A transmission bottleneck for malaria? Quantification of sporozoite expelling by *Anopheles* mosquitoes infected with laboratory and naturally circulating *P. falciparum* gametocytes

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Abstract

It is currently unknown whether all *Plasmodium falciparum* infected mosquitoes are equally infectious. We assessed sporogonic development using cultured gametocytes in the Netherlands and naturally circulating strains in Burkina Faso. We quantified the number of sporozoites expelled into artificial skin in relation to intact oocysts, ruptured oocysts, and residual salivary gland sporozoites. Sporozoites were quantified by highly sensitive qPCR; intact and ruptured oocysts by fluorescence microscopy following antibody staining of circumsporozoite protein. In laboratory conditions, higher total sporozoite burden in mosquitoes was associated with a shorter duration of sporogony ($p<0.001$). Overall, 53% (116/216) of *P. falciparum* infected *An. stephensi* mosquitoes expelled sporozoites into artificial skin. The geometric means of expelled and residual salivary gland sporozoites were 116 (interquartile range (IQR: 33-501) and 21,016 (IQR: 9127-78,380), respectively. There was a strong positive correlation between ruptured oocyst number and salivary gland sporozoite load ($\rho=0.8$; $p<0.0001$) and a weaker positive correlation between salivary gland sporozoite load and the number of sporozoites expelled ($\rho=0.35$; $p=0.0002$). In Burkina Faso, *An. coluzzii* mosquitoes were infected by natural gametocyte carriers. Among mosquitoes that were salivary gland sporozoite positive, 97.2% (36/37) expelled sporozoites with a geometric mean of 420 expelled sporozoites (IQR: 116-2,779) and harbored a geometric mean of 35,149 residual salivary gland sporozoites (IQR: 20,310-164,900). Again, we observed a strong correlation between ruptured oocyst number and salivary gland sporozoite load ($\rho=0.9$; $p<0.0001$) and a positive correlation between salivary gland sporozoite load and the number of sporozoites expelled ($\rho=0.7$; $p<0.0001$). Mosquito salivary glands in Burkina Faso harbored 1-3 distinct parasite clones; several mosquitoes expelled multiple parasite clones during probing.

Whilst sporozoite expelling was regularly observed from mosquitoes with low infection burdens, our findings indicate that mosquito infection burden is associated with the number of expelled sporozoites. Future work is required to determine the direct implications of these findings for transmission potential.

59 ## Introduction

60 Malaria transmission to mosquitoes depends on the presence of mature gametocytes in human
61 peripheral blood that are ingested by a mosquito during blood feeding. Ingested parasites
62 undergo several developmental transformations in a process called sporogony. After ingestion,
63 gametocytes transform into male and female gametes that fuse to form a zygote. The zygote
64 differentiates into a motile ookinete that penetrates the midgut epithelium to form an oocyst.
65 Multiple rounds of mitotic replication result in the formation of sporozoites inside an oocyst.
66 Upon oocyst rupture, sporozoites are released into the haemocoel and invade the salivary glands
67 [1]. These sporozoites penetrate the distal portion of the two lateral and medial lobes of the
68 glands and accumulate extracellularly inside secretory cavities before entering the salivary ducts
69 [2]. Despite the large number of sporozoites in the cavities only a small proportion pass through
70 the proximal part of the lobes where the salivary ducts become narrow [3,4], and only tens or low
71 hundreds of sporozoites are assumed to be inoculated per mosquito bite [5]. Developmental
72 bottlenecks during sporogony, as well as the size of the sporozoite inoculum, remain
73 incompletely understood [5].

74 The density of gametocytes in human peripheral blood is an important determinant of human to
75 mosquito transmission. Though infections with parasite densities below the microscopic
76 threshold for detection can infect mosquitoes [6,7] the likelihood and infection intensity increases
77 with the number of ingested gametocytes [4]. Because of the abundance of low-density
78 gametocyte carriers among infected populations, these are considered important drivers of
79 malaria transmission [8-10]. Importantly, this conclusion is based on the assumption that all
80 infected mosquitoes are equally infectious regardless of oocyst densities.

81 In apparent support of this assumption single oocyst infections can result in thousands of
82 salivary gland sporozoites [11,12]. A positive correlation between oocyst densities and salivary gland
83 sporozoites was previously observed in *P. falciparum* [13-15] as well as in *P. vivax* [16], suggesting that
84 on average 1000-2000 sporozoites reach the salivary glands per single ruptured oocyst. The few
85 studies that quantified sporozoite inoculum by allowing mosquitoes to salivating into capillary
86 tubes containing mineral oil, sucrose solution, or blood [17-19], estimated median inocula ranging
87 between 8 to 39 sporozoites with a minority of mosquitoes expelling >100 sporozoites (reviewed
88 in [5]).

89 While these studies provide some insights into sporozoite expelling and transmission dynamics,
90 they do not reflect natural feeding conditions. Also, microscopy techniques used to quantify
91 sporozoites may have underestimated the number of sporozoites [20]. Subsequent studies with
92 rodent *Plasmodium* models allowed natural mosquito probing through skin and showed a
93 positive association between sporozoite density in salivary glands and expelled sporozoite
94 numbers [20,21]. A recent study using a *P. yoelii* malaria model demonstrated that mosquitoes with
95 over 10,000 salivary gland sporozoites were 7.5 times more likely to initiate a malaria infection in
96 mice [21]. This finding has not been replicated for human malarias but is broadly consistent with
97 post-hoc analysis of infection likelihood in controlled human malaria infections (CHMI), where
98 only mosquitoes that had >1000 *P. falciparum* salivary gland sporozoites remaining after probing
99 (the residual sporozoite load) were capable of establishing an infection in malaria-naïve
100 volunteers [22].
101 If low oocyst/low sporozoite densities in mosquitoes are unlikely to initiate infections in
102 humans, this may have profound consequences for our understanding of transmission [23]. If
103 mosquitoes with low infection burdens have limited transmission potential then the rationale for
104 targetting low-density infections in humans that give rise to low infection burdens in mosquitoes
105 [4] may be diminished.
106 Here, we examined the progression of sporozoite development and the number of sporozoites
107 expelled into artificial skin by individual *Anopheles* mosquitoes infected either with *P. falciparum*
108 gametocyte cultures or in experimental infections with naturally circulating parasite strains. We
109 directly assessed the association between oocyst burden, salivary gland infection intensity and
110 the number of sporozoites expelled.
111
112

113 ## Results

114 ### Low numbers of *P. falciparum* sporozoites are quantifiable by qPCR

115 Multicopy mitochondrial COX-1 and 18S rRNA gene targets were analysed in octuplicate on serial
116 dilutions of sporozoites to assess qPCR performance and select the target that achieved highest
117 sensitivity and most consistent sporozoite detection. COX-1 outperformed 18S in detecting
118 sporozoites (**Supplementary Figure 1**), the limit of detection (LOD) and limit of quantification
119 (LOQ) for COX-1 qPCR was determined at 20 sporozoites per sample (8/8 sample positivity with a

120 coefficient of variation <2) (**Figure 1A**). Next, we confirmed the qPCR performance in
121 combination with the matrix that was used for expelling experiments by spotting serial dilutions
122 of sporozoites in whole-blood on INTEGRA® dermal substitute artificial skin [24] prior to nucleic
123 acid extraction. The matrix had no apparent impact on sporozoite detectability and
124 quantification (**Supplementary Figure 2**).
125

126 **A comparative analysis of oocyst densities using mercurochrome staining and**
127 **immunostaining**

128 Subsequently, mosquito feeding assays were performed by offering diluted *in vitro* cultured
129 gametocytes to mosquitoes to obtain a broad range of oocyst densities. The association between
130 $\log_{10}$ oocyst intensity and infection prevalence in mosquitoes was assessed using a logistic
131 regression model (using data from 457 mosquitoes, **Figure 1B**). Mosquito infection prevalence
132 was strongly associated with oocyst intensity, corroborating earlier work[25], with a strong positive
133 sigmoidal association and a 14.68 (95% CI: 8.18-26.35, $p<0.0001$) times higher odds of infection
134 prevalence associated with a 10-fold higher oocyst density. In this analysis, oocysts were
135 enumerated microscopically following standard mercurochrome staining. We previously used
136 3SP2-Alexa 488 anti-circumsporozoite (CSP) immunostaining to visualize ruptured and intact
137 oocysts [26]. The concordance between oocyst prevalence by standard oocyst mercurochrome
138 staining (day 8 post infection) and anti-CSP immunostaining on day 18 post infection (**Figure 1C**)
139 was investigated. For this, oocyst density distributions by both methods were compared within
140 batches of mosquitoes that were fed on cultured gametocytes during the same standard
141 membrane feeding assay (**Figure 1D**). We observed no statistically significant difference in oocyst
142 densities determined by day 8 mercurochrome staining (median 5 oocysts, IQR: 2-20, N=252)
143 and day 18 immunolabelling (median 6 oocysts, IQR: 2-14, N=167; Student t-test on log densities,
144 $p=0.944$).
145

146 **Highly infected mosquitoes become salivary gland sporozoite positive earlier**

147 Following assay validation, the extrinsic incubation period (EIP) was compared between
148 mosquitoes with low and high oocyst densities. Batches of high and low infected mosquitoes
149 were generated using standard membrane feeding assay with standard concentrations of
150 cultured gametocytes or culture material that was 5- or 10-fold diluted (**Figure 1B**). On day 8

