Impact of mosquito bites on asexual parasite density and gametocyte prevalence in asymptomatic chronic Plasmodium falciparum infections and correlation with IgE and IgG titers.

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1 Impact of mosquito bites on asexual parasite density and gametocyte prevalence in asymptomatic

2 chronic *Plasmodium falciparum* infections and correlation with IgE and IgG titres

3

4 Running title: Mosquito bites and chronic *P. falciparum* infections

5

6 Ramatoulaye Lawaly[1], Lassana Konate[2], Laurence Marrama[3], Ibrahima Dia[1], Diawo Diallo[1],

7 Fatoumata Diène Sarr[3], Bradley S. Schneider[4†], Isabelle Casademont[4], Mawlouth Diallo[1], Paul T.

8 Brey[5], Anavaj Sakuntabhai[4,6], Salah Mecheri[7], Richard Paul[1,4‡].

9

10 [1] Institut Pasteur de Dakar, Laboratoire d'Entomologie Médicale, BP 220, Dakar, Senegal.

11 [2] Faculté des Sciences et Techniques, UCAD, BP 5005, Dakar, Senegal.

12 [3] Institut Pasteur de Dakar, Unité d'Epidémiologie, Dakar, Senegal.

13 [4] Institut Pasteur, Unité de Génétique Fonctionnelle de Maladies Infectieuses, 25 rue de Dr. Roux, F-

14 75724, Paris cedex 15, France.

15 [5] Institut Pasteur du Laos, 1 rue Louis Pasteur, Post Office Box 3560, Vientiane, Lao PDR.

16 [6] Center of Excellence for Vector and Vector-Borne Diseases, Faculty of Science, Mahidol University,

17 Bangkok, Thailand.

18 [7] Institut Pasteur, Unité des Réponses Précoces aux Parasites et Immunopathologie, 25 rue de Dr.

19 Roux, F-75724, Paris cedex 15, France.

[†] Current address : Global Viral Forecasting, 1 Sutter Street, Suite 600, San Francisco, 94104, California, USA

[‡] Corresponding author. Mailing address: Institut Pasteur, Unité de Génétique Fonctionnelle de Maladies Infectieuses, 25 rue de Dr. Roux, F-75724, Paris cedex 15, France. Phone: 33(0)140613626; Fax: 33(0)145688229; Email: rpaul@pasteur.fr

20 **ABSTRACT**

21 An immuno-modulatory role of arthropod saliva is well-documented, but the evidence for an
22 effect on *Plasmodium* spp. infectiousness remains controversial. Mosquito saliva may orient the
23 immune response towards a Th2 profile, thereby priming a Th2 response against subsequent
24 antigens, including *Plasmodium*. Orientation towards a Th1 *vs.* a Th2 profile promotes IgG and IgE
25 proliferation respectively, where the former are crucial in the development of an efficient anti-
26 parasite immune response. Here we assessed the direct effect of mosquito bites on *Plasmodium*
27 *falciparum* asexual parasite density and the prevalence of gametocytes in chronic, asymptomatic
28 infections in a longitudinal cohort study of seasonal transmission. We additionally correlated these
29 parasitological measures with IgE and IgG anti-parasite and anti-salivary gland extract titres.
30 Mosquito biting density was positively correlated with asexual parasite density, but not asexual
31 parasite prevalence, and was negatively correlated with gametocyte prevalence. Individual anti-
32 salivary gland IgE titres were also negatively correlated with gametocyte carriage and were strongly
33 positively correlated with anti-parasite IgE titres, consistent with the hypothesis that mosquito bites
34 pre-dispose individuals to develop an IgE anti-parasite response. We provide evidence that mosquito
35 bites have an impact on asymptomatic infections and differentially so for asexual and sexual parasite
36 production. Increased research focus on the immunological impact of mosquito bites during
37 asymptomatic infections is warranted, to establish whether strategies targeting the immune response
38 to saliva can reduce the duration of infection and onward transmission of the parasite.

39

40 **INTRODUCTION**

41 Parasitic microorganisms, such as *Plasmodium* spp., use a variety of mechanisms to subvert the host
42 immune defences (31). Manipulation of the host to reduce an effective immune response is one such
43 method by which parasitic microorganisms can successfully exploit its host (1, 30). For arthropod-
44 borne organisms, an immuno-modulatory role of arthropod saliva has been reported for arboviruses
45 (24, 46) and protozoa including *Leishmania* (3, 14), *Trypanosoma* (32) and *Plasmodium* (13). Whilst prior
46 exposure to arthropod saliva can exacerbate the infection, immunity against saliva antigens has also
47 been shown to protect against a severe outcome of disease in both *Leishmania* (23) and *Plasmodium*
48 (16). Interestingly, immunity to saliva does not impact upon sporozoïte infectivity (26).

