosquitoes
373 show reduced attraction to vertebrate odours and reduced avidity to feed, and, in contrast,
374 sporozoite-infected individuals show enhanced attraction to vertebrate odours and enhanced
375 avidity to feed (Hurd, 2003; Lefèvre and Thomas, 2008; Cator et al., 2012; Caljon et al.,
376 2016). These behavioural alterations likely increase parasite transmission, provided that
377 mosquito feeds are taken on a suitable vertebrate host species for the parasite. Our results
378 indicate that, regardless of parasite developmental stage, *P. falciparum* infection did not alter
379 mosquito activation rate, a surrogate of mosquito motivation to feed. While this finding
380 contrasts with earlier studies (Rossignol et al., 1986; Cator et al., 2013; Smallegange et al.,
381 2013), it supports two recent other studies, including one on *An. coluzzii* experimentally
382 infected with sympatric wild isolates of *P. falciparum* (Cornet et al., 2013b; Vantaux et al.,
383 2015), and suggests that manipulation of mosquito activity and response to host odours may
384 not be a universal phenomenon.The expression of parasite-induced behavioural alterations,
385 like any other phenotypic traits, may depend on local coevolutionary processes (Thompson,
386 2005). Hence, natural selection might not favour the evolution of manipulation in the studied
387 populations if, for example, mosquito behaviour already ensures high parasite transmission or
388 if the mosquito vector has evolved resistance (Daoust et al. 2015). Further investigations

389 using sympatric and allopatric host-parasite combinations will be essential to integrate these
390 local co-adaptation phenomena.

391 Our results also showed similar attractiveness of host odours to infected and
392 uninfected mosquitoes. The overall expression pattern of mosquito host choice was consistent
393 with a high degree of anthropophily in both infected and uninfected *An. coluzzii*, *An. gambiae*
394 and *An. arabiensis*. The only significant effect of infection on mosquito odour choice was
395 seen in sporozoite-infected *An. gambiae* which were less repelled by calf odours compared to
396 oocyst-infected counterparts (Figure 3b). This result contrasts with the hypothesis of a
397 parasite manipulation of mosquito odour preference and the expectation that sporozoite-
398 infected individuals should be steered away from the odours of inappropriate vertebrate hosts.
399 Although the precise reason behind this effect is currently unclear, it might be a mosquito
400 response, and interactions among mosquito resources, infection and requirement of a blood-
401 meal can be suspected. Because *Plasmodium* parasites utilize mosquito resources to develop
402 and mature, sporozoite-infected *An. gambiae* females might be less choosy regarding the
403 nature of the blood source, hence explaining this increased attraction to calf odour. At this
404 stage, this remains speculative and further experiments are required to investigate the
405 mechanisms underlying this effect and to explain why this pattern was not observed in *An.*
406 *coluzzii* and *An. arabiensis.*

407 Our prediction was that sporozoites of *P. falciparum* may have evolved the ability to
408 influence mosquito preference in a way that increases contact with humans - the appropriate
409 vertebrate host of *P. falciparum* - by inducing in the mosquito vector a sensory bias for host
410 odours that are correlated with suitability for the parasite. When simultaneously exposed to
411 odours from calf and human, 71% and 76% of both sporozoite-infected and uninfected *An.*
412 *coluzzii* and *An. gambiae* were retrieved from the human trap, hence confirming the
413 anthropophilic behaviour of these species (Costantini et al. 1996; Costantini et al. 1999;