151 post infection, 20 mosquitoes were dissected and batches that had ≥70% oocyst infection
152 prevalence and means of ≤5 or >20 oocysts were selected for subsequent dissections (**Figure 1B**).
153 On days 9, 10 and 11, salivary glands and the remaining mosquito body (that included the
154 mosquito midgut) were collected separately and analysed for sporozoite density by COX-1 qPCR.
155 Mosquitoes were then binned into four categories of sporozoite infection intensity, defined as
156 the sum of mosquito body and salivary gland sporozoite density (**Figure 2A**). This total sporozoite
157 density was examined in relation with the likelihood of being salivary gland sporozoite positive
158 and thus having completed sporogonic development.
159
160 On day 9 post-infection, 54.3 % of highly infected mosquitoes (>50,000 sporozoites) were
161 salivary glands sporozoite positive (**Figure 2A**) and had 3.17 (CI 95%: 0.7-14.4, p=0.4278) times
162 the odds of being salivary gland positive compared to low infected mosquitoes with <1000
163 sporozoites (27.3% salivary gland sporozoite positive) (**Supplementary Table S1**). By day 10 post-
164 infection, 82.2 % of mosquitoes with 10,000-50,000 (10k-50k) sporozoites were salivary glands
165 positive and had 11.56 (CI 95%: 1.83, 73.25, p=0.0449) times the odds of being salivary glands
166 positive compared to low infected mosquitoes with <1000 sporozoites (28.6 % salivary gland
167 sporozoite positive). On day 11 post-infection, all 15 highly infected mosquitoes (>50,000
168 sporozoites) were salivary gland sporozoite positive and meaningful odds ratios and 95% CIs
169 could not be determined. When considering the entire period over which EIP experiments were
170 conducted, mosquitoes with > 50,000 sporozoites had a 13.44 times higher odds of being
171 salivary glands positive compared to low infected mosquitoes (<1000 sporozoites; 95% CI: 4.02-
172 44.88, p<0.0001) (**Supplementary Table S2**). Mosquitoes harbouring 10,000-50,000 sporozoites
173 had a 5.98 times higher odds of being salivary glands positive when compared to low infected
174 mosquitoes (CI 95%: 1.88-19.07, p=0.0119). These data demonstrate that EIP is shorter in high
175 infected compared to low infected mosquitoes in a temperature and humidity controlled
176 environment.
177

178 **Sporozoite densities increase with oocyst age**

179 To quantify the number of sporozoites per oocyst, individual oocysts were isolated from midguts
180 on days 9 and 10 post infection [27] and stained with 1% mercurochrome. The median sporozoite
181 density was 10,485 (IQR: 9171.3-12,322.5; 12 examined mosquitoes) per oocyst for day 9 and

182 15,390 (IQR: 10,600-20,887, 19 examined mosquitoes,) for day 10 (**Figure 2B**, p=0.04995, by
183 Welsch two sample t-test on log_{10}-transformed data).
184

185 **Oocyst density, salivary gland density, and the size of the sporozoite inoculum are**
186 **positively associated in mosquitoes infected with cultured gametocytes**

187 We performed artificial skin feeding experiments with individual mosquitoes on day 15 post
188 infection to assess sporozoite expelling. To avoid interference of residual blood with oocyst
189 immunolabeling, we assessed mosquito oocyst density (ruptured and intact oocysts) and
190 sporozoite density in the salivary glands on day 18, allowing 3 days for bloodmeal digestion. This
191 approach allowed us to determine the density of intact and ruptured oocysts and associate this
192 to sporozoite density in the same mosquito. It was noted that a minority of oocysts failed to
193 rupture during this time span; 5% (93/1854) of all oocysts were visually intact and 88.3%
194 (166/188) of examined mosquitoes had at least one unruptured oocyst on day 18. While we
195 observed good concordance between oocyst densities by mercurochrome staining on day 8 and
196 immunostaining on day 18 post infection (**Figure 1D**), oocysts sporadically did not take up the
197 3SP2-Alexa488 anti-CSP antibody labelling (**Supplementary Figure 3**). In 54% (12/22) of
198 mosquitoes without evidence of ruptured oocysts, we observed salivary gland sporozoites.
199 Nevertheless, there was a strong positive association observed between ruptured oocysts and
200 salivary gland sporozoite load (ρ=0.80, p<0.0001; N=185) (**Figure 3B**). When intact oocysts were
201 also included, this association was nearly identical (ρ=0.80, p<0.0001; N=185) (**Supplementary**
202 **Figure 4A**). We estimated a median of 4951 (IQR: 3016-8318) salivary gland sporozoites per
203 ruptured oocyst in *An. stephensi*. Next, the association between sporozoite density and the
204 number of expelled sporozoites (inoculum size) was determined. For this, individual mosquitoes
205 in miniature cages were allowed to probe for a maximum of 8 minutes on blood-soaked artificial
206 skin (**Supplementary Figure 5**). The skin on the feeder (surface feeding area 201 mm^2) was used
207 as input for DNA extraction with excess skin discarded, after we confirmed that there was no
208 evidence for sporozoite migration outside this area (**Supplementary information S1**).
209 Among all mosquitoes used in skin feeding experiments, 53% (116/216) expelled sporozoites at
210 any parasite density, and 45% (97/216) expelled sporozoites above our threshold for reliable
211 detection and quantification of 20 sporozoites/skin (**Supplementary Figure 6**). In line with
212 previous work with rodent malaria species *P. berghei* [28], sporozoite expelling was observed in

213 mosquitoes that did not take a bloodmeal; 33% (5/15) of mosquitoes that probed but failed to
214 take a blood meal expelled sporozoites (sporozoite range 5-1802). To examine sporozoite
215 expelling in relation to infection burden, mosquitoes were binned into four categories of salivary
216 gland infection intensity that was estimated by combining the residual sporozoite load in the
217 salivary glands and the sporozoites successfully expelled into the skin. In this way also heavily
218 infected mosquitoes that expelled the majority of sporozoites were categorized as heavily
219 infected. We observed a strong positive association between oocyst sheets and total salivary
220 gland sporozoite load (Spearman's correlation coefficient (ρ) $= 0.80$, p<0.0001; N=111; **Figure**
221 **3B**). When examining this association for different ranges of oocyst intensity (<5, <10, <20
222 oocysts), correlation estimates remained highly similar and statistical significance was retained
223 (**Supplementary Table S3**).
224 We observed no statistically significant association between salivary gland infection intensity and
225 the prevalence of expelling sporozoites (**Figure 3A;** 95% CI: 0.74 - 0.85); p=0.1880). Among
226 mosquitoes that expelled sporozoites, the geometric mean number of expelled sporozoites was
227 126 (IQR: 30-501) while the highest number of sporozoites detected in skin was 4166. We
228 observed a weak but statistically significant positive association between total sporozoite load
229 and the number of expelled sporozoites (ρ=0.35, 95% CI: 0.17 - 0.50; p=0.0002; N=112; **Figure**
230 **3C**). When examining this association for different ranges of total sporozoite load (<10,000;
231 <50,000; <100,000 sporozoites), correlation estimates remained highly similar although this
232 correlation lost statistical significance when only including low total sporozoite loads <10,000
233 sporozoites (ρ=0.29; 95% CI: -0.07 - 0.58, p=0.1094) (**Supplementary Table S3**). When we
234 included 26 observations from mosquitoes that did not expel any sporozoites we observed no
235 statistically significant association between total sporozoite load and the number of expelled
236 sporozoites (ρ=0.016, 95% CI: -0.12, 0.16; p=0.8321; **Supplementary Figure 7**).
237 We observed no evidence for a sharp increase in sporozoite expelling at sporozoite densities
238 ≥10,000, as was previously described in rodent malaria models [21]; 28% (53/186) of our
239 mosquitoes harbored sporozoites below this density. Among these low-infected mosquitoes,
240 64% (34/53) expelled sporozoites and the median number of expelled sporozoites was 67 (IQR:
241 13-128).
242

243 **Natural infected mosquitoes in Burkina Faso show comparable correlation between**
244 **oocyst density, salivary gland density and sporozoite inoculum**

245 Seven gametocyte donors (age 5-15 years) were recruited in Balonghin, Burkina Faso. Their
246 blood was offered to locally reared *An. coluzzii* via membrane at the gametocyte density
247 observed and following gametocyte enrichment by magnetic activated cell-sorting whereby
248 gametocyte concentration was increased ~3-4-fold (**Supplementary Table S4**). Five donors
249 infected mosquitoes in at least one of these two conditions (**Figure 4A**); as expected mosquito
250 infection rates and oocyst densities were significantly increased after gametocyte enrichment
251 (**Supplementary Table S4**). Mosquito batches with ≥50% infection prevalence were used for
252 artificial skin feeding experiments as described above. From a total of 53 mosquitoes, salivary
253 gland sporozoites were detected in 69% (37/53). 31 mosquito midguts were available for
254 immunolabelling of which 67.7% (23/31) had oocyst sheets detected by immunolabelling on day
255 19 and nearly half of these (48% [15/31]) had <5 oocyst sheets. Two mosquito midguts were
256 negative by immunolabeling while their residual salivary gland sporozoite loads were 7958 and
257 14,750 respectively, suggesting oocyst staining failure or rupture followed by complete oocyst
258 disappearance. 87.8% (370/421) of all detected oocysts ruptured while 39% (9/23) of oocyst
259 positive mosquitoes harbored at least one intact oocyst (range 1-19). Failure to rupture was
260 uncommon in low infected mosquitoes (≤5 oocysts, N=80) where only 2 midguts had 1 intact
261 oocyst. Among 53 mosquitoes used for artificial skin feeding on day 16 post-infection, 89%
262 (33/37) salivary gland sporozoite positive mosquitoes with a median of 45,100 residual salivary
263 gland sporozoites (IQR: 20,310-164,900) expelled a median of 1035 sporozoites (IQR: 171-2969).
264 We estimated a median of 6350 (IQR: 4225-8475) salivary gland sporozoites per ruptured oocyst
265 in these experiments. Three skin samples were positive by COX-1 qPCR (range 1-64) while
266 salivary glands were negative, suggesting either all sporozoites were expelled or a technical
267 failure in DNA extraction from the salivary glands. AMA-1 amplicon sequencing was conducted
268 on extracted salivary glands and artificial skins for mosquitoes that expelled sporozoites at any
269 quantity. Among 22 mosquitoes that were infected from three gametocyte donors we identified
270 10 unique clones; 68.2% (15/22) of the infected mosquitoes that were tested harbored
271 multiclonal infections. Following probing and successful sporozoite expelling, 10 skin samples
272 contained more than one *P. falciparum* clone (45.5%; 10/22) (**Figure 5**).

