

1 **Field evidence for manipulation of mosquito host selection by the human malaria parasite,**
2 ***Plasmodium falciparum***

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17

18 **Abstract**

19 Whether the malaria parasite *Plasmodium falciparum* can manipulate mosquito host choice in
20 ways that enhance parasite transmission toward humans is unknown. We assessed the influence of
21 *P. falciparum* on the blood-feeding behaviour of three of its major vectors (*Anopheles coluzzii*,
22 *An. gambiae* and *An. arabiensis*) in Burkina Faso. Host preference assays using odour-baited traps
23 revealed no effect of infection on mosquito long-range anthropophily. However, the identification
24 of the blood meal origin of mosquitoes showed that females carrying sporozoites, the mature
25 transmissible stage of the parasite, displayed a 24% increase in anthropophagy compared to both
26 females harbouring oocysts, the parasite immature stage, and uninfected individuals. Using a
27 mathematical model, we further showed that this increased anthropophagy in infectious females
28 resulted in a > 250% increase in parasite transmission potential, everything else being equal. This
29 important epidemiological consequence highlights the importance of vector control tools targeting
30 infectious females.

31

32 **Introduction**

33 There is mounting evidence that malaria parasites affect phenotypic traits of their vectors and hosts
34 in ways that increase contacts between them, hence favouring parasite transmission (Hurd 2003,
35 Koella 2005, Lefèvre and Thomas 2008). In addition to increased vertebrate attractiveness to
36 mosquito vectors (Lacroix *et al.* 2005, Cornet *et al.* 2013, Batista *et al.* 2014, De Moraes *et al.*
37 2014, Busula *et al.* 2017, Emami *et al.* 2017), another frequently reported parasite-induced change
38 is the alteration of vector motivation and avidity to feed (Cator *et al.* 2012, Stanczyk *et al.* 2017).
39 Mosquitoes infected with *Plasmodium* sporozoites (the mosquito to human transmission stage) can
40 indeed display increased (i) responses to host odours (Rossignol *et al.* 1986, Cator *et al.* 2013), (ii)

41 landing and biting activity (Rossignol *et al.* 1984, Rossignol *et al.* 1986, Wekesa *et al.* 1992,
42 Anderson *et al.* 1999, Koella *et al.* 2002, Smallegange *et al.* 2013), (iii) number of feeds (Koella
43 *et al.* 1998) and (iv) blood volume intake (Koella and Packer 1996, Koella *et al.* 1998, Koella *et*
44 *al.* 2002). In contrast, mosquitoes infected with oocysts (the immature non-transmissible stage of
45 the parasite), are less likely to attempt to feed (Anderson *et al.* 1999, Koella *et al.* 2002, Cator *et*
46 *al.* 2013). Since biting is risky (e.g., host defensive behaviours can kill the vector and its parasite),
47 reduced feeding attempts would be beneficial to the parasite during the non-transmissible stage as
48 this would reduce mortality before the parasite reaches maturity and is ready to be transmitted
49 (Schwartz and Koella 2001).

50 These “stage-dependent” behavioural alterations likely increase parasite transmission
51 (Dobson 1988, Cator *et al.* 2014), provided that mosquito feeds are taken on a suitable vertebrate
52 host species for the parasite. While malaria vectors can usually feed on a range of different
53 vertebrate species (Takken and Verhulst 2013), the malaria parasites they transmit are often highly
54 host-specific, infecting only one or a few vertebrate species (Perkins 2014). For example *P.*
55 *falciparum*, which causes the most severe form of human malaria, displays an extreme form of
56 specificity and can develop and reproduce in hominids only (predominantly in humans and to a
57 lesser extent in chimpanzees, bonobos, and gorillas) (Prugnolle *et al.* 2011, Rayner *et al.* 2011,
58 Ngoubangoye *et al.* 2016), such that any mosquito bite on another vertebrate species would be a
59 dead-end for the parasite. In contrast, the vectors of *P. falciparum* can feed on a wide range of
60 vertebrate host species in the wild depending on the geographic area and the relative abundance of
61 humans and other vertebrates (Costantini *et al.* 1999, Takken and Verhulst 2013). Accordingly, *P.*
62 *falciparum* could modify its vector choice in ways that enhance transmission toward humans
63 and/or reduce mosquito attraction to other unsuitable host species (i.e. specific manipulation). A

64 previous study testing this hypothesis found no effect of *P. falciparum* infection on host preference
65 of three major vector species, *An. coluzzii*, *An. gambiae*, and *An. arabiensis* (Nguyen *et al.* 2017).
66 However, this study examined the odour-mediated mosquito host preference in laboratory
67 conditions using a dual-port olfactometer, not the final realised host choice which is of primary
68 importance for parasite transmission.

69 Here, we assessed the influence of *P. falciparum* on *An. coluzzii*, *An. gambiae* and *An.*
70 *arabiensis* blood-feeding behaviour in three villages in Burkina Faso. First, odour-baited traps, set
71 side by side in a choice arrangement and releasing either human or calf odours were used to
72 determine odour-mediated mosquito host preference (Experiment 1). Second, indoor-resting
73 blood-fed mosquito females were collected and the origin of their blood meal was identified to
74 determine mosquito host selection (Experiment 2). Third, we quantified the epidemiological
75 consequences of variation in the patterns of host selection using a compartmental model for
76 *Plasmodium* transmission between humans and mosquitoes.

77

78 **Material and methods**

79 ***Collection sites***

80 The study was conducted in three villages in South-Western Burkina Faso: Soumousso
81 (11°23'14"N, 4°24'42"W), Klesso (10°56'40.5"N, 3°59'09.9"W) and Samendeni (11°27'14.3"N,
82 4°27'37.6"W) (Figure supplement S1). The three villages are located in an area characterized by
83 wooded savannah, where *Anopheles* females only have access to temporary, rain-filled puddles
84 and quarries that permit larval development during the rainy season from June to November. The
85 dry season extends from December to May. In these rural villages, domestic animals (including
86 cattle, goats, sheep, pigs, chickens, donkeys, dogs) are usually kept in compounds in open