414 Dekker et al. 2001; Takken and Verhulst 2013). Such anthropophily already ensures relatively
415 high probability of transmission toward the appropriate host, perhaps making parasite
416 manipulation of mosquito host choice useless in this system (i.e. weak selective pressures for
417 its evolution). In other words, it is possible that, in this system, the balance between the
418 transmission benefits of an increased preference for humans (e.g. from an anthropophily of 70
419 to 90%) and the costs associated with manipulation (Poulin, 1994) explain why this change
420 has not evolved. Alternatively, it is possible that the parasite's ability to modify its mosquito
421 host choice did evolve but was not expressed here because of our experimental design.
422 First, while our olfactometer allows the study of long-range odour-mediated
423 attractiveness, the full sequence of mosquito host-seeking process also includes short-range
424 stimuli. Odour-mediated preference is critical at the initial step of host location; however final
425 host decision might be influenced by cues other than odours, thereby determining alternative
426 patterns of host preference. Indeed, olfactometer obviates stimuli such as visual cues, heat,
427 moist, convective currents, and host movements. Under this scenario, sporozoite-infected *An.*
428 *gambiae* would present similar host preference as uninfected counterparts in the early stages
429 of the host-seeking process, when it mostly responds to host odours (i.e. long-range host
430 preference), and then display increased attraction to humans at a shorter range, when other
431 cues become more important (i.e. short-range host preference). In addition, we did not control
432 the quantity of emitted host odour which could also affect mosquito behavioural responses
433 (Costantini et al., 1996).
434 Second, our experiments were conducted between 6.30.pm and 11.30.pm while
435 mosquito activation spans from 6.00.pm until early morning the following day with a peak of
436 activity occurring around midnight. This activity peak is correlated with human resting
437 behaviour to presumably maximize mosquito fitness (Lehane 2005). During this time frame,
438 human are less defensive, possibly facilitating feeding and reduce mosquito mortality. Malaria

parasites may have evolved the ability to finetune manipulation to the temporal behaviour of both the vectors and the vertebrate host. Under this scenario, manipulation of host choice might occur later in the night (e.g. during the peak of mosquito activity), and we hence may have missed it.

Third, *An. gambiae* and *An. coluzzii* mosquitoes from colonies continually replenished with F1 from wild-caught females were used here and it will also be important to use F0 from field mosquitoes since rearing insects in the laboratory for many generations is unlikely to represent the genetic diversity observed in nature.

Fourth, the uninfected control mosquitoes were fed on the same blood as infected mosquitoes but in which gametocytes were heat-inactivated. This procedure allows avoiding confounding effects of different blood origins on mosquito fitness and behavioural responses. However, these heat-killed gametocytes might trigger a mosquito immune response, which in turn, might affect mosquito behaviour. Although no study has, to our knowledge, directly explored whether heat-killed gametocytes can stimulate a mosquito immune response, there is evidence that alive gametocytes blood triggers a mosquito immune response that does not occur with heat-killed gametocytes (Mendes et al. 2008, 2011). In addition, the differences in immune gene expression between mosquitoes challenged with alive and dead *P. falciparum* gametocytes is similar to that observed between mosquitoes challenged with alive *P. berghei* gametocytes and mosquitoes that received a parasite-free blood-meal (Mendes et al. 2008, 2011). These results suggest that heat-killed *P. falciparum* gametocytes and parasite-free blood-meal may induce similar mosquito immune response. However, a study showed that a challenge with heat-killed *Escherichia coli* can generate mosquito behavioural changes similar to that observed in mosquitoes infected with rodent malaria parasites (Cator et al 2013). Overall, future studies should ideally include two control groups: heat-inactivated gametocytes from the same carrier (to avoid effects of different blood-meal sources on

464 mosquito behaviour) and (ii) uninfected blood from a parasite-free donor (to avoid possible
465 effects of heat-killed gametocytes on mosquito behaviour).

466 Finally, it is possible that the expression of parasite manipulation of host preference is
467 more pronounced in some mosquito-parasite combinations than in others. In particular, we
468 predicted that the expression of parasite manipulation of vector host choice might be more
469 obvious in *An. arabiensis*, a presumably more zoophilic/opportunistic vector species (Takken
470 and Verhulst, 2013). When simultaneously exposed to calf and human odours, we found that
471 90 % of *An. arabiensis* were retrieved from the human trap. This result contrasts with most
472 existing studies on *An. arabiensis* host preference, which report an overall high degree of
473 zoophily (Takken and Verhulst, 2013). Using odour-baited entry traps in Tanzania, Mahande
474 et al. (2007), for example, showed a 90% zoophily in *An. arabiensis*. In contrast, of an
475 estimated 1,800 field *An. arabiensis* collected using the same technique in Central Burkina
476 Faso, slightly more than 8% were collected in the calf-baited trap, suggesting a high degree of
477 anthropophily (Costantini et al., 1998). Our findings support the idea that West African
478 populations of *An. arabiensis* may be generally more anthropophilic than East African ones
479 (Costantini et al., 1999) and emphasizes that the anthropophilic/zoophilic label given to
480 malaria mosquito species must be carefully interpreted and refer to populations rather than
481 whole taxonomic unit.