273 During the artificial skin feeding, 30% (16/53) of probing mosquitoes did not ingest blood of
274 which 68% (11/16) expelled sporozoites (range 1-11,970). There appeared to be a trend towards
275 higher prevalence of expelling with increasing sporozoite density (**Figure 4B**). There was a strong
276 association between ruptured oocyst density and total salivary gland sporozoite density (ρ=0.84,
277 95% CI: 0.80 - 0.95; p<0.0001) (**Figure 4C**); when intact oocysts were also included the
278 association was very similar (ρ=0.86, p<0.0001; N=30) (**Supplementary Figure 4B**). When
279 examining these associations for different ranges of oocyst intensity (<5, <10, <20 oocysts),
280 correlation estimates remained highly similar and statistical significance was retained
281 (**Supplementary Table S3**). There was also a strong positive association between total sporozoite
282 load and the number of sporozoites expelled (ρ=0.71, 95% CI: 0.52 - 0.82; p<0.0001) (**Figure 4D**).
283 When examining this association between total sporozoite load and expelling for different
284 ranges of total sporozoite load (<10,000, <50,000, <100,000 sporozoites), correlation estimates
285 remained similar and statistically significant (**Supplementary Table S3**).
286

287 ## Discussion

288 We examined *P. falciparum* sporogony in different mosquito species by employing sensitive
289 molecular and staining techniques in conjunction with mosquito probing experiments on
290 artificial skin. Through the visualization of ruptured oocysts and the simultaneous quantification
291 of sporozoites that are expelled by individual mosquitoes, we observed that 95% of oocysts
292 rupture to release sporozoites and that higher salivary gland sporozoite load is associated with
293 shorter time to colonization of salivary glands and larger inoculum size.
294

295 The proportion infectious mosquitoes is a central component of malariometric indices, both in
296 terms of quantifying the force of infection and the human infectious reservoir. The
297 entomological inoculation rate (EIR) is defined as the number of infectious bites per person per
298 time-unit and is the product of human biting rate and the proportion of sporozoite-positive
299 mosquitoes [29,30]. While EIR is a common measure of human malaria exposure, mosquito
300 infection prevalence is used in this calculation and thus assumes all sporozoite-positive
301 mosquitoes are equally infectious [31]. Similarly, assessments of the human infectious reservoir for
302 malaria typically take the number of oocyst-positive mosquitoes as measure of transmission [10,32]
303 and thereby not only assume that all oocysts will lead to salivary gland sporozoites but also that

304 all oocyst positive mosquitoes have equal transmission potential. Recent work with a rodent
305 malaria model challenged this central assumption[21]; we provide the first direct evidence for *P.*
306 *falciparum* that sporozoite burden may indeed be a relevant determinant in efficient sporozoite
307 expelling.
308
309 In the current study, we assessed sporozoite expelling by mosquitoes carrying low and high
310 infection burdens. Our findings confirm that the vast majority (~95%) of oocysts rupture to
311 release sporozoites. This estimate is higher than a previous study with cultured gametocytes
312 (~72%) [15] that did not provide a second bloodmeal that may accelerate oocyst maturation [33]; a
313 second bloodmeal also better mimics natural feeding habits where multiple bloodmeals are
314 taken within the period required for sporogony. Moreover, we observed a strong positive
315 association between sporozoite salivary gland load and ruptured oocyst density in mosquitoes
316 infected with both cultured and naturally circulating gametocytes, with similar median numbers
317 of 4951 (IQR: 3016-8318) and 6350 (IQR: 4225-8475) salivary gland sporozoites per ruptured
318 oocyst in *An. stephensi* and *An. coluzzii* respectively. Previous studies have shown that despite
319 the substantial release of sporozoites per oocyst, only a proportion of sporozoites successfully
320 reach the salivary glands [11,34]. In line with this, we observed considerably higher sporozoite
321 estimates in intact oocysts (10,485-15,390 sporozoites per oocyst). Whilst these sporozoite
322 estimates are higher than commonly reported [5], an early study from the 1960s reported
323 microscopy-detected sporozoite densities above 9000 sporozoites per oocyst [12] and a recent
324 study using qPCR similarly observed up to 12,583 sporozoites from a single oocyst [35]. These high
325 sporozoite numbers per oocyst also mean that, different from *P. yoellii* [21] and *P. berghei* [22], only
326 a minority of infected mosquitoes had sporozoite densities below the threshold values
327 previously reported in relation to a very low likelihood of achieving secondary infections. Even
328 among infected *An. stephensi* mosquitoes with only 1 ruptured oocyst, 92.9% (26/28) had >1000
329 and 35.7% (10/28) had >10,000 sporozoites in their salivary glands. In our experiments in natural
330 gametocyte carriers in Burkina Faso, only 2 mosquitoes were observed with single oocysts and
331 both had >10,000 salivary gland sporozoites. Although *P. falciparum* sporozoite densities below
332 these lower thresholds were thus uncommon, we observed that 39% of sporozoite-positive
333 mosquitoes across a wide range of infection densities failed to expel sporozoites upon probing.
334 This finding broadly aligns with an earlier study of Medica and Sinnis that reported that 22% of *P.*

335 *yoelii* infected mosquitoes failed to expel sporozoites [20]. For highly infected mosquitoes, this
336 inefficient expelling has been related to a decrease of apyrase in the mosquito saliva [36,37].
337
338 Importantly, we observed a positive association between salivary gland sporozoite density and
339 the number of expelled sporozoites. For unknown reasons, this association was markedly
340 stronger in experiments where *An. coluzzii* mosquitoes were infected using blood from
341 gametocyte carriers who were naturally infected with *P. falciparum* in Burkina Faso, compared
342 to *An. stephensi* mosquitoes infected with cultured gametocytes. In our experiments with
343 natural gametocyte carriers, sporozoite density appeared associated with both the likelihood of
344 expelling any sporozoites and the inoculum size. The most heavily infected mosquitoes expelled
345 ~14-fold higher sporozoite numbers compared to the lowest quartile. In addition, we found
346 evidence that the extrinsic incubation period (EIP) – the period required for a mosquito to
347 become salivary gland sporozoite positive – is shorter for heavily infected mosquitoes. Previous
348 studies have not found consistent effects of parasite burden on EIP [38,39] but, unlike our
349 experiments, were also not specifically designed to examine this association across a broad
350 range of infection intensities. Our associations of a shorter EIP in highly infected mosquitoes and,
351 in separate experiments, a larger sporozoite inoculum size for highly infected mosquitoes make
352 mosquito infection intensity a plausible factor in determining onward transmission potential to
353 humans. Heavily infected mosquitoes may be infectious sooner and be more infectious. On the
354 other side of the infection spectrum, it is conceivable that submicroscopic gametocyte carriers
355 that typically result in low oocyst burdens in mosquitoes give rise to infected mosquitoes with a
356 reduced transmission potential. This would greatly reduce their importance for sustaining
357 malaria transmission and make it less important to identify individuals with low parasite
358 densities for malaria elimination purposes [40]. At the same time, we occasionally observed high
359 sporozoite inocula from mosquitoes with low infection intensity. This argues against a clear
360 threshold sporozoite or oocyst density below which mosquitoes are truly irrelevant for
361 transmission. Moreover, the high number of sporozoites per oocyst make it plausible that even
362 the low oocyst burdens that are typically observed from asymptomatic parasite carriers (in the
363 range of 1-5 oocysts/infected gut [8,32]) would be sufficient to result in salivary gland sporozoite
364 loads that are sufficient to result in secondary infections.
365

366 Our study leaves a number of questions and has several limitations. While the use of two
367 mosquito and gametocyte sources was a relevant strength of our study; an uncertainty relates to
368 the choice of artificial skin that has a realistic 1.33 mm thickness but is arguably less natural than
369 microvascularized skin with all the natural cues for mosquito probing. We initially considered
370 filter paper cards to study expelling [41] (**Supplementary Figure 8**) but this approach was
371 abandoned because of a marked loss in sensitivity due to incomplete DNA recovery (~10-fold
372 signal reduction) [42] and since it is definitely not a natural skin mimic. Whilst genuine skin might
373 have improved natural feeding behaviour, probing and blood-feeding were highly efficient in our
374 model and we see no reasons to assume bias in the comparison between high- and low-infected
375 mosquitoes. Our assessments of EIP and of sporozoite expelling did not demonstrate the viability
376 of sporozoites. Whilst the infectivity of sporozoites at different time-points post infection has
377 been examined previously [43], these experiments have never been conducted with individual
378 mosquitoes. In vitro experiments that aim to determine the infectivity of single mosquito bites
379 would ideally retain the skin barrier that may be a relevant determinant for invasion capacity
380 and use primary hepatocytes. These experiments were beyond the scope of the current work
381 and would also not provide conclusive evidence on the likelihood of achieving secondary
382 infections. Given striking differences in sporozoite burden between different *Plasmodium*
383 species – low sporozoite densities appear considerably more common in mosquitoes infected
384 with *P. yoelii* and *P. berghei* [5,22,44] , and the inherent limitations of in vitro studies, the association
385 between sporozoite inoculum size and the likelihood of achieving secondary infections may be
386 best examined in controlled human infection studies. CHMI experiments can be specifically
387 designed to estimate the likelihood that probing by (individual) high- and low infected
388 mosquitoes results in blood-stage infection in malaria naïve volunteers. Whilst laborious, CHMI
389 studies are unique in allowing definitive evidence on possible differences in infectiousness
390 between high and low infected mosquitoes.
391
392 In conclusion, we observed that the majority of oocysts rupture and contribute to salivary gland
393 infection load. We further observe that this sporozoite load is highly variable and an important
394 determinant of the number of sporozoites that is expelled into the skin upon probing.
395

396 ## Methods

397 ### *P. falciparum in vitro* culture and mosquito infection

398 *Plasmodium falciparum* gametocytes, NF54 (West-Africa) and NF135 (Cambodia) [45,46] were
399 cultured in an automated culture system [47] and maintained as previously described at
400 Radboudumc, Nijmegen [48]. *An. stephensi* mosquitoes, Nijmegen Sind-Kasur strain [49], were reared
401 at 30°C and 70-80% humidity with a 12-hour reverse day/night cycle. To have a range of
402 infection intensities in mosquitoes, undiluted and diluted cultured gametocytes (0.3%-0.5%
403 gametocytes) were generated in heparin blood. 100-150, 1-3-days old female mosquitoes were
404 fed using glass membrane mini-feeders [50].