49 It is recognised that the type of immune balance driven by the parasite operates at a very early stage
50 post parasite delivery. The response of sentinel cells, such as dendritic cells, thus determines the
51 evolution of the immune response and can lead to protection, tolerance or immuno-pathology (2).
52 Saliva contains pharmacologically active proteins and peptides (43), which provoke a localised allergic
53 reaction in the skin, and injection of saliva into the skin during a mosquito bite induces the
54 production of IgE and IgG antibodies (8, 9), as well as dermal hypersensitivity reactions (21, 42).
55 This suggests that the saliva can orient the immune response towards a Th2 profile. Dendritic cells
56 that are oriented to a Th2 phenotype by an antigen are more susceptible to orient the immune
57 response towards a Th2 profile when confronted by a second antigen (12). Thus, saliva could orient
58 the response mounted against the arthropod-borne pathogen. Orientation of the immune response
59 towards a Th1 profile is crucial for immunity to intracellular pathogens (35), whereas orientation
60 towards a Th2 profile drives immunity to extracellular pathogens and antigens resulting in class
61 switching giving rise to IgE-producing B cells (55).

62 The acquisition of immunity to the human lethal malaria parasite *Plasmodium falciparum* develops
63 very slowly and is not sterilising. Even in zones where the transmission intensity is high, the
64 development of immunity only results in a premunition leading to a reduction in the number of
65 clinical episodes and the progressive control of parasite density. Cytophilic immunoglobulins (IgG1
66 & IgG3), which are capable of eliminating the parasites by opsonisation, play an important role in
67 this premunition (51). Although individuals living in malaria endemic regions have elevated total and
68 *P. falciparum* parasite-specific IgE levels, the role of this class of immunoglobulin is unclear. Elevated
69 levels are observed in severe acute clinical episodes, suggesting a pathogenic role of IgE (38),
70 whereas high levels in asymptomatic infections are seemingly protective against subsequent clinical
71 episodes (4).
72 Studies on immuno-modulation have focussed on the direct interaction between the host and
73 pathogen during the infectious process and immediate consequences thereafter (e.g. 25, 26, 50).
74 Surprisingly, however, no attention has been paid to the longer-term consequences of immuno-
75 modulation that impact upon an existing infection. In malaria endemic areas of highly seasonal
76 transmission, individuals can carry *P. falciparum* parasites without symptoms for the duration of the
77 non-transmission season. Production of gametocytes, specialised sexual parasite stages, is required
78 for transmission from man to the mosquito. Gametocyte production is associated with non-specific
79 immune responses occurring during febrile episodes of symptomatic infections. Specific immune
80 responses have also been suggested to induce gametocyte production. Gametocytes are induced
81 following the addition of lymphocytes from naturally infected Gambian children, but not after
82 addition of the same components from European controls (48). Furthermore, there is some
83 suggestion that parasites increase their conversion rate to gametocytes in individuals with acquired
84 immunity (18).

85 Here we examine the immuno-modulatory impact of mosquito saliva in a malaria endemic setting
86 and address the hypothesis that saliva impacts upon existing malaria infections over the long-term
87 through orientation of the Th1/Th2 response as revealed through specific IgG3 and IgE. Specifically,
88 we carried out a family-based longitudinal cohort study in a region of endemic *P. falciparum* malaria in
89 Senegal to determine whether mosquito biting intensity and individual immunoglobin profiles are
90 associated with quantitative parasite phenotypes in chronic, long-term, asymptomatic infections.

91

92 **MATERIALS AND METHODS**

93 **Study sites, subjects and ethical clearance**

94 A family-based longitudinal cohort study was performed in 2005 in Gouye Kouly (N 14°43, W
95 16°36), Senegal. Family structures were constructed by using a questionnaire, interviewing each
96 individual or key representatives of the household to obtain both demographic information such as
97 birth date, age, sex and genetic relationships between children, their parents, and sometimes their
98 grandparents or non-relatives in the same household, and other households. The population was
99 composed of 482 individuals, of which 387 (80.3%) were enrolled into the study. The majority of
100 individuals were Serere ethnic group. Transmission is highly seasonal in this site with an
101 Entomological Inoculation Rate measured at approximately 2 infectious bites per person per year. *P.*
102 *falciparum* prevalence rates in humans varied from 8% to 15% in the dry and wet season. The project
103 protocol and objectives were carefully explained to the assembled village population and informed
104 consent was individually obtained from all subjects by signature on a voluntary consent form written
105 in both French and in Wolof. The request for volunteers to perform mosquito landing catches, as a
106 specific task within the protocol, was made and discussed during the village meeting and with each
107 individual prior to obtaining written consent. Such volunteers were not placed under
108 chemoprophylaxis given the long length of the study and the estimated high frequency of Glucose-6-