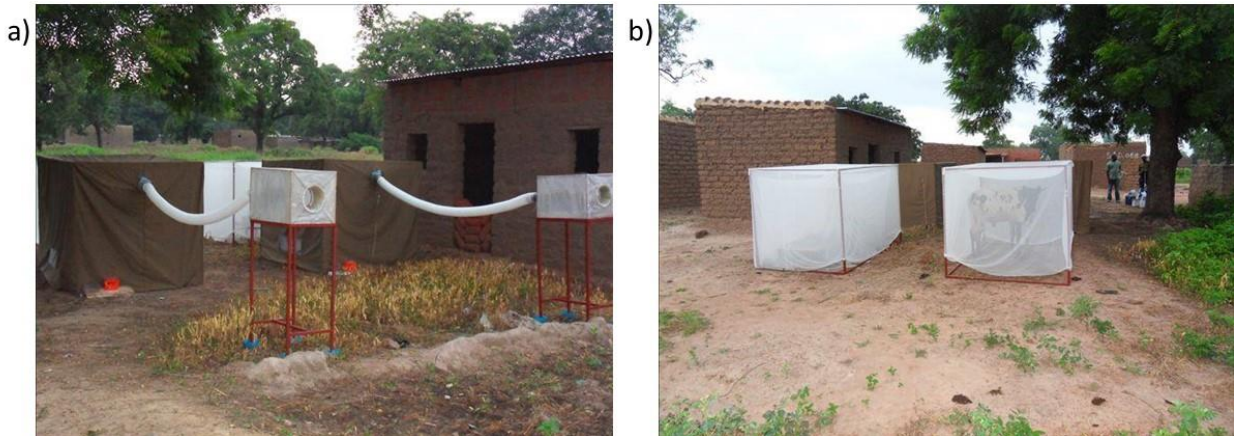
87 conditions but a few households use separate roofed shelters for sheep, goats, pigs and chickens.
88 Most houses are mud-walled with roofs of iron sheets or thatch, but a few houses are made of
89 bricks.

90 ***Experiment 1: Mosquito host preference***

91 Two odour-baited entry traps (OBETs as in Costantini *et al.* 1996, Costantini *et al.* 1998, Lefèvre
92 *et al.* 2009) and two odour-baited double net traps (BNTs as in Tangena *et al.* 2015) baited with
93 calf and human odours were used to assess the host preference of field populations of mosquitoes
94 in Samandeni and Klesso villages (Figure 1). The two OBETs were connected to a tent (Lxlxh:
95 250x150x150 cm) by air vent hoses (Scanpart®, DxL=10*300cm; Figure 1a). The odours of the
96 two hosts were drawn by a 12-V fan from the tents and into the OBETs by the air vent hoses,
97 coming out of the traps at a speed of 15cm/s (± 2 cm/s), as measured with a Testo 425-Compact
98 Thermal Anemometer (Testo, Forbach, France) equipped with a hot wire probe [range: 0 to +
99 20m/s, accuracy: $\pm (0.03 \text{ m/s} + 5\% \text{ of mv})$]. Host-seeking mosquitoes responding to the host cues
100 flew up the odour-laden streams and entered one of the two traps. The two odour-baited double
101 net traps (BNTs) consisted of an untreated bed net (Lxlxh: 300x250x185 cm) from which each
102 corner was raised 20 cm above ground and a smaller untreated bed net (Lxlxh: 190x120x150 cm)
103 protecting the human volunteer in the human baited trap (Figure 1b).

104 In both OBETs and BNTs, the human volunteers rested on a metal-framed bed (Lxl:
105 190x80 cm) and were protected from mosquito bites. OBETs and BNTs were operated from 19:00
106 to 05:30 hours, for 3 nights in June 2013, and 13 nights in September 2013 in Samandeni. The
107 BNTs only were set-up for 6 nights in September in Klesso. Different combinations of live calves
108 and humans were used as odour sources on each testing day to obviate any individual effect. Calves
109 of about similar size and weight as human volunteers were used to equalize the quantity of emitted

110 odours. Trapped mosquitoes were retrieved in the morning using mouth aspirators. They were kept
111 in a 20x20x20 cm cage with a humid towel on top and brought back to the laboratory for further
112 processing (see below).



113
114 **Figure 1.** Traps baited with calf and human odours used to assess the host preference of field
115 populations of mosquitoes in Samandeni and Klesso villages. **a)** Two odour-baited entry traps
116 (OBETs) were connected to a tent by air vent hoses. **b)** Two odour-baited double net traps (BNTs).

117

118 ***Experiment 2: Mosquito blood-feeding pattern***

119 Indoor resting blood-fed mosquitoes were collected between 7 am and 9 am by insecticide spray
120 catches as in Lefèvre et al. (2009) to determine the origin of their blood-meal. Briefly, white sheets
121 were spread over the floor surface and the furniture inside houses. The houses were then sprayed
122 with an insecticide (Kaltox®: allethrin 0.27%, tetramethrin 0.20 %, permethrin 0.17%, propoxur
123 0.68%) to knock down the mosquitoes. Fifteen minutes after spraying, blood-fed *An. gambiae s.l.*
124 mosquitoes were collected from the white sheet using forceps and placed on moist filter paper
125 inside labeled petri dishes.

126

127 In Samandeni and Klesso, mosquito collections were carried out in the rainy season only (4 days
128 in June 2013, and 13 days in September 2013 in Samandeni, and 6 days in September 2015 in
129 Klesso), whereas in Soumouso they were conducted in both the rainy and the dry season (26 days
130 between January and November 2009). In Soumouso, human dwellings (from 10
131 neighbourhoods) only were sampled whereas animal sheds and unoccupied houses were also
132 sampled in Samandeni and Klesso. A total of 27 human dwellings, 7 unoccupied houses and 20
133 animal sheds were sampled in Samandeni. A total of 7 human dwellings, 7 unoccupied houses and
134 9 animal sheds were sampled in Klesso. All mosquitoes were kept in a Petri dish with a humid
135 paper towel to facilitate later dissection and brought back to the laboratory for further processing
136 (see below).

137

138 *Laboratory processing of samples*

139 A total of 3447 blood-fed *Anopheles gambiae s.l.* collected indoors (Experiment 2) and 674
140 females collected in the choice traps (Experiment 1) were processed. In addition, a subset of 276
141 females collected indoors was used to determine parity (parous versus nulliparous) based on the
142 condition of ovarian tracheoles in order to control for age. Similarly, a subset of 418 individuals
143 was used to determine different species within the *Anopheles gambiae sensu stricto* complex (i.e.
144 distinguishing *Anopheles arabiensis*, *Anopheles coluzzii* and *Anopheles gambiae*) using routine
145 PCR-RFLP based on segregating SNP polymorphisms in the X-linked ribosomal DNA InterGenic
146 Spacer region as described in Santolamazza *et al.* (2008).