405 ### Mosquito feeding on gametocyte carriers who were naturally infected with *P.*
406 ### *falciparum*

407 *P. falciparum* gametocyte donors were recruited at schools in Saponé Health District, 45
408 kilometres southwest of Ouagadougou. Following informed consent, finger prick blood was
409 examined for gametocytes by counting against 500-1000 white blood cells (WBCs) in thick blood
410 films. The gametocyte counts were done by two independent microscopists and expressed as
411 density/µL by assuming 8,000 WBCs per µL of blood (**Supplementary Table S5**). If gametocyte
412 densities were above 16 /µL, 2-5mL of venous blood was drawn by venepuncture in Lithium
413 heparin tubes (BD Vacutainers, ref. 368496) and transported to Centre National de Recherche et
414 de Formation sur le Paludisme (CNRFP) insectary in Ouagadougou in thermos flasks filled with
415 water at 35.5°C [51].
416 700 µL of whole blood was used for immediate feeding, that was performed as described
417 elsewhere, using 3 to 5 day-old *Anopheles coluzzii* mosquitoes per glass mini feeder (Coelen
418 Glastechniek, The Netherlands) that was attached to a circulating water bath set up at 38°C
419 (Isotemp®, Fisher Scientific) [52,53]. 50 mosquitoes per cup (starved for 12 hours) were allowed to
420 feed in the dark for 15-20 minutes through a Parafilm membrane. To increase mosquito infection
421 prevalence and intensity, 1mL of blood was used to enrich gametocytes with magnetic cells
422 sorting columns (MACS), as described by Graumans et al. [54] (**Supplementary Figure 9**). In this
423 process, a pre-warmed 23G hypodermic needle (Becton Dickinson, Franklin Lakes, NJ) was
424 attached to an LC column on a QuadroMACS™ separator (MiltenyiBiotech, UK) that was placed
425 inside a temperature-monitored incubator (37°C). The flow-through was collected in a 15ml

426 Falcon tube. Following hydration with 1ml RPMI, 1ml donor blood (Lithium Heparin) was added
427 to MACS column. The column was rinsed with 2ml warm RPMI. The 15mL Falcon tube was
428 replaced with a new tube, and the needle was removed. Enriched (bound) gametocytes were
429 washed off the column with 4ml of warm RPMI, the plunger was used to press the last 1mL of
430 medium through the column. The two 15ml Falcon tubes, one with the blood mixed to RPMI and
431 the second with the gametocyte suspension were spun down at 2000rpm for 5 minutes in a
432 temperature-controlled centrifuge at 37°C (Eppendorf 5702 R). RPMI supernatant was removed
433 from both tubes with 3ml disposable Pasteur pipettes. To prepare the mosquito blood meal, the
434 small visible pellet of concentrated gametocytes (~30µl), was resuspended with 150µl of
435 prewarmed malaria naïve serum (Sanquin Bloodbank, Nijmegen, The Netherlands) with a blunt
436 needle and 200µl of the patients packed red cells were added and mixed. About 350µl
437 gametocyte enriched blood meal was added to a water-jacket glass feeder as described above.
438

439 **Mosquito husbandry and oocyst detection by mercurochrome staining**

440 In both insectaries, at Radboudumc (The Netherlands) and at CNRFP (Burkina Faso), following
441 membrane feeding unfed mosquitoes were immediately removed from cups with an aspirator.
442 On day 4-6 post infection mosquitoes were given a second blood meal to synchronize oocyst
443 development. Mosquitoes were kept at 27–29°C in the insectaries on 5-10% glucose and
444 dissected 7-8 days to assess infection prevalence. 20 mosquito midguts were stained with 1%
445 mercurochrome and oocysts were examined and confirmed by two independent microscopists
446 at 400X under an optic microscope (CX 40 Olympus). If oocysts prevalence was above 40%,
447 infected mosquitoes were transferred to the bio-secure insectary in Nijmegen, whilst in
448 Ouagadougou cups with infected mosquitoes were placed into secured metal cages
449 (30x30x30cm) and kept in a temperature and relative humidity controlled environment (27–29°C
450 and 70-80% HR) with double screened doors to prevent escaping sporozoite positive
451 mosquitoes.
452

453 **Extrinsic incubation period**

454 The extrinsic incubation period (EIP) defined as duration of sporogony was estimated in *An.*
455 *stephensi* mosquitoes in order to assess i) the best day to perform the expelling experiment, and
456 ii) if EIP differed in low vs high infected mosquitoes. Cages containing 150 mosquitoes were fed

457 with *P. falciparum* NF54 and NF135 gametocytes, and 20 midguts were dissected on day 6-8 post
458 infection (PI) to assess oocyst infection intensity. Groups of low (mean of 5 oocysts) and high
459 (mean of 20 oocysts) infected mosquitoes (prevalence of infection >70%) were maintained in a
460 secure insectary until salivary glands dissections were performed on day 9-10-11. Salivary glands
461 for individual mosquitoes were collected in 1,5mL Eppendorf tubes containing 180µl oocyst lysis
462 buffer (NaCl 0.1M: EDTA 25mM: TRIS-HCl 10mM), and stored at -20°C for further molecular
463 analysis. Mosquito bodies were first homogenised by beat-beating as previously described [55], in
464 100µl PBS.

465 **Sporozoite expelling experiments**

466 15 and 16 days post feeding, at Radboudumc and CNRFP respectively, infected mosquitoes were
467 used to quantify the number of expelled sporozoites. To prevent contamination, all instruments
468 and equipment were cleaned from nucleic acids by 30-min exposure to sodium hypochlorite
469 (10% in H2O), rinsed with water and paper dried on the day before the experiment. New gloves
470 were used each time the experiments were performed. Integra dermal substitute (Dermal
471 Regeneration Template, single layer 20x25cm, ref number 68101), hereafter referred to as
472 artificial skin, was cut into 3.5cm squares. Squares were transferred to Petri dishes filled with
473 sterile nuclease-free water (VWR, E476), and left overnight at room temperature (RT).
474 Mosquitoes were individually collected in small Perspex cages (5x5x7cm, covered with netting
475 material on the top and bottom sides) (**Supplementary Figure 5C**). Mosquitoes were starved 14-
476 16 hours prior to feeding. On the day of the expelling experiment, artificial skin was transferred
477 with gloves to an inverted positioned glass membrane mini-feeder (convex bottom, 15mm
478 diameter, ref 70172000) connected to a heated circulating water bath (CORIO C-B5, Julabo) set
479 to 39°C. A rubber band was wrapped around the feeder to secure the artificial skin. Paper tissue
480 was gently pressed on the skin four times to absorb water. 100 µL of naïve donor blood (EDTA,
481 BD Vacutainers, ref. 367525) was pipetted on the circular artificial skin area and spread evenly
482 across the surface with the horizontal side of the tip. The feeder was then turned around and
483 placed on top of the cage, without touching the netting, with a maximum of eight minutes for
484 mosquito probing. Following mosquito probing, a scalpel (Dalhausen präzisa plus, no 11) was
485 used to cut the artificial skin above the rubber band around the entire feeder. The artificial skin
486 was transferred with tweezers to a 1,5mL Eppendorf tube containing 180µl oocyst lysis buffer,

487 and stored at -20°C. After feeding, mosquitoes were transferred to metal cages. Mosquitoes
488 were kept at 27-29°C in the insectaries on 5% glucose.

489 **Immunolabelling of intact and ruptured oocysts**

490 Mosquitoes were allowed to digest blood for three days to prevent interference with
491 immunolabelling; mosquito salivary glands and midguts were dissected on day 18 PI for *An.*
492 *stephensi* (Nijmegen), and day 19 PI for *An. coluzzii* (Burkina Faso). Salivary glands were collected
493 in 1,5mL Eppendorf tubes containing 180µl oocyst lysis buffer (NaCl 0.1M: EDTA 25mM: TRIS-HCl
494 10mM), and stored at -20°C for further molecular analysis. Midguts were dissected in 20 µl
495 phosphate buffered saline (PBS, pH 7.2) without mercurochrome. For experiments performed in
496 Nijmegen, *An. stephensi* midguts were transferred to a fresh drop of (1:400) 3SP2-Alexa488 anti-
497 CSP antibodies and incubated for 30 minutes at RT in a slide humidity incubation box. Following
498 staining, midguts were washed twice with 10µl of PBS for 10 minutes. Midguts were transferred
499 to glass slides and secured with a cover slip. Intact/degenerated and ruptured oocysts were
500 counted using an incident light fluorescence microscope GFP filter at 400X. Due to lack of
501 fluorescence microscopes at CNRFP, we combined a formalin fixation method with
502 immunostaining. *An. coluzzii* midguts were transferred into individual screw cap tubes
503 (Eppendorf) filled with 400µl of 4% formalin and stored at 4°C until shipped to the Netherlands.
504 Midguts stored in 4% formalin were collected by using a p1000 Gilson pipette with the point of
505 the tip cut and placed on a slide. Midguts were rinsed from formalin 3 times in PBS 1X-Tween
506 0.05% by moving the midgut with a needle from drop to drop. They were then transferred to a
507 fresh drop of 3SP2-Alexa488 anti-CSP antibodies (1:400) and incubated for 20 minutes. Midguts
508 were rinsed in PBS and examined as described above (**Supplementary Figure 10**).
509

510 **Sample extraction, sporozoite quantification by qPCR and amplicon deep sequencing**