109 Phosphate Dehydrogenase deficiency in the region. The installation of a health clinic in the study site
110 enabled treatment of all clinical cases of malaria with appropriate antimalarial treatment according to
111 the recommendation of the Malaria Division, Ministry of Public Health. The protocol was approved
112 by the Ethical Committee of the Ministère de la Santé du Senegal.
113
114 **Blood sample collection for *P. falciparum* parasite analyses**
115 An intensive sampling schedule was implemented: prior to the rains in June and then every week
116 for 8 weeks following the onset of the rains (first week of July) and after the end of the transmission
117 season in November. At each time point a thick blood smear was taken from all individuals. In the
118 June survey and every two weeks from July, approximately 300-500 μL of blood were taken by finger
119 prick from each individual in an EDTA microtainer (Sarstedt), of which 200 μL were mixed with in
120 one mL TRIzol® (Invitrogen), kept on dry ice and then frozen at -80°C for RNA extraction. The
121 remainder of the sample was used for DNA extraction and serological analyses. Following DNA
122 extraction and PCR amplification, all samples that were found to be positive for *P. falciparum* were
123 then analysed for the presence of gametocytes by RT-PCR. The cohort was randomly divided into
124 two groups (by household) such that half the cohort provided such a blood sample every week of the
125 8-week continuous survey. A final survey on all the population was carried out after the rainy season
126 in November, at which time another 300-500 μL blood sample was taken for immunoglobulin
127 analysis. In all cases parasite positivity was established as follows. Thick and thin blood smears were
128 prepared and stained by 3% Giemsa stain. Blood smears were examined under an oil immersion
129 objective at ×1000 magnification by the trained laboratory technicians and 200 thick smear fields
130 were examined before being declared negative for asexual or gametocyte stage parasites. The total
131 number of leucocytes and parasites were counted and a parasite/leucocyte ratio established. The

132 number of parasites per microlitre was then estimated on the basis of a mean number of 8000
133 leucocytes per microlitre of blood.

134 **PCR and RT-PCR for *P. falciparum* gametocyte detection**

135 DNA was extracted from all samples using the standard phenol-chloroform extraction method and
136 amplified using the ssrRNA gene nested PCR method of Snounou *et al.*, 1993 (49). RNA extraction
137 was performed from the TRIzol® (Invitrogen) conserved samples of those found positive, following
138 the protocol recommended by the manufacturer. The extracted RNA was directly analysed or stored
139 at -80ºC. The RT-PCR was carried out as described previously (27). Briefly, "*Plasmodium falciparum*
140 meiotic recombination protein DMC1-like protein" gene (AF356553) was selected because it is
141 exclusively expressed in gametocytes (28) and contains introns. Primers were thus selected spanning
142 an exon-exon junction, amplifying a 101 bp segment, in the middle of which a probe was designed,
143 using Primer3 software (44). Primer sequences were: forward primer GAM8_F 5'
144 ATATCGGCAGCGAAAATGTGT 3'; reverse primer GAM8_R 5'
145 GACAATTCCCCTCTTCCACTGA 3' and probe GAMPRO 5'

146 (6-Fam)TGCCCTTCTCGTAGTTGATTCGATTATT(BHQ1) 3'. cDNA was synthesised and the
147 reaction primed with GAM8_R. Eight µL of extracted RNA was mixed with buffer, dNTPs (final
148 concentration 1 mM), RNase-free water, AMV Reverse transcriptase (20U; Promega) and
149 Ribonuclease inhibitor (20U; Promega). Amplification cycle conditions were: 10 min at 65°C, 60 min
150 at 42°C, 5 min at 95°C. Quantification of cDNA was carried out using a fluorescent probe assay.
151 Two µL of synthesised cDNA was mixed with 2X mastermix (ABGene), GAM8_R (final
152 concentration: 400 nM), GAM8_F (final concentration: 400 nM), GAM8_PRO (final concentration:
153 300 nM) and sterile water. The reaction was analysed with a Rotor Gene® real-time PCR machine
154 (Corbett Research). Each sample was analysed in triplicate. A dilution series containing 1000, 100, 10,
155 1 and 10^{-1} gametocytes /µL were used as controls.

156 **Entomological surveys**

157 Indoor and outdoor mosquito landing catches on volunteers were performed in 5 locations within
158 the village for 2 nights (from 7pm to 7am) every week during the rainy season and 2 nights monthly
159 for the rest of the year. The five sites were selected to provide good coverage of the village. Species
160 identification was performed on each individual mosquito the following day. Anophelines were
161 identified using the key of Diagne *et al.*, (1994)(15). The PCR technique of Paskewitz & Collins
162 (1990) (36) was used to differentiate the members of the *Anopheles gambiae* species complex. The
163 culicines were identified using the key of Edwards (1941)(19).

164

165 **Salivary gland extract preparation for ELISA**

166 Salivary glands of *Anopheles gambiae* (Yaounde strain) were dissected under sterile conditions, placed
167 in 1×PBS and sonicated 5 × 4 min. The solution was then centrifuged at 8,000 × *g* for 15 min at
168 4°C. The protein concentration was determined by Nanodrop and diluted in 1×PBS to a
169 concentration of 5 μg / mL.