147 *Anopheles gambiae sl.* females were dissected in a drop of phosphate buffered saline (PBS)
148 (pH 7.2). Blood-fed midguts were gently squeezed under a stereomicroscope (magnification 35x,
149 Leica EZ4D, Wetzlar, Deutschland) to get the blood out, which was mixed with PBS, absorbed on

150 a filter paper, and then kept at -20°C until identification by an enzyme-linked-immunosorbent
151 assay (ELISA) for Soumouosso and Samendeni samples (Beier *et al.* 1988) and by multiplex PCR
152 for Klesso samples (Kent and Norris 2005). Each blood meal was discriminated between human,
153 cattle, goat/sheep, chicken, dog, pig, and horse/donkey origins. ELISA-based determination of
154 mosquito blood meal origin was performed using anti-human IgG-, anti-bovine IgG-, anti-pig IgG,
155 anti-chicken IgG-, anti-goat IgG-, anti-sheep IgG-, anti-dog IgG-, and anti-horse IgG-peroxidase
156 conjugates (A8794, A5295, A5670, A9046, A5420, A3415, A6792, A6917, Sigma-Aldrich).
157 PCR-based determination of the mosquito blood meal origin targeting the vertebrate host
158 cytochrome B was performed as described by Kent and Norris (2005), with the following
159 modifications: (i) Three additional primers were designed from available Genbank sequences to
160 target the following potential hosts: chicken470F (Genbank accession number: AB044986.1),
161 sheep695F (KY662385.1), donkey574F (FJ428520.1); (ii) for each individual, two multiplex
162 reactions were performed to avoid cross-reactions between primers and to optimize the
163 determination. In the multiplex reaction #1, UNREV1025, Chicken470F, Sheep695F, Goat894F
164 and Donkey574F primers were used at an amplification temperature of 49.2 °C. In the multiplex
165 reaction #2, UNREV1025, Dog368F, Human741F, Cow121F and Pig573F primers were used at
166 an amplification temperature of 58°C. Blood meal origin diagnostic was based on the PCR
167 products expected sizes as follow: donkey (460bp), sheep (340bp), chicken (290bp), goat (150bp),
168 dog (680bp), cow (561bp), pig (453bp), human (334bp).

169 The extracted midguts were then stained with 1% Mercurochrome® solution to detect with
170 a microscope (magnification 400x, Leica ICC50, Wetzlar, Deutschland) the presence and number
171 of *Plasmodium* spp. oocysts. PCR on a subset of oocyst-infected individuals (20 midguts of a total
172 of 118 oocyst-infected individuals) confirmed that these oocysts all belonged to *P. falciparum*.

173 The head and thorax of individual mosquitoes were stored at -20°C in 1.5 mL Eppendorf tubes.
174 Sporozoite infection with *P. falciparum* was determined by ELISA using peroxidase-conjugated
175 *Plasmodium falciparum* circumsporozoite protein monoclonal antibody for the Soumouso
176 samples (Wirtz *et al.* 1987) and by qPCR for the samples from Samendeni and Klesso (Boissière
177 *et al.* 2013). The quantification of *P. falciparum* sporozoites in salivary glands was determined by
178 qPCR using 7500 Fast Real time PCR System (Applied Biosystems, Foster City CA, USA). The
179 mosquito heads and thoraxes were crushed individually and DNA extracted as previously
180 described (Morlais *et al.* 2004). For sporozoite quantification, we targeted the fragment of subunit
181 1 of the mitochondrial cytochrome c oxidase gene (cox 1) using the forward and reverse primer
182 sequences, qPCR-PfF 5'-TTACATCAGGAATGTTATTGC-3' and qPCR-PfR 5'-
183 ATATTGGATCTCCTGCAAAT-3, respectively. The reaction was conducted in a 10 μL final
184 volume containing: 1 μL of DNA template, 1x HOT Pol EvaGreen qPCR Mix Plus ROX, and
185 600nM of each primer. Amplification was started by an initial activation step at 95°C for 15min
186 and 40 cycles of denaturation at 95°C for 15s and annealing / extension at 58°C for 30s. Detection
187 was conducted during the last step (Boissière *et al.* 2013). Quantification was based on a standard
188 curve built from four serial dilutions (12%) of an asexual parasite culture. We made dilutions
189 ranging from 60 to 60,000 genome/ μl of DNAs from a standard culture. The first dilution (10^{-1})
190 was used as a positive control. The standard curve ($y = -3.384X + 35.874$) was obtained by linear
191 regression analysis of Ct values (Cycle threshold) *versus* log₁₀ genome copy number of parasite
192 culture.

193 This protocol allowed us to gather the following information for each collected individual
194 mosquito: immature *Plasmodium* infection status (presence of oocysts in the midgut); mature *P.*

195 *falciiparum* infection status (presence of sporozoites in salivary glands); source of blood meal or
196 trap (calf/human) chosen; shelter type (human dwellings, unoccupied houses, animal sheds).

197

198 ***Statistical analyses***

199 *Experiment 1: Mosquito host preference* -The anthropophily index (AI) was expressed as
200 the number of *Anopheles gambiae s.l.* caught in the human-baited trap over the total number of
201 mosquitoes caught in both human- and calf- baited traps. We tested the effect of infection status
202 (uninfected, infected with the oocyst immature stages and infected with the sporozoite
203 transmissible stages), collection method (OBET vs. BNT), and their interaction on AI using a
204 General Linear Model (GLM) with a binomial error structure.

205 *Experiment 2: Mosquito blood-feeding pattern* -The human blood index (HBI) was expressed
206 as the number of *Anopheles gambiae s.l.* fed on humans including mixed human-animal blood
207 meals over the total number of blood-fed *Anopheles gambiae s.l.*. We tested the effect of
208 *Plasmodium* infection status (uninfected, oocyst-infected, sporozoite-infected individuals - 25
209 individuals with both oocysts and sporozoites were included in the sporozoite infected group and
210 excluding these individuals from the analysis yielded similar results), village (Soumouso, Samendeni, Klesso), shelter type (human dwelling, unoccupied house, animal shed) and relevant
211 two-way interactions (infection status by shelter type and infection status by village) on HBI using
212 a GLM with a binomial error structure. The effect of species (*Anopheles gambiae*, *An. coluzzii* and
213 *An. arabiensis*), infection status, shelter type, and their interactions on HBI was assessed using the
214 subset of females identified to the molecular level using a GLM with a binomial error structure.
215 The effect of parity (nulliparous vs. parous) on HBI was assessed on a subset of females using a
216 GLM with a binomial error structure.
217

218 We also verified for both AI and HBI whether choice significantly differed from a random
 219 distribution between humans and animals or whether mosquitoes displayed a statistically
 220 significant attraction to one type of blood meal or trap.