511 Serial dilutions of *P. falciparum* sporozoites were generated to prepare standard curves for
512 qPCR. Therefore pooled salivary glands from highly infected mosquitoes were collected in a glass
513 pestle grinder that contained 500µl PBS. The sample was homogenised and subsequently diluted
514 100 times in PBS. Sporozoites were transferred to a hemocytometer and counted under a phase
515 contrast light microscopy (400x magnification), by two independent microscopists. Serial
516 dilutions were prepared in PBS, using glass test tubes and low binding tips. For each
517 concentration, 100µl was filled out in a 1,5ml Eppendorf tube. Eppendorf tubes were stored at -

518 70°C for at least one day before sample processing. Prior to DNA extraction, 30µl proteinase K
519 (Qiagen, cat no. 19133) was added to Eppendorf tubes containing collected artificial skin and
520 salivary glands samples. PBS was added to all samples to have an equal volume (410µL) before
521 incubation overnight at 56°C. The following day, total nucleic acids (NA) were extracted with the
522 automated MagNA Pure LC instrument (Roche) using the MagNA Pure LC DNA Isolation Kit –
523 High performance (Roche, product no. 03310515001), and eluted in 50µL. Samples were used
524 immediately or stored at -20°C. *P. falciparum* sporozoites were quantified by qPCR, targeting the
525 mitochondrial gene COX-1. A previously published primer set [56] was modified to improve
526 template annealing, forward primer 5'-CATCAGGAATGTTATTGCTAACAC-3' and reverse primer 5'-
527 GGATCTCCTGCAAATGTTGGGTC-3', resulting in an amplicon length of 112bp. A probe was
528 designed for amplicon detection 6FAM-ACCGGTTTTAACTGGAGGAGTA-BHQ1. qPCR reactions
529 were prepared with TaqMan Fast Advanced Master Mix (Applied Biosystems, ref 4444557). For
530 each reaction was used 12.5µl mix, 0.4µl primers (stock 50µM, final concentration 800nM), 0.1µl
531 probe (stock 100µM, final concentration 400nM) 7µl PCR grade water and 5µl template DNA. In
532 each run, standard curves and negative controls (water) were included. Melt curves were visually
533 inspected. Samples were run on a Bio-Rad CFX 96 real time System at 95°C for 15 sec, followed
534 by 30 cycles of 95°C for 15 sec, 60°C for 60 seconds. To identify unique clones in mosquitoes
535 infected by gametocyte donors with naturally acquired *P. falciparum* gametocytes, samples were
536 genotyped by apical membrane antigen 1 (AMA-1) amplicon sequencing as previously described
537 [57].

538 **Statistical analysis**

539 Statistical analyses were performed in R, version 3.1.12 [58]. Associations between $\log_{10}$ oocyst
540 intensity and infection prevalence was modelled using a logistic regression model (using N=457,
541 **Figure 1B**). The difference in mean $\log_{10}$ oocyst densities between staining types were compared
542 using a t-test (t = 0.070075, df = 405, p-value = 0.9442, N=406, **Figure 1D**). The association
543 between total sporozoite density and experiment day with the prevalence of salivary gland
544 sporozoite was modelled using a mixed effects logistic regression with a random intercept for
545 the different experiments (using N=266, **Figure 2A**). Welsch t-test was used to compare
546 sporozoite density between days 9 and 10 (t = -2.0467, df = 28.66, p=0.04995, N=31, **Figure 2B**).
547 The association between total sporozoite load and sporozoite expelling prevalence was modelled
548 using a logistic regression (using N=186, **Figure 3A**).

549 Spearman's correlation coefficient (ρ) was used to assess the association between oocyst sheets
550 and salivary gland sporozoite load (one outlier not included), (ρ=0.80, 95% CI: 0.74, 0.85;
551 p<0.0001; N=111, **Figure 3B**); the association between total sporozoite density and the number
552 of sporozoites that was expelled into the artificial skin (ρ=0.35, 95% CI: 0.17 - 0.50; p=0.0002;
553 N=112, **Figure 3C**) (one outlier not included); the associations between ruptured oocyst density
554 and total sporozoite load, and between total sporozoite load and skin expelling (ρ=0.9, 95% CI:
555 0.80, 0.95; p<0.0001; N=25, **Figure 4C**) and (ρ=0.71, 95% CI: 0.52 - 0.82; p<0.0003; N=25, **Figure**
556 **4D**).

557 **Ethics declarations**

558 The study protocol in Burkina Faso was approved by the London School of Hygiene and Tropical
559 Medicine ethics committee (Review number: 14724), the Centre National de Recherche et de
560 Formation sur le Paludisme institutional review board (Deliberation N°
561 2018/000,002/MS/SG/CNRFP/CIB) and the Ethics Committee for Health Research in Burkina Faso
562 (Deliberation N° 2018–01-010). Experiments with in vitro cultured parasites and *An. stephensi*
563 mosquitoes at Radboud university medical center were conducted following approval from the
564 Radboud University Experimental Animal Ethical Committee (RUDEC 2009-019, RUDEC 2009-
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566 **Consent for publication**

567 All authors have given their consent for this publication.

568 **Competing interests**

569 The authors declare that they have no competing interests.

570

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Contributions

Conceptualization and study design were done by C.A., W.G., P.S., K,L. and T.B. Formal analysis, visualization, data curation were done by J.R. and T.B. Methodology was performed by C.A., W.G, K.L., G.J. S.H; Z.S and G.M performed samples collection and sporozoite expelling in Burkina Faso. Manuscript drafting, review and editing was by C.A.,W.G, J.R, K.L. and T.B. Supervision and project administration was done by K.L, T.B, G.G, M.G, A.B.T. Resources, funding acquisition by T.B. All authors reviewed the manuscript.

600 ## References

601 1 Mueller, A. K., Kohlhepp, F., Hammerschmidt, C. & Michel, K. Invasion of mosquito salivary glands
602 by malaria parasites: prerequisites and defense strategies. *International journal for parasitology*
603 **40**, 1229-1235, doi:10.1016/j.ijpara.2010.05.005 (2010).
604 2 Wells, M. B. & Andrew, D. J. Anopheles Salivary Gland Architecture Shapes Plasmodium
605 Sporozoite Availability for Transmission. *MBio* **10**, doi:10.1128/mBio.01238-19 (2019).
606 3 Frischknecht, F. *et al.* Imaging movement of malaria parasites during transmission by Anopheles
607 mosquitoes. *Cell Microbiol* **6**, 687-694, doi:10.1111/j.1462-5822.2004.00395.x (2004).
608 4 Bradley, J. *et al.* Predicting the likelihood and intensity of mosquito infection from sex specific
609 Plasmodium falciparum gametocyte density. *Elife* **7**, doi:10.7554/eLife.34463 (2018).
610 5 Graumans, W., Jacobs, E., Bousema, T. & Sinnis, P. When Is a Plasmodium-Infected Mosquito an
611 Infectious Mosquito? *Trends Parasitol* **36**, 705-716, doi:10.1016/j.pt.2020.05.011 (2020).
612 6 Schneider, P. *et al.* Submicroscopic Plasmodium falciparum gametocyte densities frequently
613 result in mosquito infection. *Am J Trop Med Hyg* **76**, 470-474 (2007).
614 7 Bousema, J. T. *et al.* Moderate effect of artemisinin-based combination therapy on transmission
615 of Plasmodium falciparum. *J Infect Dis* **193**, 1151-1159, doi:10.1086/503051 (2006).
616 8 Andolina, C. *et al.* Sources of persistent malaria transmission in a setting with effective malaria
617 control in eastern Uganda: a longitudinal, observational cohort study. *The Lancet. Infectious*
618 *diseases* **21**, 1568-1578, doi:10.1016/s1473-3099(21)00072-4 (2021).
619 9 Slater, H. C. *et al.* The temporal dynamics and infectiousness of subpatent Plasmodium falciparum
620 infections in relation to parasite density. *Nat Commun* **10**, 1433, doi:10.1038/s41467-019-09441-
621 1 (2019).
622 10 Ouedraogo, A. L. *et al.* Dynamics of the Human Infectious Reservoir for Malaria Determined by
623 Mosquito Feeding Assays and Ultrasensitive Malaria Diagnosis in Burkina Faso. *The Journal of*
624 *infectious diseases* **213**, 90-99, doi:10.1093/infdis/jiv370 (2016).
625 11 Rosenberg, R. & Rungsiwongse, J. The number of sporozoites produced by individual malaria
626 oocysts. *The American journal of tropical medicine and hygiene* **45**, 574-577 (1991).
627 12 Pringle, G. A count of the sporozoites in an oöcyst of plasmodium falciparum. *Transactions of The*
628 *Royal Society of Tropical Medicine and Hygiene* **59**, 289-290, doi:10.1016/0035-9203(65)90009-x
629 (1965).
630 13 Vaughan, J. A., Noden, B. H. & Beier, J. C. Population dynamics of Plasmodium falciparum
631 sporogony in laboratory-infected Anopheles gambiae. *J Parasitol* **78**, 716-724 (1992).
632 14 Miura, K. *et al.* Strong concordance between percent inhibition in oocyst and sporozoite
633 intensities in a Plasmodium falciparum standard membrane-feeding assay. *Parasit Vectors* **12**,
634 206, doi:10.1186/s13071-019-3470-3 (2019).
635 15 Stone, W. J. *et al.* The relevance and applicability of oocyst prevalence as a read-out for mosquito
636 feeding assays. *Sci Rep* **3**, 3418, doi:10.1038/srep03418 (2013).
637 16 Solarte, Y. *et al.* Plasmodium vivax sporozoite production in Anopheles albimanus mosquitoes for
638 vaccine clinical trials. *Am J Trop Med Hyg* **84**, 28-34, doi:10.4269/ajtmh.2011.09-0499 (2011).
639 17 Beier, J. C., Davis, J. R., Vaughan, J. A., Noden, B. H. & Beier, M. S. Quantitation of Plasmodium
640 falciparum sporozoites transmitted in vitro by experimentally infected Anopheles gambiae and
641 Anopheles stephensi. *Am J Trop Med Hyg* **44**, 564-570, doi:10.4269/ajtmh.1991.44.564 (1991).
642 18 Beier, J. C. *et al.* Quantitation of malaria sporozoites transmitted in vitro during salivation by wild
643 Afrotropical Anopheles. *Medical and veterinary entomology* **5**, 71-79, doi:10.1111/j.1365-
644 2915.1991.tb00523.x (1991).
645 19 Rosenberg, R., Wirtz, R. A., Schneider, I. & Burge, R. An estimation of the number of malaria
646 sporozoites ejected by a feeding mosquito. *Trans R Soc Trop Med Hyg* **84**, 209-212,
647 doi:10.1016/0035-9203(90)90258-g (1990).
648 20 Medica, D. L. & Sinnis, P. Quantitative dynamics of Plasmodium yoelii sporozoite transmission by
649 infected anopheline mosquitoes. *Infect Immun* **73**, 4363-4369, doi:10.1128/IAI.73.7.4363-
650 4369.2005 (2005).