170 **Parasite (*P. falciparum*) preparation for ELISA**

171 *P. falciparum* (strain 89F5, Palo Alto) *in vitro* intra-erythrocytic cultures that were schizont-rich were
172 mixed with water (1 packed cell volume of red blood cells to 4 volumes of water) and an equal
173 volume of lysis buffer (10 mM Tris pH8, 0.4 M NaCl, EDTA 10 mM, 2% triton X-100) and
174 incubated at 4°C for 15 min. The solution was then centrifuged at 4°C for 10 min at 8,000 × *g* and
175 the supernatant reserved for analysis.

176 **IgE ELISA**

177 Salivary gland (or parasite) extract was diluted in 0.1 M $NaHCO_3$ (pH 9.6), 50 μL added per well
178 and incubated at 37°C for 1 hour. Plates were then washed 3× in wash buffer (1×PBS/0.05%
179 Tween-20). 100 μL blocking buffer (1×PBS/1% Bovine Serum Albumin) was added per well,

180 incubated at 37°C for 1 hour and then washed 3×. Serial dilutions of approximately 5% of pre-
181 season plasma samples enabled the optimal dilution for measurement of IgE titres. Each plasma
182 sample was diluted 1/5 in 100 μL blocking buffer and 50 μL add per well for 2 duplicate wells. Plates
183 were incubated overnight with gentle agitation at room temperature. The next day, plates were
184 washed 5× and 50 μL goat IgG anti-human-IgE immunoglobulin (pre-coupled with alkaline
185 phosphatase) diluted 1/400 in blocking buffer (Sigma-Aldrich, Saint Quentin Fallavier, France) was
186 added per well and incubated for 2 hours at 37°C. Plates were then washed 5× and 50 μL PNPP (4-
187 nitrophenylphosphate disodium salt hexahydrate)(Sigma-Aldrich, Saint Quentin Fallavier, France),
188 the substrate of alkaline phosphatase, dissolved in 0.1 M Tris-HCl pH 8.8 at 1 mg / mL, was added
189 per well. Plates were placed in the dark for 3 hours and the reaction terminated with 25 μL 1 N
190 NaOH and read at 410 nm.

191 **IgG ELISA**

192 Salivary gland (or parasite) extract was diluted in 0.1 M $NaHCO_3$ (pH 9.6), 50 μL added per well
193 and incubated at 37°C for 1 hour. Plates were then washed 3× in wash buffer (1×PBS/0.05%
194 Tween-20). 100 μL blocking buffer (1×PBS/1% Bovine Serum Albumin) was added per well,
195 incubated at 37°C for 1 hour and then washed 3×. Again serial dilutions of approximately 5% of pre-
196 season plasma samples enabled the optimal dilution for measurement of IgG titres. Each plasma
197 sample was diluted 1/50 (for IgG 3 (mouse monoclonal ZG4) & IgG 4 (mouse monoclonal RJ4)) or
198 1/100 (IgG total (mouse monoclonal R10Z8E9)) in 100 μL blocking buffer and 50 μL add per well
199 for 2 duplicate wells. All monoclonals from Skybio Ltd., Wyboston, Bedfordshire, United Kingdom.
200 Plates were incubated overnight at 4°C. The next day, plates were washed 5× and 50 μL mouse anti-
201 human-IgG (diluted 5/1000 in blocking buffer for IgG 3 & 4; 2/1000 for IgG total.) and incubated
202 for 2 hours with gentle agitation at room temperature. Plates were then washed 5× and 50 μL rabbit
203 anti-mouse coupled with HRP (Horse Radish Peroxidase) (DAKO Ltd., Trappes, France) at 1/2000

204 in blocking buffer was added per well. Plates were then washed 5× and 50 μL O-phenylenediamine
205 (Sigma-Aldrich, Saint Quentin Fallavier, France), the substrate of HRP, dissolved in citrate buffer
206 (5.1 mL 0.1 M citric acid, 14.9 mL 0.1 M Na_3Citrate, pH 5.1) at 1 mg / mL plus 2 μL / mL H_2O_2
207 (Sigma-Aldrich, Saint Quentin Fallavier, France) was added per well. Plates were then placed in the
208 dark for 7-10 min and the reaction stopped with 25 μL 1 N HCl and read at 490 nm.