221 For model selection, we used the stepwise removal of terms, followed by likelihood ratio tests
 222 (LRT). Term removals that significantly reduced explanatory power ($P < 0.05$) were retained in the
 223 minimal adequate model (Crawley 2007). All analyses were performed in R v.3.0.3.

224

225 *Mathematical model*

226 In order to explore the epidemiological consequences of variation in HBI, we built a
 227 compartmental model for *Plasmodium* transmission between humans and mosquitoes (Keeling and
 228 Rohani 2008):

$$\frac{dS_m}{dt} = \mu N_m - ab \frac{S_m}{N_m} I_h \varepsilon_s - \mu S_m$$

$$\frac{dE_m}{dt} = ab \frac{S_m}{N_m} I_h \varepsilon_s - (\mu + \gamma) E_m$$

$$\frac{dI_m}{dt} = \gamma E_m - \mu I_m$$

$$\frac{dS_h}{dt} = -ac \frac{S_h}{N_h} I_m \varepsilon_i + \delta I_h$$

$$\frac{dI_h}{dt} = ac \frac{S_h}{N_h} I_m \varepsilon_i - \delta I_h$$

229

230

231 Susceptible mosquitoes (S_m) are born at rate μ and become exposed (E_m) according to their biting
 232 rate (a), their probability to get infected (b) and the HBI of susceptible mosquitoes (ε_s). Then,
 233 exposed mosquitoes become infectious (I_m) according to their extrinsic incubation period (γ).

234 Mosquito population die at rate (μ). N_m is the number of mosquitoes. Susceptible humans (S_h) get
235 infected according to mosquito biting rate, the probability to develop infection (c) and the HBI of
236 infectious mosquitoes (ϵ_i). N_h is the number of humans. Then, infectious humans remain infectious
237 (I_h) during a period equals to $1/\delta$ on average. See parameter values in table supplement S1 (Roux
238 *et al.* 2015, Vantaux *et al.* 2016). In our simulation we based the HBI of exposed mosquitoes (ϵ_s)
239 on the confidence intervals of oocyst-infected mosquitoes that were experimentally measured in
240 this study. Then we explored the impact of the HBI of infectious mosquitoes (ϵ_i , during the
241 sporozoite stage) on the Entomological Inoculation Rate (EIR), representing the number of
242 infectious bites received by a human during one year (Smith and Ellis McKenzie 2004), as defined
243 by:

$$EIR = ma \frac{I_m}{N_m}$$

244

245

246

247 where m is the ratio between mosquitoes and humans, and other parameters are as above. We kept
248 an identical human population size of 100 individuals and only varied mosquito densities to
249 assume different ratio values (m) between mosquitoes and humans (low: $m=1$, medium: $m=10$ and
250 high: $m=100$) in order to explore the impact of different HBIs on the EIR in relation to mosquito
251 densities. Then, the mathematical model was simulated for one season in order to estimate the
252 proportion of infectious mosquitoes.

253 ***Ethics***

254 Ethical approval was obtained from the Centre Muraz Institutional Ethics Committee under
255 agreement no. 0003-2009/CE-CM and A0003-2012/CE-CM.

256

257 **Results**

258 **Experiment 1: Mosquito host preference** – To assess the inherent mosquito host preference of
259 field populations of mosquitoes, we used two odour-baited entry traps (OBETs) and two odour-
260 baited double net traps (BNTs) releasing either calf or human odours. The anthropophily index
261 (AI) was expressed as the number of *Anopheles gambiae s.l.* caught in the human-baited trap over
262 the total number of mosquitoes caught in both human- and calf- baited traps. The infection status
263 was successfully determined in 584 out of the 674 mosquitoes (86.6%) collected in the OBETs
264 (383 individuals) and BNTs (201 individuals). Uninfected, oocyst-infected and sporozoite-
265 infected females displayed similar host preferences ($X^2_2 = 3.6$, $P = 0.17$, Figure supplement S2, AI
266 uninfected females: $63.3 \pm 4\%$, $N=531$, $OR=0.58$, $95\% CI = 0.53-0.63$, $P < 0.0001$; AI oocyst-
267 infected females: $55.2 \pm 18\%$, $N=29$, $OR=0.81$, $95\% CI = 0.56-1.18$, $P=0.58$; AI sporozoite-
268 infected females: $45.8 \pm 20\%$; $N=24$, $OR=1.18$, $95\% CI = 0.78-1.78$, $P=0.7$). There was no effect
269 of collection method on AI (OBETs: $64 \pm 5\%$, BNTs: $59 \pm 7\%$; $X^2_1 = 1.5$, $P = 0.21$), indicating
270 that both methods are comparable to assess mosquito host preference. There was no interaction
271 between mosquito infection and collection method ($X^2_2 = 0.26$, $P = 0.9$; Figure supplement S2).