482 We observed no parasite manipulation of mosquito odour-mediated host choice in the
483 natural associations between *P. falciparum* and three of its major vector species, *An. coluzzii*,
484 *An. gambiae*, and *An. arabiensis*. All three species were rather anthropophilic regardless of
485 their infectious status. Further work is required to explore whether *P. falciparum* is able to
486 modify its mosquito vertebrate choice in a way that increase transmission toward suitable host
487 species. While our study examined the odour-mediated long-range mosquito host choice,
488 determining the origin of blood-meals retrieved from uninfected, oocyst-infected and

sporozoite-infected mosquitoes in the field may reveal the existence of specific manipulation. Future studies on specific manipulation in other vector systems would provide important information on the ecology and epidemiology of vector-borne diseases. A recent study suggested that the rodent- or bird-specialized *Borrelia* genospecies were unable to alter attraction of the generalist tick *Ixodes ricinus* to mouse odour (Berret and Voordouw, 2015). However, this study used ticks collected from the field and was not able to establish a causal relationship between *Borrelia* infection and attraction to mouse odour (Berret and Voordouw, 2015). Other possibly good model systems to study specific manipulation in vector-borne diseases are tsetse fly-transmitted trypanosomes. For example, *Glossina palpalis gambiensis* has a broad range of hosts in central Africa (humans, reptiles, bushbuck, and ox) and is the main vector of *Trypanosoma brucei gambiense* responsible for the medically important Human African trypanosomiasis. We would predict that once infected, flies are more attracted by human cues than by those of other vertebrates. Finally, parasite manipulation of mosquito host choice could theoretically occur at the intraspecific level (among different human individuals), with infected vectors biting more than expected less-immune hosts.

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710 **Figure legends**

711

712 **Figure 1.** Schematic representations of (a) the dual-choice olfactometer and (b) the
713 behavioural assays. C1 represents the tent containing calf 1, H1 represents the tent containing
714 human volunteer 1, N represents the tent with outdoor air (control), O represents the
715 Olfactometer, Y corresponds to mosquito infected status, Φ corresponds to mosquito
716 uninfected status, R and Y represent the colours of the mosquitoes which are red and yellow
717 respectively. The position of the tents was switched among replicates to account for side
718 effect. Test period 1 and 2 correspond to the oocyst and sporozoite developmental stages in
719 infected mosquitoes, respectively.

720

721 **Figure 2.** Mosquito activation rate, expressed as the proportion of mosquitoes caught in both
722 collecting boxes out of the total number released in the downwind box for each treatment
723 combination. (a) *Anopheles coluzzii*, (b) *Anopheles gambiae*, (c) *Anopheles arabiensis.*
724 Numbers inside the bars indicate the total number of mosquitoes released across all runs.
725 Error bars show the 95% confidence interval. C-O: for calf odour *vs* outdoor air combination,
726 H-C: human odour *vs* calf odour combination, H-O: human odour *vs* outdoor air combination.
727 Test Period 1 and Test Period 2 correspond to the oocyst and sporozoite stages in infected
728 mosquitoes, respectively.

729

730 **Figure 3.** Mosquito odour-mediated choice - expressed as the proportion of mosquitoes
731 caught in one collecting box out of the total number retrieved from both collecting boxes. (a)
732 *An. coluzzii*, (b) *An. gambiae* (c) *An. arabiensis.* Data show proportion ± 95% confidence
733 interval across all runs. Numbers indicate the total numbers of mosquitoes in both traps across

734 all runs. The annotation human, calf, control corresponds to source of odour the mosquitoes
735 chose Test Period 1 and Test Period 2 correspond to the oocyst and sporozoite stages in
736 infected mosquitoes, respectively. (* indicates significant bias toward an odour source; *: $P <$
737 0.05, **: $P < 0.01$, ***: $P < 0.001$).