209 **Statistical analyses**

210 Statistical analyses and model fitting were conducted using the statistical package Genstat 7.1 (22).
211 Mean biting rates (averaged over the two days of each weekly or monthly survey) were not normally
212 distributed (Shapiro-Wilk test P<0.001) and thus were boxcox transformed. Immunoglobulin titres
213 were similarly not normally distributed (Shapiro-Wilk test P<0.001) and thus were also boxcox
214 transformed. When parasite density was used as an explanatory variable it was Ln+1 transformed.
215 For the analysis of the effect of mosquito biting rate (anopheline only or all mosquitoes) on
216 individual parasite density, a Generalized Linear Mixed Model (GLMM) with a Poisson error
217 structure (loglinear regression) was fitted with individual person as a factor in the random model, to
218 account for multiple measures at different time points on the same individual. Additional fitted
219 explanatory variables were gender and age as a continuous variable. For the analysis of the effect of
220 mosquito biting rate (anopheline only or all mosquitoes) on parasite prevalence rates and the
221 presence/absence of gametocytes in individual blood smears, binomial error structures were
222 implemented (thus a logistic regression). (Ln+1) transformed parasite density was fitted as an
223 additional explanatory factor in the gametocyte prevalence analysis.

224 Analyses of the effect of immunoglobulin titre on parasite density, parasite prevalence rates and the
225 proportion of parasite infections with gametocytes were similarly performed fitting GLMM with
226 Poisson or binomial error structure. Immunoglobulin titre measured in June, prior to transmission
227 season was used. In addition, because the duration of gametocyte carriage for a single infection in

228 endemic settings can last up to 30 days (6, 17), a more conservative analysis on gametocyte positivity
229 was performed: i.e. we analysed the effect of immunoglobulin titre on whether an individual ever
230 carried gametocytes by fitting a Generalized Linear Model (GLM) and weighting for the number of
231 samples for each person. F-statistics in the GLM and Wald statistics, which approximate to a χ^2
232 distribution, in the GLMM were established.

233 The following data exclusion criteria were implemented prior to analyses: (i) data from the week of
234 and three weeks following any individual that had suffered a clinical malaria episode; (ii) data from
235 the 2 weeks following an absence from the village. These exclusion criteria enable the analysis of only
236 confirmed asymptomatic infections.

237

238 **RESULTS**

239 **Parasite and gametocyte prevalence rates and asexual parasite density**

240 During nine weeks of survey, a total of 185 samples were found to be positive for *P. falciparum* by
241 thick smear alone (N=178) or smear and PCR (N=185). Mean parasite density was 4.5 parasites /µl
242 (S.D. 1.4, N=178), increasing from a mean of 3.5 (SD 0.2) pre-transmission season to a mean of 5.0
243 (SD 0.5) during the transmission season. 145 people were positive at least once (range 1-4, median 1).
244 There were only 8 smears positive for gametocytes, zero of which occurred in the pre-transmission
245 season (June). Same day RNA samples for RT-PCR detection of gametocytes were available for 121
246 of the 185 parasite positive samples from 82 of the 145 individuals. 79 of these samples from 49
247 individuals were gametocyte positive; thus 42 samples from 33 individuals were gametocyte negative.
248 For those parasite positive samples for which a same day RNA sample was not available, RNA
249 samples from the week before and the week after were analysed. Two of these 119 samples were
250 found to be gametocyte positive, both from the same individual. In addition, 42 randomly selected
251 RNA samples that were parasite negative were tested for gametocytes; none were found to be

252 positive. 88% of parasite positive infections prior to the rains had gametocytes (none were smear
253 positive) and comprised 24 of the 81 total gametocyte positive infections. Of the remaining
254 gametocyte positive samples, 30 were from apparently new infections, 12 from infections from
255 previous weeks that were at that time gametocyte negative and 15 that were at that time gametocyte
256 positive.

257 **Mosquito biting rates and effect on parasitological measures**

258 All species mosquito biting rate ranged from 14 bites per person per night during the dry season
259 survey to over 100 during the rains. Anopheles biting rates ranged from zero during the dry season
260 to 3 bites/person/night during the rains. The most prevalent mosquitoes were *Aedes aegypti* and *Aedes*
261 *furcifer*, accounting for 71% of all the mosquitoes captured and *Culex tritaeniorhyncus* and *Culex*
262 *quinquefasciatus* accounting for 22%. Other species captured were *Aedes metallicus*, *Aedes unilineatus*,
263 *Aedes vittatus*, *Aedes neavei*, *Aedes decens*, *Aedes vexans*, *Aedes argenteopunctatus*, *Aedes sudanensis Culex lutzia*
264 *tigripes, Culex perfuscus, Culex antennatus, Culex univitatus*. The only anopheline species observed was
265 *Anopheles arabiensis*, accounting for 3% of all the mosquitoes captured. Despite some local variation,
266 coefficient of variation (CV) in biting density, whether for culicines or anophelines, was <1,
267 suggesting low variance in mosquito biting rates across the five sampling stations in the study site.