272

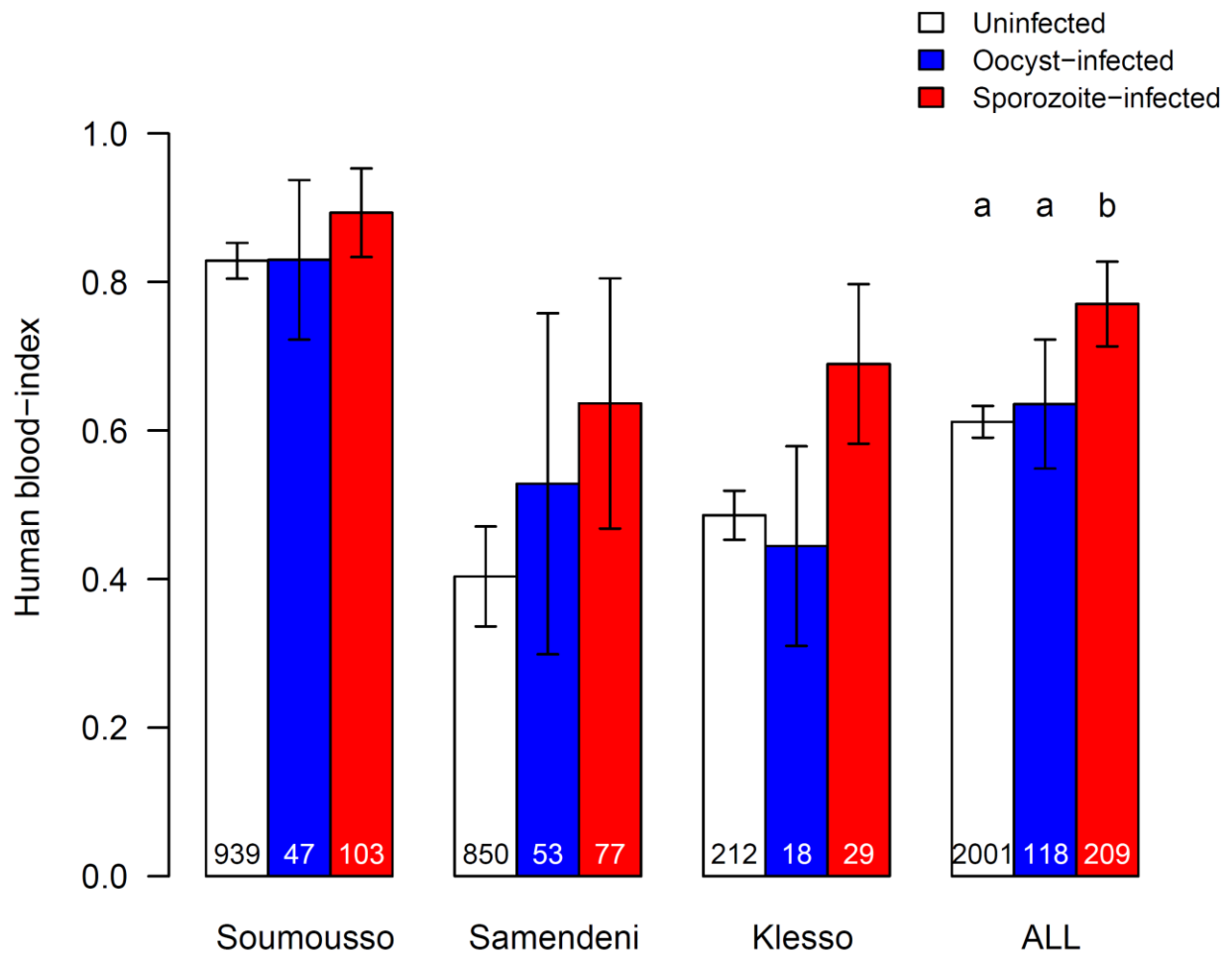
273 **Experiment 2: Mosquito blood-feeding pattern** – To assess the realized host selection of
274 *Anopheles gambiae s.l.*, the blood meal origins of indoor-resting females were identified. The
275 human blood index (HBI) was expressed as the number of females fed on humans (including mixed
276 human-animal blood meals) over the total number of blood-fed females. Of the 3447 blood-fed
277 *Anopheles gambiae s.l.* collected indoors, the blood meal origin was successfully identified in 2627
278 samples (76%). Among these 2627 samples, infection status was successfully determined in 2328

279 mosquitoes (88.6%). The following analyses are restricted to these 2328 females. HBI was
280 significantly affected by mosquito infection status ($X^2_2 = 13.007$, $P = 0.0015$; Figure 2) with a 24%
281 increase in HBI in sporozoite-infected females compared to both their oocyst-infected and
282 uninfected counterparts (sporozoite-infected: $77 \pm 5.7\%$; $N=209$, deviation from random feeding:
283 $OR=0.3$, 95% $CI = 0.25-0.35$, $P < 0.0001$; oocyst-infected females: $63.6 \pm 5.7\%$, $N=118$, $OR=0.57$,
284 95% $CI = 0.47-0.69$, $P = 0.004$; uninfected females: $61.1 \pm 2.1\%$; $N=2001$, $OR=0.64$, 95% $CI =$
285 $0.61-0.66$, $P < 0.0001$). However, because sample size in the uninfected group ($N=2001$) was
286 higher than that of both sporozoite-infected ($N= 209$) and oocyst-infected groups ($N=118$), we ran
287 a second set of analyses using a subset of 150 randomly selected uninfected individuals. This
288 approach normalizes statistical power to test for statistically significant differences in HBI across
289 heterogeneous sample sets. The randomisation was repeated 100 times and the analysis confirmed
290 a significantly higher anthropophagy in sporozoite-infected individuals compared to both oocyst-
291 infected individuals and uninfected individuals in 100% of these randomisations (mean (X^2_2) =
292 12.7, $CI(X^2_2) = (7.54-21.59)$, mean (P) = 0.0043, $CI(P) = (0.00002-0.023)$); Tukey post-hoc tests:
293 sporozoite-infected *vs.* oocyst-infected individuals, this pair-wise comparison was significantly
294 different in 100 % of the randomisations: mean(P) = 0.02577, $CI(P) = (0.02559-0.02591)$;
295 sporozoite-infected *vs.* uninfected individuals, this pair-wise comparison was significantly
296 different in 90% of the randomisations: mean (P) = 0.023, $CI(P) = (5e-07 - 3e-01)$; oocyst-infected
297 *vs.* uninfected individuals, this pair-wise comparison was significantly different in 0 % of the
298 randomisations: mean (P) = 0.78, $CI(P) = (0.07-0.99)$).

299 The HBI of sporozoite-infected mosquitoes was higher than that of oocyst-infected and
300 uninfected females regardless of the village considered (infection status: village interaction: $X^2_4 =$

301 2.3, $P = 0.68$, Figure 2) or the shelter type in which mosquito females were collected (infection
302 status: shelter type interaction: $X^2_4 = 0.7$, $P = 0.95$, Figure supplement S3).

303 HBI was also significantly influenced by shelter type ($X^2_2 = 145.92$, $P < 0.0001$). Females
304 collected in animal sheds were significantly less likely to have fed on human hosts ($22.3 \pm 4\%$)
305 than females collected in unoccupied houses ($40.9 \pm 6.8\%$; Chi-square post-hoc test: $X^2_1 = 21.6$, P
306 < 0.0001) or in human dwellings ($74.5 \pm 2\%$; Chi-square post-hoc test: $X^2_1 = 385$, $P < 0.0001$).
307 Females collected in human dwellings were also significantly more likely to have fed on human
308 hosts than females collected in unoccupied houses (Chi-square post-hoc test: $X^2_1 = 96$, $P < 0.0001$).
309 HBI was significantly affected by the village ($X^2_2 = 139.5$, $P < 0.0001$). However, in Soumouso
310 only human dwellings were sampled confounding the effect of village and shelter type in this case.
311 Therefore, we carried out an analysis on the human dwellings only to compare HBIs in the three
312 villages. Mosquitoes were significantly less anthropophagic in Samendeni ($56.5 \pm 4\%$), compared
313 to Soumouso ($83.5 \pm 2.2\%$; Chi-square test: $X^2_1 = 138.8$, $P < 0.0001$) and Klesso ($77.3 \pm 9\%$; Chi -
314 square test: $X^2_1 = 12.7$, $P = 0.0004$). HBIs in Soumouso and Klesso were not significantly different
315 ($83.5 \pm 2.2\%$ vs. $77.3 \pm 9\%$ respectively; Chi-square test: $X^2_1 = 1.8$, $P = 0.18$).