21 Aleshnick, M., Ganusov, V. V., Nasir, G., Yenokyan, G. & Sinnis, P. Experimental determination of the force of malaria infection reveals a non-linear relationship to mosquito sporozoite loads. *PLoS pathogens* **16**, e1008181, doi:10.1371/journal.ppat.1008181 (2020).

22 Churcher, T. S. *et al.* Probability of Transmission of Malaria from Mosquito to Human Is Regulated by Mosquito Parasite Density in Naive and Vaccinated Hosts. *PLoS Pathog* **13**, e1006108, doi:10.1371/journal.ppat.1006108 (2017).

23 WHO. *Meeting report of the WHO Evidence Review Group on Low-Density Malaria Infections*, <https://www.who.int/publications/m/item/WHO-HTM-GMP-MPAC-2017.10> (2017).

24 Agostinis, C. *et al.* Differential Capability of Clinically Employed Dermal Regeneration Scaffolds to Support Vascularization for Tissue Bioengineering. *Biomedicines* **9**, doi:10.3390/biomedicines9101458 (2021).

25 Churcher, T. S. *et al.* Measuring the blockade of malaria transmission--an analysis of the Standard Membrane Feeding Assay. *Int J Parasitol* **42**, 1037-1044, doi:10.1016/j.ijpara.2012.09.002 (2012).

26 Stone, W. J. R. *et al.* The relevance and applicability of oocyst prevalence as a read-out for mosquito feeding assays. *Scientific reports* **3**, 3418, doi:10.1038/srep03418 (2013).

27 Soontarawirat, I. *et al.* Plasmodium vivax genetic diversity and heterozygosity in blood samples and resulting oocysts at the Thai–Myanmar border. *Malaria Journal* **16**, 355, doi:10.1186/s12936-017-2002-x (2017).

28 Matsuoka, H., Yoshida, S., Hirai, M. & Ishii, A. A rodent malaria, Plasmodium berghei, is experimentally transmitted to mice by merely probing of infective mosquito, Anopheles stephensi. *Parasitol Int* **51**, 17-23, doi:10.1016/s1383-5769(01)00095-2 (2002).

29 Tusting, L. S., Bousema, T., Smith, D. L. & Drakeley, C. Measuring changes in Plasmodium falciparum transmission: precision, accuracy and costs of metrics. *Advances in parasitology* **84**, 151-208, doi:10.1016/B978-0-12-800099-1.00003-X (2014).

30 Shaukat, A. M., Breman, J. G. & McKenzie, F. E. Using the entomological inoculation rate to assess the impact of vector control on malaria parasite transmission and elimination. *Malar J* **9**, 122, doi:10.1186/1475-2875-9-122 (2010).

31 Smith, D. L. *et al.* Ross, macdonald, and a theory for the dynamics and control of mosquito-transmitted pathogens. *PLoS pathogens* **8**, e1002588, doi:10.1371/journal.ppat.1002588 (2012).

32 Goncalves, B. P. *et al.* Examining the human infectious reservoir for Plasmodium falciparum malaria in areas of differing transmission intensity. *Nat Commun* **8**, 1133, doi:10.1038/s41467-017-01270-4 (2017).

33 Shaw, W. R. *et al.* Multiple blood feeding in mosquitoes shortens the Plasmodium falciparum incubation period and increases malaria transmission potential. *PLoS pathogens* **16**, e1009131, doi:10.1371/journal.ppat.1009131 (2020).

34 Hillyer, J. F., Barreau, C. & Vernick, K. D. Efficiency of salivary gland invasion by malaria sporozoites is controlled by rapid sporozoite destruction in the mosquito haemocoel. *Int J Parasitol* **37**, 673-681, doi:10.1016/j.ijpara.2006.12.007 (2007).

35 Wang, C. Y. T. *et al.* Assessing Plasmodium falciparum transmission in mosquito-feeding assays using quantitative PCR. *Malar J* **17**, 249, doi:10.1186/s12936-018-2382-6 (2018).

36 Rossignol, P. A., Ribeiro, J. M. & Spielman, A. Increased intradermal probing time in sporozoite-infected mosquitoes. *The American journal of tropical medicine and hygiene* **33**, 17-20, doi:10.4269/ajtmh.1984.33.17 (1984).

37 Thievent, K., Zilio, G., Hauser, G. & Koella, J. C. Malaria load affects the activity of mosquito salivary apyrase. *J Insect Physiol* **116**, 10-16, doi:10.1016/j.jinsphys.2019.04.003 (2019).

38 Guissou, E. *et al.* Intervention reducing malaria parasite load in vector mosquitoes: No impact on Plasmodium falciparum extrinsic incubation period and the survival of Anopheles gambiae. *PLoS Pathog* **19**, e1011084, doi:10.1371/journal.ppat.1011084 (2023).

39 Stopard, I. J., Churcher, T. S. & Lambert, B. Estimating the extrinsic incubation period of malaria using a mechanistic model of sporogony. *PLoS Comput Biol* **17**, e1008658, doi:10.1371/journal.pcbi.1008658 (2021).

702 40 Slater, H. C. *et al.* Assessing the impact of next-generation rapid diagnostic tests on Plasmodium
703 falciparum malaria elimination strategies. *Nature* **528**, S94-101, doi:10.1038/nature16040 (2015).
704 41 Brugman, V. A. *et al.* Detection of malaria sporozoites expelled during mosquito sugar feeding.
705 *Scientific reports* **8**, 7545, doi:10.1038/s41598-018-26010-6 (2018).
706 42 Holzschuh, A. & Koepfli, C. Tenfold difference in DNA recovery rate: systematic comparison of
707 whole blood vs. dried blood spot sample collection for malaria molecular surveillance. *Malar J* **21**,
708 88, doi:10.1186/s12936-022-04122-9 (2022).
709 43 Yang, A. S. P. *et al.* AMA1 and MAEBL are important for Plasmodium falciparum sporozoite
710 infection of the liver. *Cell Microbiol* **19**, doi:10.1111/cmi.12745 (2017).
711 44 Ahmed, A. *et al.* Invasive Malaria Vector Anopheles stephensi Mosquitoes in Sudan, 2016-2018.
712 *Emerging infectious diseases* **27**, 2952-2954, doi:10.3201/eid2711.210040 (2021).
713 45 Conway, D. J. *et al.* Origin of Plasmodium falciparum malaria is traced by mitochondrial DNA.
714 *Molecular and biochemical parasitology* **111**, 163-171 (2000).
715 46 Teirlinck, A. C. *et al.* NF135.C10: a new Plasmodium falciparum clone for controlled human
716 malaria infections. *J Infect Dis* **207**, 656-660, doi:10.1093/infdis/jis725 (2013).
717 47 Ponnudurai, T., Lensen, A. H., Leeuwenberg, A. D. & Meuwissen, J. H. Cultivation of fertile
718 Plasmodium falciparum gametocytes in semi-automated systems. 1. Static cultures. *Trans R Soc*
719 *Trop Med Hyg* **76**, 812-818 (1982).
720 48 van de Vegte-Bolmer, M. *et al.* A portfolio of geographically distinct laboratory-adapted
721 Plasmodium falciparum clones with consistent infection rates in Anopheles mosquitoes. *Malar J*
722 **20**, 381, doi:10.1186/s12936-021-03912-x (2021).
723 49 Feldmann, A. M. & Ponnudurai, T. Selection of Anopheles stephensi for refractoriness and
724 susceptibility to Plasmodium falciparum. *Med Vet Entomol* **3**, 41-52, doi:10.1111/j.1365-
725 2915.1989.tb00473.x (1989).
726 50 Ponnudurai, T. *et al.* Infectivity of cultured Plasmodium falciparum gametocytes to mosquitoes.
727 *Parasitology* **98 Pt 2**, 165-173 (1989).
728 51 Soumare, H. M. *et al.* Maintaining Plasmodium falciparum gametocyte infectivity during blood
729 collection and transport for mosquito feeding assays in the field. *Malar J* **20**, 191,
730 doi:10.1186/s12936-021-03725-y (2021).
731 52 Ouédraogo, A. L. *et al.* A protocol for membrane feeding assays to determine the infectiousness
732 of P. falciparum naturally infected individuals to Anopheles gambiae. (2013).
733 53 Musiime, A. K. *et al.* Is that a real oocyst? Insectary establishment and identification of
734 Plasmodium falciparum oocysts in midguts of Anopheles mosquitoes fed on infected human
735 blood in Tororo, Uganda. *Malar J* **18**, 287, doi:10.1186/s12936-019-2922-8 (2019).
736 54 Graumans, W. *et al.* Plasmodium falciparum Gametocyte Enrichment in Peripheral Blood Samples
737 by Magnetic Fractionation: Gametocyte Yields and Possibilities to Reuse Columns. *Am J Trop Med*
738 *Hyg* **100**, 572-577, doi:10.4269/ajtmh.18-0773 (2019).
739 55 Graumans, W. *et al.* Semi-high-throughput detection of Plasmodium falciparum and Plasmodium
740 vivax oocysts in mosquitoes using bead-beating followed by circumsporozoite ELISA and
741 quantitative PCR. *Malar J* **16**, 356, doi:10.1186/s12936-017-2011-9 (2017).
742 56 Boissiere, A. *et al.* Application of a qPCR assay in the investigation of susceptibility to malaria
743 infection of the M and S molecular forms of An. gambiae s.s. in Cameroon. *PloS one* **8**, e54820,
744 doi:10.1371/journal.pone.0054820 (2013).
745 57 Briggs, J. *et al.* Sex-based differences in clearance of chronic Plasmodium falciparum infection.
746 *Elife* **9**, doi:10.7554/eLife.59872 (2020).
747 58 Team, R. C. *The R Project for Statistical Computing* <https://www.r-project.org/.> (2019).