268 The number of anopheline bites per person was found to be positively associated with a significant
269 increase in parasite density for the same week (P=0.001), explaining 9% of the overall variation
270 (Table 1). Age was negatively associated with parasite density (P=0.002). Although similar
271 associations were found when using anopheline biting density from the week or 2 weeks before, they
272 were less significant. Age was marginally negatively associated with parasite prevalence rates
273 (P=0.012); by contrast, there was no effect of mosquito biting rates. The proportion of parasite
274 positive infections that were also gametocyte positive was found to be negatively associated with
275 anopheles biting rates of that same week (P<0.001), explaining 10% of the observed variation in

276 gametocyte rates. Parasite density was also negatively correlated with gametocyte presence (P<0.001);
277 there was no effect of age (P=0.50). As before, the association was less significant when using
278 anopheline biting density from the week or 2 weeks before. When using all species mosquito biting
279 density, the same patterns were found, but were consistently less significant. All species biting rates
280 were highly positively correlated with anopheline biting rates (r=0.85).

281 **Impact of immunoglobulin titres on parasitological measures**

282 We then tested the effect of immunoglobulin titres before the transmission season on parasite
283 phenotypes, both including and excluding the impact of anopheline biting observed above. Pre-
284 season IgE anti-salivary gland extract (anti-SG) titres were strongly negatively associated with the
285 proportion of parasite positive infections also harbouring gametocytes (P=0.004) (Fig. 1) and this
286 association was increased when co-analysing with the number of anopheline bites (P=0.002)(Table 2).
287 Similarly, these titres were negatively associated with individuals ever carrying gametocytes when
288 parasite positive (P=0.003). No other tested immunoglobulin titres against salivary glands or
289 parasites had any significant association with any parasite phenotype. The effect of an
290 immunoglobulin class may be influenced by the titres of other Igs. Notably the effect of IgE titres
291 can be strongly muted by IgG4 titres. Ratios of anti-SG IgG4 to IgE were not found to be associated
292 with any parasite phenotype.

293 **Immunoglobulin profiles and correlation among anti-salivary gland extract and ant-**
294 **parasite immunoglobulin titres**

295 IgE anti-parasite titres were highly positively correlated with IgE anti-SG titres (r=0.72) (Fig. 2).
296 There were no other strong correlations (r>0.5) among immunoglobulins. IgE and IgG4 anti-SG
297 decreased with age (P=0.002 and P<0.001 respectively), whereas IgG3 anti-parasite titres increased
298 with age (P<0.001); IgE anti-parasite showed a trend to decrease with age, but this was not
299 significant (P=0.15). There were several striking seasonal changes in immunoglobulin titres. Notably

300 IgE & IgG4-anti-SG increased as did anti-parasite IgG3. Paradoxically, however, anti-parasite IgE
301 levels diminished. The differences in anti-parasite IgE titre (November *vs.* June) strongly correlated
302 with the IgG3 anti-parasite titre in June (r=0.77); i.e. those individuals who showed the lowest
303 decrease in anti-parasite IgE titre had the highest June IgG3 anti-parasite titre.

304

305 **DISCUSSION**

306 In this study, we observed that mosquito biting density was strongly positively associated with
307 parasite density but with no impact on parasite prevalence rates. This seasonal trend has been noted
308 before in a very different setting in Liberia (34). Although new infections could lead to such
309 increased parasite density, the absence of any change in parasite prevalence rates and the association
310 with mosquito biting rates of the same week, rather than the week before (given the development
311 period of at least 1 week in the liver) argue against this. Furthermore, this confirms observations in
312 an experimental mouse model, which found that mosquito bites accelerated malaria parasite asexual
313 replication rate even during primary infections (5), although this was not confirmed (47).

314 We found a strong negative impact of mosquito bites on the production of gametocytes, but which
315 was notably due to the large percentage of dry season (when anophelines were absent and other
316 mosquito spp. at much reduced abundances) infections that carried gametocytes. Time since
317 infection (41) and time since treatment (29) have previously been highlighted as increasing
318 gametocyte carriage in symptomatic infections. The relevance of this to chronic, asymptomatic
319 infections is unclear. We are unable to differentiate the age of infection and the much reduced level
320 of mosquito biting during the dry season in this study. However, as discussed below, the strong
321 negative correlation between anti-SG titres and the occurrence of gametocytes does suggest some
322 influence of mosquito bites on gametocyte production.