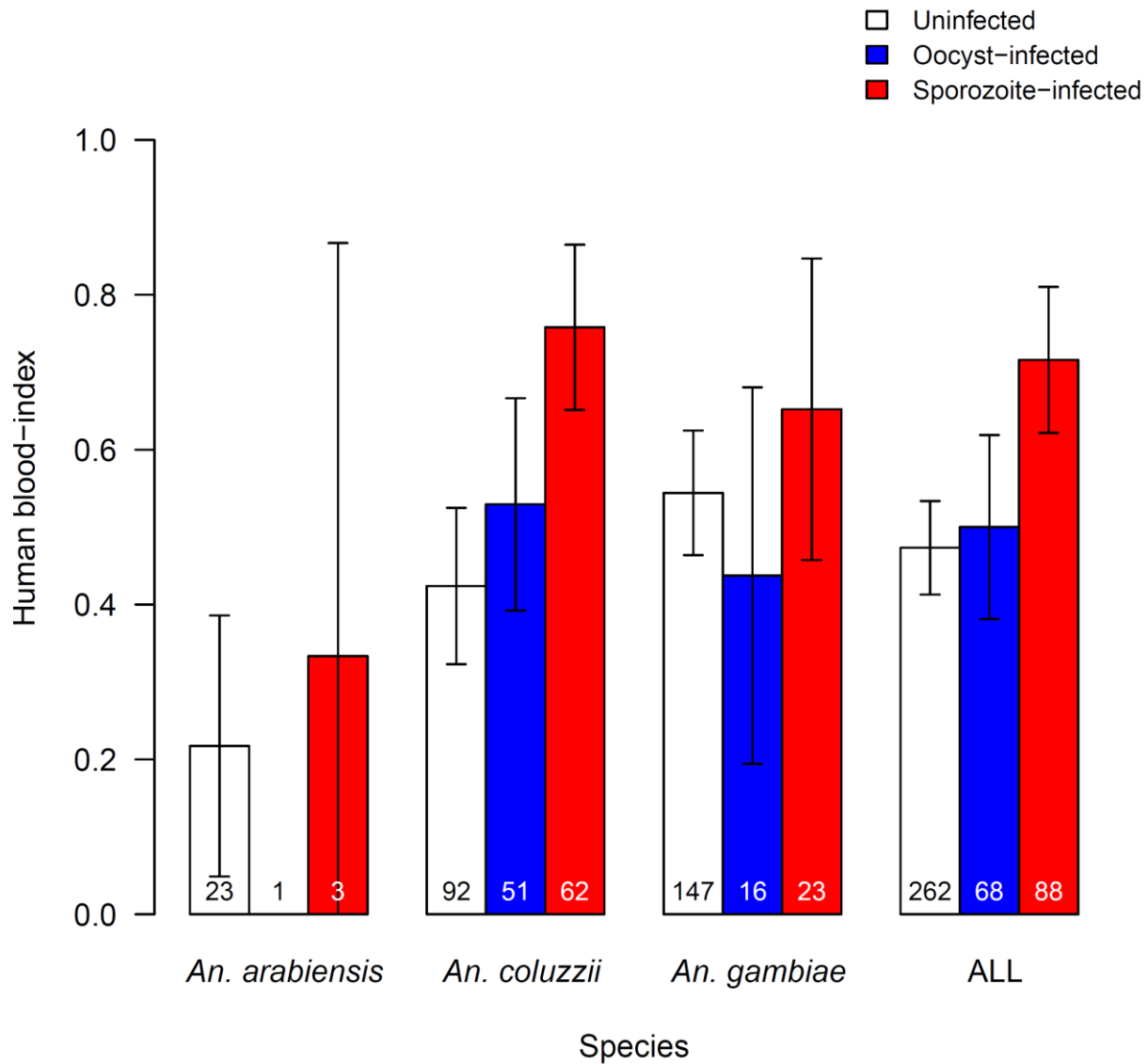


316

317 **Figure 2.** Effect of infection status on the human-blood index of *Anopheles gambiae s. l.* females
 318 expressed as the number of females fed on humans out of the total number of blood-fed females
 319 for the three sampled villages. Data show proportion \pm 95% confidence intervals. Numbers in bars
 320 indicate the total numbers of mosquitoes. Different letters indicate differences between infection
 321 status (Chi-square post-hoc tests: sporozoite-infected vs. oocyst-infected females $X^2_1=6.1$,
 322 $P=0.013$; sporozoite-infected vs. uninfected females $X^2_1=19.4$, $P<0.0001$; oocyst-infected vs.
 323 uninfected females $X^2_1=0.18$, $P= 0.67$).

324

325 A significant species variation in HBI was observed ($X^2_2 = 10.2$, $P = 0.006$; Figure 3) with
326 *Anopheles arabiensis* being significantly less anthropophagic ($22.2 \pm 15\%$, $N=27$, $OR=3.5$, 95%
327 $CI = 2.2-5.56$, $P = 0.007$) than *An. gambiae* ($54.8 \pm 7.1\%$; $N=186$, $OR=0.82$, 95% $CI = 0.71-0.95$,
328 $P = 0.19$) and *An. coluzzii* ($55.1 \pm 6.8\%$; $N=205$, $OR=0.81$, 95% $CI = 0.71-0.94$, $P=0.14$). Although
329 HBI varied among mosquito species, sporozoite-infected individuals displayed the highest
330 anthropophagy regardless of the species considered (infection status: species interaction: $X^2_4 = 4$,
331 $P = 0.42$; Figure 3 and supplementary material).