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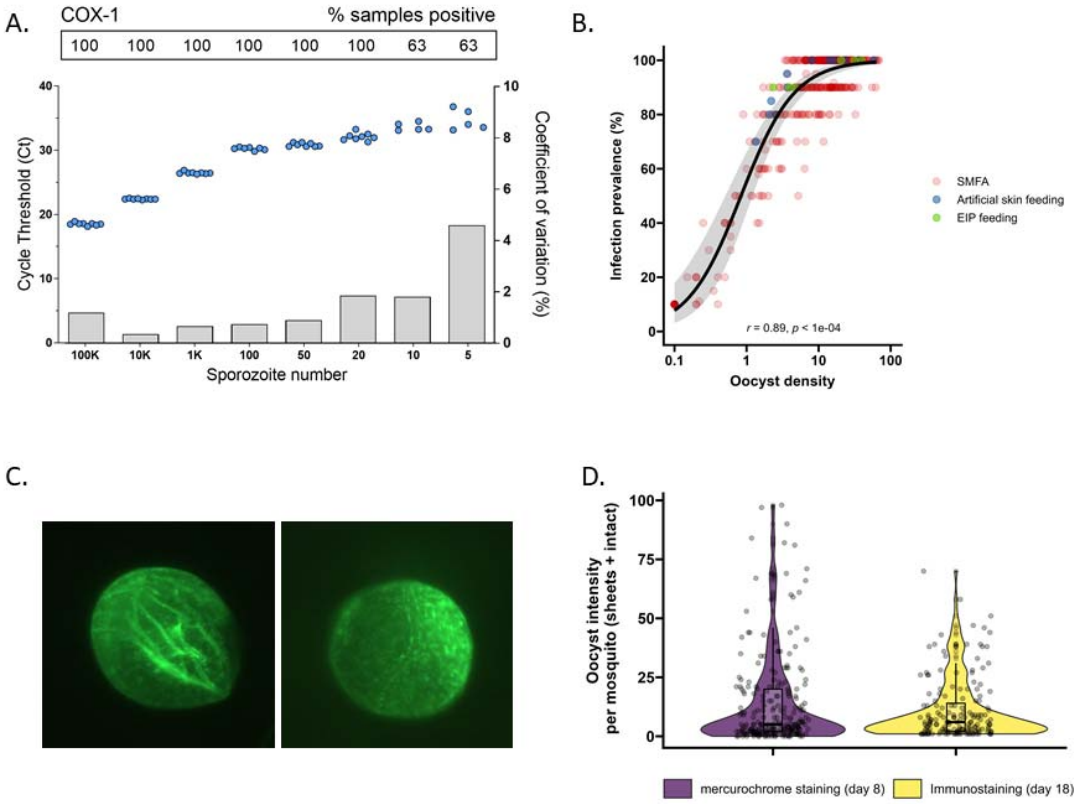
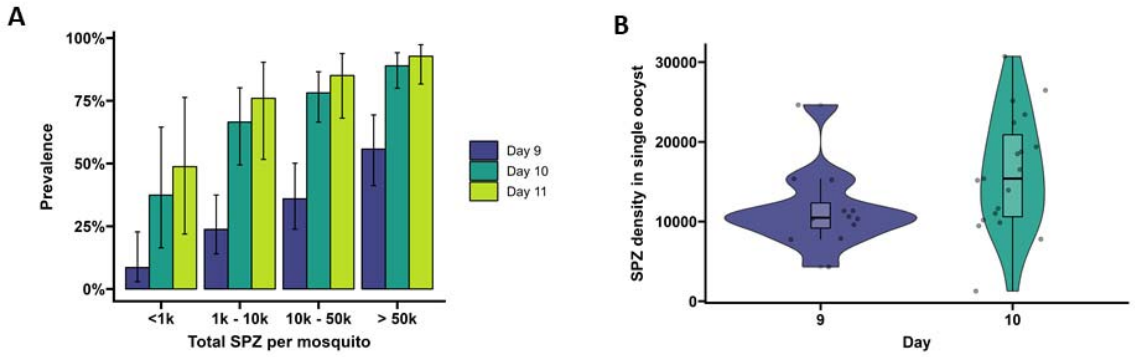


Figure. 1 The detectability of sporozoites by molecular methods and oocysts by immunolabeling. A. qPCR performance for *P. falciparum* sporozoites. Serial dilutions of sporozoites (x-axis) were prepared in PBS and assessed in octuplicate on a single plate to determine qPCR COX-1 limit of detection and quantification. Dots represent sample cycle threshold (left y-axis), and bars coefficient of variation (right y-axis). For each serial dilution Ct sample positivity is shown as the percentage (%) of total tested. **B.** The relationship between oocyst density versus infection prevalence from 457 membrane feeding experiments using cultured gametocytes. Colours represent regular feeds (red) and those selected for performing experiments on the extrinsic incubation period (EIP; green) or sporozoite expelling experiments (blue). **C**. Immunofluorescence staining with 3SP2-Alexa 488 anti-CSP. Empty sheet and intact oocyst. **D.** Violin plots of oocysts staining - day 8 post infection by mercurochrome (purple) and - day 18 by 3SP2-Alexa 488 anti-CSP immunostaining (yellow) for cultured parasites. Box plots show interquartile range, whiskers show the 95% intervals.



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765 **Figure. 2 Extrinsic Incubation Period in high versus low infected mosquitoes. A.** Total sporozoites (SPZ)
766 per mosquito in body plus salivary glands (x-axis) were binned by infection load <1k; 1k-10k; 10k-50k;
767 >50k and plotted against the proportion of mosquitoes (%) that were sporozoite positive (y-axis) as
768 estimated from an additive logistic regression model with factors day and SPZ categories. 120, 120, 40
769 mosquitoes were dissected on day 9 (blue), 10 (dark green), 11 (light green) respectively (**Supplementary**
770 **information S2**). Error bars show the 95% confidence intervals. **B.** Violin plots of sporozoite density (SPZ)
771 in single oocysts dissected on day 9 (purple) and day 10 (green). The box indicates the interquartile range
772 (IQR) (Q1 and Q3 quartiles) and the median. Lines extending Q1 and Q3 quartiles indicate the range of the
773 data within 1.5 times IQR.

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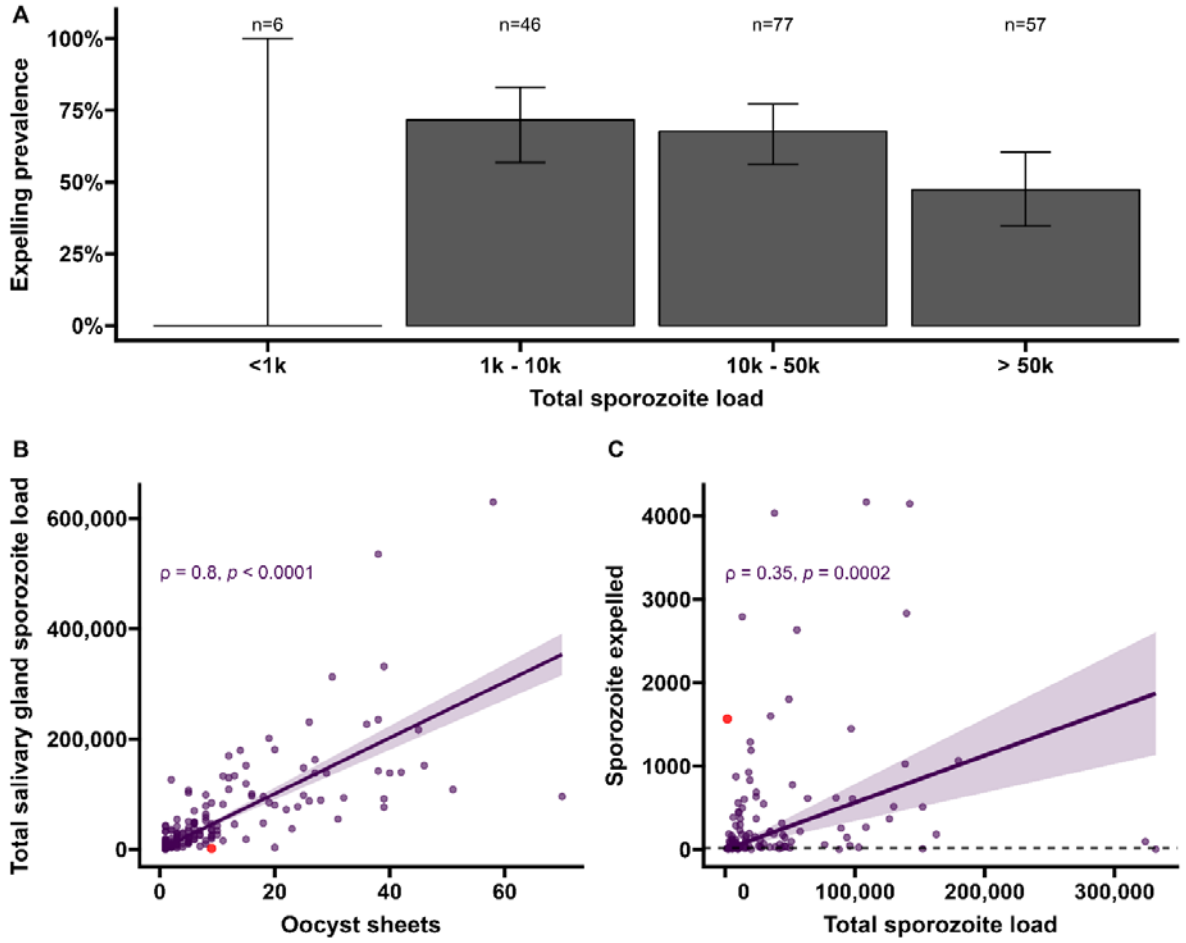