323 Individual anti-SG IgE titre was also found to be strongly positively correlated with IgE anti-
324 parasite titre. This is consistent with the hypothesis that mosquito bites pre-dispose individuals to
325 develop an IgE anti-parasite response, potentially by orientation of the immune response to a Th2
326 profile (53). Such an orientation of the immune response would be expected to lead to a reduced
327 Th1 type environment resulting in a lower acquisition of asexual parasite-targeting effectors and thus
328 a more fertile ground for asexual parasite survival. This is consistent with suggestive evidence that
329 individuals with higher acquired immunity induce a higher level of gametocyte conversion in
330 infecting parasites (7). Although IgG3 anti-parasite titres did not impact upon gametocyte prevalence
331 or parasite density, they increased with age, which itself had a significant negative impact on parasite
332 density. Interestingly, IgG3 anti-parasite titres were negatively correlated with the seasonal decrease
333 in IgE anti-parasite titres. Such a seasonal decrease might be indicative of exhaustion of circulating
334 IgE, potentially being bound to effector cells. If this is the case, then there is clearly competitive
335 interference of anti-parasite IgE by anti-parasite IgG3, with potential consequences on the parasite.
336 There is conflicting evidence concerning a role for mosquito saliva in facilitating the initiation of an
337 infection by *Plasmodium* sporozoites (26, 50). Here, we provide evidence that mosquito saliva has a
338 demonstrable impact on the parasite during the chronic asymptomatic stage of infection. We
339 previously proposed that, in malaria endemic regions of seasonal mosquito activity, such chronic
340 infection parasites may respond to the effects of anopheline bites by producing gametocytes in order
341 to transmit rapidly after the expansion of the anopheline population (37). The general effect of all
342 mosquito spp. bites on parasite phenotypes and the predominance of culicine mosquitoes observed
343 here, suggests that parasites may respond generally to increased mosquito bites. Parasites need to
344 produce gametocytes to transmit to mosquitoes and they are generated from the circulating asexual
345 parasite population. Consequently, parasites are faced with a trade-off between, on the one hand,
346 producing sufficient asexual parasites to maintain an effective population size to withstand

347 immunological destruction, and, on the other, generating sufficient gametocytes to be able to
348 transmit. Chronic infection parasites persist at very low densities, often only detectable by PCR. The
349 parasite must therefore generate gametocytes (at a density of at least 1/μl) from a very low density
350 asexual population. Accelerated parasite replication following anopheline mosquito bites would
351 provide parasites with a sufficient biomass to generate gametocytes at high enough densities to
352 ensure transmission, a phenomenon observed in the mouse model studies (5). An alternative
353 explanation for such accelerated parasite replication is that it is a parasite response enabling it to
354 outcompete co-infecting clones either for resources (i.e. direct competition) or for "enemy-free"
355 space (i.e. avoid the immunological consequences induced by another clone - apparent competition
356 (33)). Investment in asexual stages would thus be at a cost to gametocyte production, hence the
357 observed negative impact of parasite density on gametocyte prevalence.

358 The role of IgE in the immuno-allergic response is well documented but their role in the outcome
359 of malaria infection remains controversial and poorly understood. The levels of *P. falciparum*-specific-
360 IgE are elevated during a malaria episode and it has been proposed that they play a pathogenic role
361 for severe episodes (20, 39, 40), whereas in asymptomatic infections the IgE levels were associated
362 with protection (4). As described above, an important role for the Th1/Th2 balance in the outcome
363 of infection has been suggested by several studies (20). A recent genome wide linkage study
364 identified several loci that were linked to asymptomatic parasite densities (45) and all these loci have
365 been previously linked to asthma/atopic disease or related phenotypes (e.g. 52, 54). The acquisition
366 of premunition following successive infections may therefore include the development of immuno-
367 tolerance as well as immuno-protection.

368 Strategies that reduce the development of effective immune responses will not only enable an
369 increased duration of the concurrent infection, but also potentially enable re-infection of the same
370 host by the same strain. The extent to which parasites actively manipulate the Th1/Th2 balance,

371 rather than simply profit from the allergenic nature of mosquito saliva is not clear. Parasites are
372 known to alter expression of several salivary gland proteins during their development within the
373 salivary gland (10), but their immunogenicity has not been characterized and would in any case only
374 be relevant for the initial invasion of the host. It seems more likely that the parasite is profiting from
375 the allergenic nature of mosquito bites to then induce a Th2 response against itself. Whilst repeated
376 infections will eventually lead to the development of an effective immune response, it will be
377 substantially delayed. A single infection by a clone of *P. falciparum* can last up to 2 years, a duration
378 which may be facilitated by such immuno-modulation in addition to mechanisms such as antigenic
379 variation (11). Sterilising immunity, if ever achieved, takes a lifetime of regular exposure to infection
380 and a single strain can infect the same individual twice. It is thus possible that immuno-modulation
381 can enable repeated infection of the same host and hence is a key mechanism for maintaining a
382 permissive host population.
383 In conclusion, this work contributes to the on-going debate concerning targeting of mosquito
384 saliva components as a strategy of malaria control (16, 26). In contrast to the focus on the initial
385 stages of infection in naïve hosts, our work suggests that there may be longer term effects of
386 mosquito saliva that promote parasite persistence in chronic infections. Thus, whilst preventing
387 infections is optimal, mechanisms aimed at reducing the duration of infection will contribute to
388 reducing prevalence and onward transmission of the parasite.
389

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395

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566

567 **Table 1.** The effect of anopheline biting density on *P. falciparum* phenotypes: (A) Parasite density;

568 (B) Parasite prevalence rates; (C) proportion of infections harbouring gametocytes. [a]