332

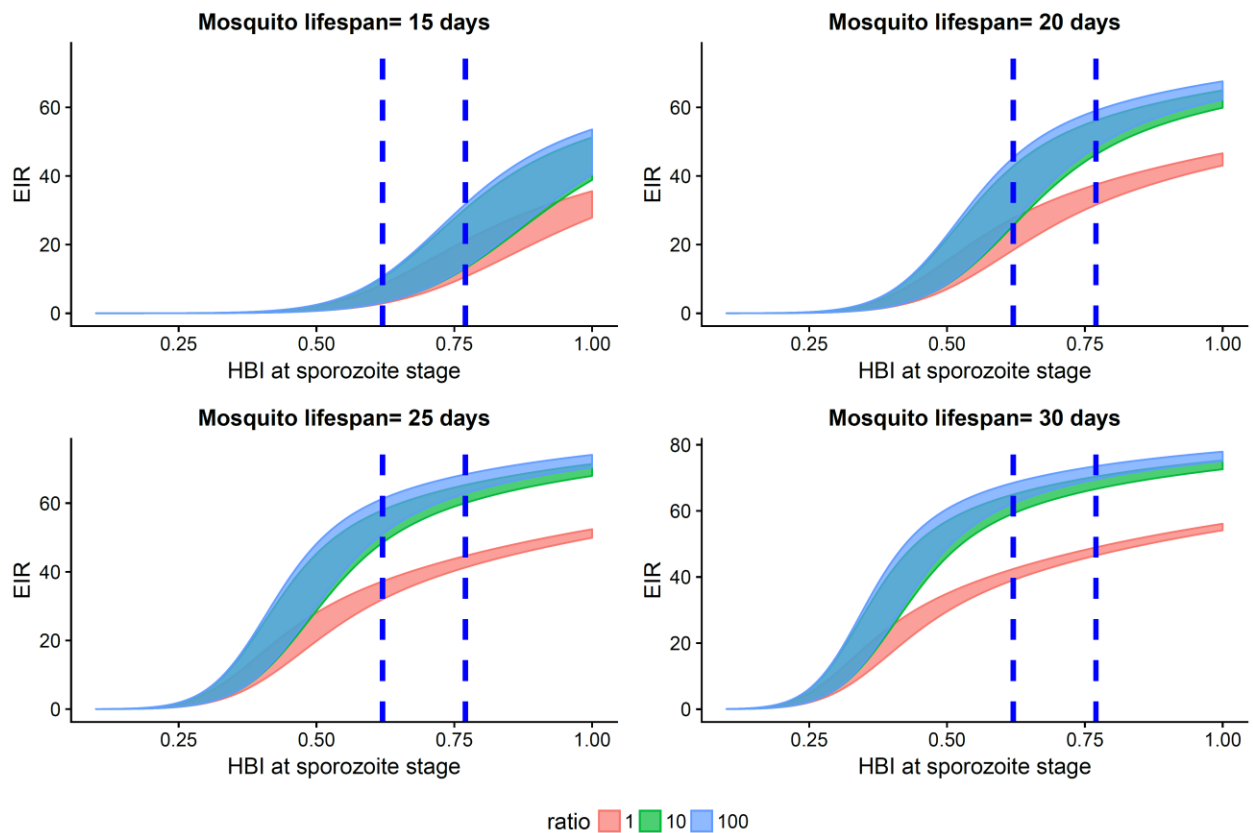
333 **Figure 3.** Effect of infection status and mosquito species on the human-blood index expressed as
 334 the proportion of females fed on humans or humans and animals out of the total of blood-fed
 335 females. Data show proportion \pm 95% confidence intervals. Numbers in bars indicate the total
 336 numbers of mosquitoes.

337

338 Finally, HBI was not significantly affected by parity, a proxy used to estimate mosquito age
339 (nulliparous females: $49.53 \pm 9\%$, parous females: $45.6 \pm 7.5\%$; $X^2_1 = 0.4$, $P = 0.52$).

340

341 ***Epidemiological consequences*** – To investigate the epidemiological impact of a higher HBI in
342 infectious females compared to oocyst-infected and uninfected females, we built a mathematical
343 model based on the experimental values observed in this study. This model assessed the impact of
344 different HBIs on the Entomological Inoculation Rate (EIR, number of infectious bites received
345 by a person during one year) at different mosquito lifespans and densities. In order to consider the
346 heterogeneity of HBI values on epidemiological consequences, the HBI of susceptible mosquitoes
347 was based on the average value whereas the HBI of exposed mosquitoes were assumed to be
348 uniformly distributed within the confidence intervals of the HBI of oocyst-infected mosquitoes
349 that were experimentally measured in this study. Then, the impact of HBI variation in infectious
350 (sporozoite-infected) mosquitoes on parasite transmission potential was explored fully (Figure 4).
351 For an average mosquito lifespan of 15 days (Figure 4a), an HBI of 0.62 in infectious mosquitoes
352 (similar to that of susceptible mosquitoes) resulted in an EIR of 4 at a low ratio of 1 (1 mosquito
353 per human), while an HBI of 0.77 (as observed here in infectious mosquitoes) resulted in an EIR
354 of 14. In other words, a 24% increase in HBI resulted in a 250% increase in EIR, everything else
355 being equal. Transmission consequences were even larger when the human-to-mosquito ratios
356 were higher (EIR = 5 vs. EIR = 19 with a ratio of 10 or 100, i.e. a 280% increase in EIR) but the
357 size of the increase in EIR for sporozoite-infected mosquitoes declined with increasing mosquito
358 longevity (Figure 4c, 4d, and supplementary material).



359
 360 **Figure 4.** Expected epidemiological consequences of HBI variation for different values of
 361 mosquito lifespan and mosquito/human ratio. The X axis represents the range of values considered
 362 for the HBI of infectious (sporozoite-infected) mosquitoes and the Y axis is the Entomological
 363 Inoculation Rate (EIR, number of infectious bites received by a person over one year) when the
 364 **HBI of exposed mosquitoes corresponds to the confidence intervals of the HBI of oocyst-infected**
 365 **mosquitoes that were experimentally measured in this study.** The ribbons represent the possible
 366 EIR values for different HBI of sporozoite-infected mosquitoes according to the confidence
 367 interval of HBI in oocyst-infected mosquitoes ($63.6\% \pm 5.7\%$) and for different values of the
 368 mosquito to human ratio. **The dashed lines represents the average value measured for susceptible**

369 mosquitoes (0.62) and for sporozoite-infected mosquitoes (0.77). Ratio=adult mosquito/human
370 densities.