Figure 3. Sporozoite expelling in relation to infection burden in *Anopheles stephensi* mosquitoes infected with cultured gametocytes. A. Binning of mosquitoes by total sporozoite load and expelling prevalence (N=186) **B.** The number of ruptured oocysts stained by 3SP2-Alexa 488 anti-CSP and fluorescent microscopy (X-axis) in relation to total salivary gland sporozoite density (Y-axis), assessed by COX-1 qPCR; ρ=0.80 (CI: 0.74 - 0.85, p<0.0001). The red dot indicates a mosquito which had 9 ruptured oocysts but only 126 residual salivary glands sporozoites while expelled 1567 sporozoites. Considering the high number of ruptured oocysts in the midgut it is possible that some lobes of salivary glands were missed during dissection and sporozoite load was underestimated by qPCR (95% CI: 0.17, 0.50). **C.** Total sporozoite density (residual salivary gland sporozoites + sporozoites expelled, X-axis) in relation to the number of expelled sporozoites (Y-axis) by COX-1 qPCR ρ=0.35 (CI: 0.17 - 0.50, p=0.0002). The dotted line on the x- axis shows the threshold of qPCR detection of 20 sporozoites.

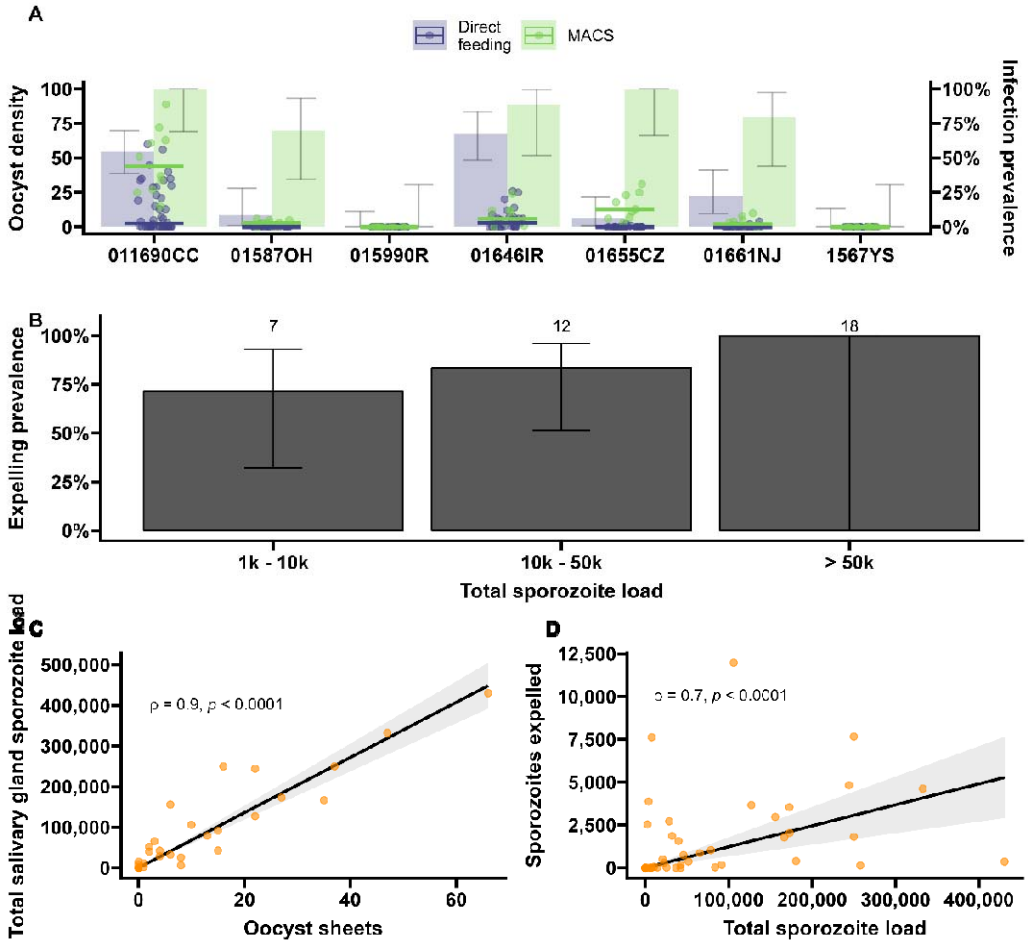

Figure 4. Sporozoite expelling in relation to infection load in *Anopheles coluzzii* mosquitoes infected by naturally acquired gametocyte infections in Burkina Faso. (**A**) Direct feeding (blue) vs magnetic-activated cell sorting (MACS; green). Bars show the infection prevalence for each of the 7 gametocyte carriers. Scatter plots with median lines show the midgut oocyst density as result of direct feeding (blue) and MACS (green). (**B**) Binning of total sporozoite load and expelling prevalence (N=25). (**C**) Scatter plot of absolute numbers of ruptured oocyst (sheet) density assessed by fluorescent microscopy vs total salivary gland sporozoite density assessed by COX-I qPCR; ρ=0.90 (95% CI: 0.80 - 0.95). The line represents the fitted linear regression line and the grey shaded area is the 95%CI. (**D**) Scatter plot of absolute numbers of total sporozoite density (residual salivary gland sporozoites + sporozoites expelled) and sporozoites expelled into the artificial skin assessed by COX-I qPCR; ρ=0.70 (CI: 0.52 - 0.82). The line represents the fitted linear regression line and the grey shaded area is the 95% CI.

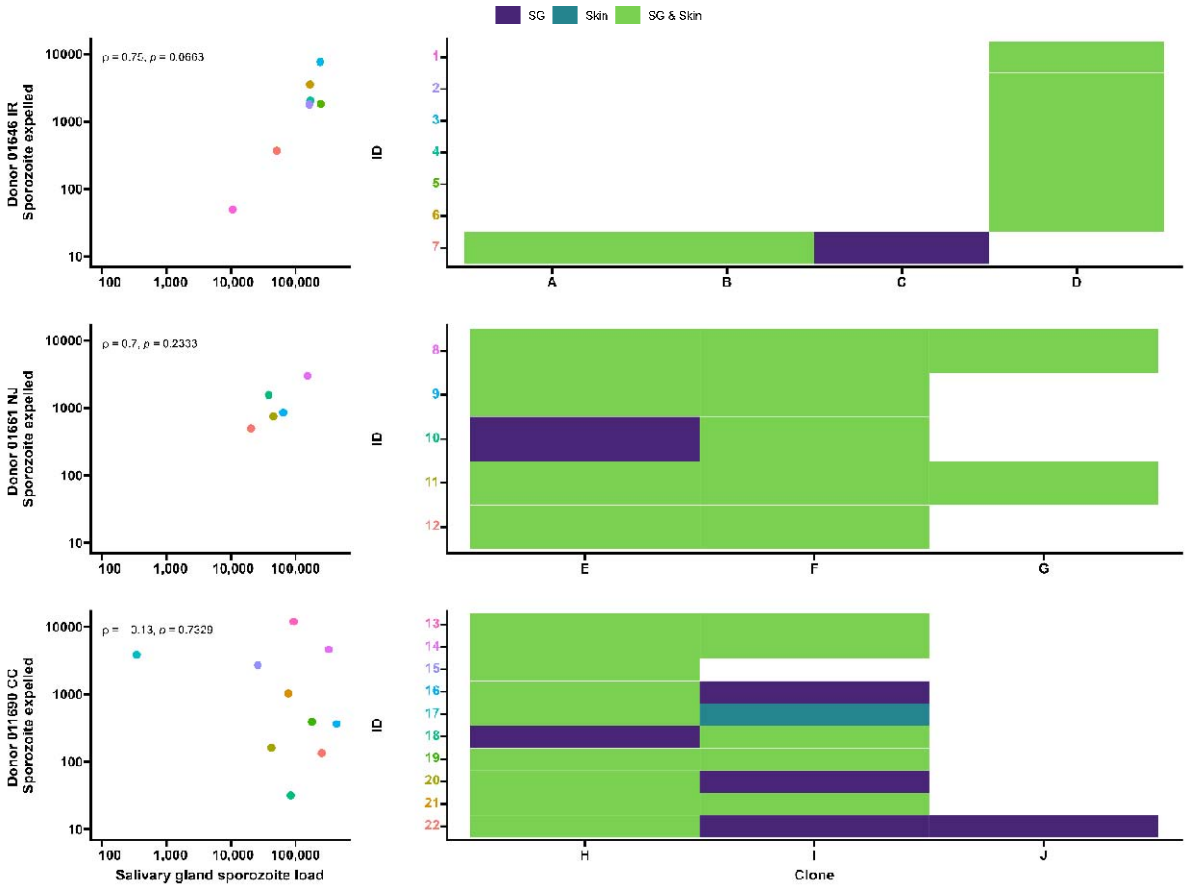

Figure 5. Clonal complexity of *P. falciparum* infections in salivary glands and artificial skins following probing by mosquitoes infected by gametocyte carriers who were naturally infected in Burkina Faso. Clonal data for three donors: 01646 IR (**top row**), 01661 NJ (**middle row**) and 011690 CC (**bottom row**). **Left panel** shows scatter plots for the association between sporozoite salivary gland load and expelled sporozoites in skin, with a Spearman's rank correlation across samples from each donor. **Right panel** shows clonal data for each donor sample in a heatmap plot. The colored numbers on y-axes correspond with the color of the sample in the scatter plot. Purple indicates the presence of a clone in the salivary gland only, turquoise indicates the presence of a clone in the skin only and green indicates the presence of a clone in both salivary gland and skin.