569

	Parameter	Wald	P-value	Parameter est. (SE)	adj. R^2
(A)	Anopheles density Same week	10.36	0.001	0.076 (0.023)	0.086
	Anopheles density week before	9.49	0.002	0.066 (0.002)	0.082
	Anopheles density 2 weeks before	3.89	0.049	0.032 (0.016)	0.055
	Age	9.62	0.002	-0.005 (0.002)	
(B)	Anopheles density Same week	1.77	0.183		
	Anopheles density week before	0.47	0.495		
	Anopheles density 2 weeks before	0.98	0.322		
	Age	6.26	0.012	-0.011 (0.004)	0.004
(C)	Anopheles density Same week	14.1	<0.001	-1.08 (0.29)	0.10
	Anopheles density week before	12.5	<0.001	-0.886 (0.250)	0.12
	Anopheles density 2 weeks before	8.26	0.004	-0.525 (0.183)	0.09
	Ln (Parasite density+1)	12.57	<0.001	-0.962 (0.271)	

570 [a]Shown are the Wald statistics, the P-value, the parameter estimates (with standard errors) from the

571 fitted model and the adjusted R^2. The statistics for the parameters Age and Ln (Parasite density+1)

572 are those in the best fit model.

573

574

575

576 **Table 2.** Effect of IgE anti-salivary gland extract titre (pre-anopheline season) on the proportion of

577 *P. falciparum* infections harbouring gametocytes: (A) Excluding the Anopheline biting rate; (B)

578 Including the Anopheline biting rate.[a]

579

	Parameter	Wald	P-value	Parameter est. (SE)	adj. R^2
(A)	IgE anti-SG	8.24	0.004	-2.77 (0.97)	0.082
	Ln (Parasite density+1)	34.9	<0.001	-2.17 (0.37)	
(B)	IgE anti-SG	9.38	0.002	-0.788 (0.257)	0.131
	Ln (Parasite density+1)	13.75	<0.001	-0.502 (0.135)	
	Anopheles density Same week	11.69	<0.001	-0.580 (0.171)	

580 [a] Shown are the Wald statistics, the P-value, the parameter estimates (with standard errors) from the

581 fitted model and the adjusted R^2. The statistics for the Ln (Parasite density+1) are those in the best

582 fit model.

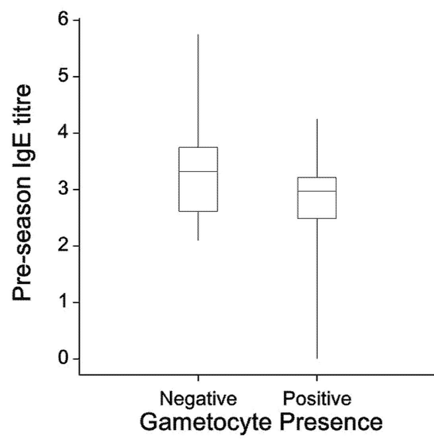
583

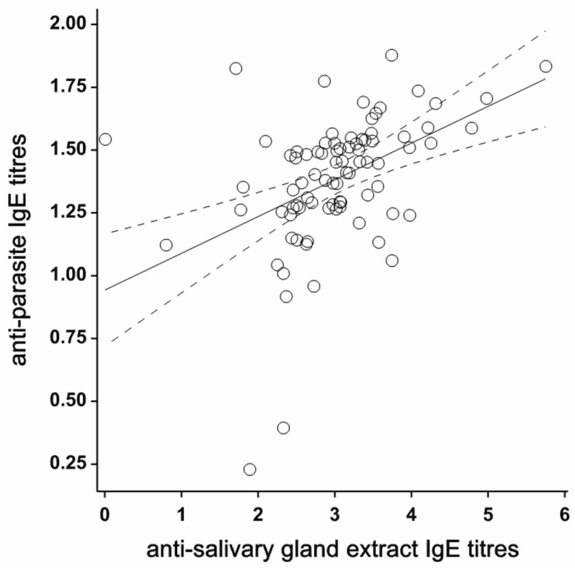
584 **Figure Legends**

585 **Figure 1**. Boxplot of pre-anopheline season IgE anti-salivary gland extract titres (boxcox
586 transformed) in individuals who had or had never carried gametocytes during a *P. falciparum* infection.
587 The box spans the interquartile range of the values, so that the middle 50% of the data lie within the
588 box, with a line indicating the median. Whiskers extend beyond the ends of the box as far as the
589 minimum and maximum values.

590

591 **Figure 2**. Correlation between anti-parasite and anti-salivary gland extract titres (boxcox
592 transformed). Shown is the linear correlation (solid line) and the 95% confidence intervals estimated
593 from a loglinear regression (dashed lines).


Pre-season IgE titre
6
5
4
3
2
1
0
Negative
Positive
Gametocyte Presence


anti-parasite IgE titres
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0
1
2
3
4
5
6
anti-salivary gland extract IgE titres