371

372 **Discussion**

373 The mosquito host preference assays (experiment 1 using OBETs and BNTs,) showed that infected
374 mosquitoes displayed similar long-range attraction toward human odour as uninfected individuals
375 regardless of parasite developmental stages (oocyst vs. sporozoite), confirming previous laboratory
376 results (Nguyen *et al.* 2017). However, consistent with the hypothesis of specific manipulation,
377 the patterns of mosquito host selection (experiment 2 based on identification of mosquito blood-
378 meal sources) showed that sporozoite-infected *An. coluzzi*, *An. gambiae* and *An. arabiensis*
379 females were more likely to have fed on human than oocyst-infected and uninfected individuals.
380 By distinguishing sporozoite and oocyst infection, we ruled out the potential confounding effect
381 of a mere intrinsic mosquito characteristic. Infected mosquitoes may indeed exhibit increased
382 anthropophagy not because of being infected but just because of an innate preference for humans,
383 thus making these mosquito individuals infected. Here, individuals infected with sporozoites
384 displayed different HBI than individuals infected with oocysts, thus ruling out this possibility.
385 Because *Plasmodium falciparum* takes about 10 to 18 days to complete its development
386 (depending on temperature, (Nikolaev 1935, Shapiro *et al.* 2017, Ohm *et al.* 2018) there is an
387 increased likelihood of sporozoite infection as mosquitoes become older. This means that mosquito
388 age could be a confounding factor of infection, with infected mosquitoes displaying increased HBI
389 not because they harbour sporozoites but because they are older. Such an age effect could be
390 mediated by specific physiological requirements in old mosquitoes or by a positive reinforcement
391 (learning / memory) of feeding on humans. Our data does not support an age effect as we did not

392 find a significant effect of parity (a proxy for age) on HBI (i.e. parous and nulliparous mosquito
393 females displayed similar anthropagy).

394 The precise mechanisms responsible for increased anthropagy in sporozoite-infected
395 mosquitoes is not yet clear, but at least three hypotheses can be proposed. First, malaria parasites
396 might manipulate mosquito short-range behaviours only, whereas at longer range when
397 mosquitoes rely mainly on CO₂ and other volatile odours (Gillies 1980, Mboera and Takken 1997,
398 Gibson and Torr 1999, Cardé and Gibson 2010), sporozoite-infected mosquitoes display similar
399 preferences to uninfected and oocyst-infected individuals. At short range, mosquitoes rely on other
400 cues including visual stimuli, moisture, heat and skin emanations (Gibson and Torr 1999, Cardé
401 and Gibson 2010, Takken and Verhulst 2013). These stimuli can be host specific, and inform of
402 host suitability for parasite development before the mosquito engages in selection and eventually
403 in feeding. In addition to a possible preferential short-range attraction of sporozoite-infected
404 mosquitoes toward host species suitable for parasite development, there could also be short-range
405 repellence by unsuitable host species.

406 Second, the parasite may induce changes in the vector such as an alteration of
407 microhabitat choice to spatially match the habitat of the suitable host. This could be achieved
408 through parasite manipulation of mosquito endophagic/philic behaviours resulting in a higher
409 degree of indoor -feeding and -resting of sporozoite-infected females. For example, infectious
410 mosquitoes may exhibit an enhanced tendency to enter (or a decreased tendency to exit) house
411 interstices regardless of emitted odours.

412 Third, the parasite may induce changes in the vector such as an alteration of time activity
413 in order to temporally match the time of rest or activity of the suitable host. Mosquitoes exhibit
414 circadian rhythms in many activities such as flight, host-seeking, swarming, egg-laying, etc.

415 (Rund *et al.* 2016). There is mounting evidence that, following bed-net introduction, malaria
416 vectors can display an increased tendency to feed outdoors (Russell *et al.* 2011) or bite earlier in
417 the evening or later in the morning (Moiroux *et al.* 2012). Accordingly, *P. falciparum* could
418 manipulate mosquito host-seeking rhythms in a way that increases bites on unprotected people.
419 Testing this hypothesis would require sampling mosquitoes at distinct periods and comparing the
420 proportion of uninfected, oocyst-infected and sporozoite-infected vectors among samples.

421 Sporozoite-induced change in mosquito host selection occurred in three major and related
422 mosquito vectors, namely *An. coluzzii*, *An. gambiae* and *An. arabiensis*. This suggests that
423 manipulation likely already occurred in the common ancestor of these three species and that the
424 parasites might exploit a physiological pathway common to all three mosquito species to modify
425 its vector host choice.

426 Transmission models generally assume that uninfected and infected vectors have similar
427 preferences for human (Smith and Ellis McKenzie 2004, Smith *et al.* 2012). This study suggests
428 that this assumption may not be valid and that these models possibly underestimate transmission
429 intensity. Our modelling approach confirms that HBI increases in infectious mosquitoes can have
430 a dramatic impact on disease transmission. In particular, if we consider mosquito lifespans relevant
431 to natural settings (i.e. 15 to 20 days; Gillies 1961, Gillies and Wilkes 1965, Saul *et al.* 1990,
432 Charlwood *et al.* 1997, Killeen *et al.* 2000), the transmission potential was almost multiplied by 3
433 when the HBI increased from 0.62 to 0.77 *i.e.* the value observed for the infectious mosquitoes in
434 this study. For many mosquito–*Plasmodium* associations including *An. gambiae s.l.*-*P. falciparum*,
435 the duration of the parasite’s development within the mosquito is as long as the insect vector’s
436 average lifespan (Gillies 1961, Gillies and Wilkes 1965, Saul *et al.* 1990, Charlwood *et al.* 1997,
437 Killeen *et al.* 2000, WHO 2014). This means that most mosquitoes do not live long enough to

438 transmit the disease, and hence that feeds taken by infectious mosquitoes on unsuitable host species
439 would have disastrous consequences for parasite fitness. The model suggests that the benefits of
440 specific manipulation should be particularly high in vectorial systems in which transmission
441 opportunities are rare (short vector lifespan, relatively long parasite development period, and
442 diverse blood sources).

443 In conclusion, our results suggest that the human malaria parasite *P. falciparum* evolved
444 the ability to enhance transmission toward humans, the appropriate host species, by increasing
445 mosquito anthropophagy (or decreasing zoophagy) with potentially profound public health
446 consequences. Future laboratory and field studies will be essential to confirm these results and to
447 better understand the epidemiological, ecological and evolutionary consequences of parasite
448 manipulation of vector behaviours.

449

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456

457 **Data availability**

458 Raw data are available on zenodo: <https://doi.org/10.5281/zenodo.1296744>.

459 Statistical analyses are available as supplementary file.

460

461 **Competing interests**

462 We have no competing interests.

463

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468

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