Gametocyte production and transmission fitness of African and Asian *Plasmodium falciparum* isolates with differential susceptibility to artemisinins

Nicholas I. Proellochs[1], Chiara Andolina[1], Jordache Ramjith[1], Rianne Stoter[1], Geert-Jan van Gemert[1], Wouter Graumans[1], Susana Campino[2], Leen N. Vanheer[2], Martin Okitwi[3], Patrick K. Tumwebaze[3], Melissa D. Conrad[4], Taane G. Clark[2], David A. Fidock[5,6], Didier Menard[7], Sachel Mok[6], Teun Bousema[1,2]

[1] Department of Medical Microbiology, Radboud University Medical Centre, Nijmegen, The Netherlands
[2] Department of Infection Biology, Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, UK
[3] Infectious Diseases Research Collaboration, Kampala, Uganda
[4] Department of Medicine, University of California, San Francisco, CA, USA
[5] Department of Microbiology and Immunology Columbia University Irving Medical Center, New York, NY, USA
[6] Center for Malaria Therapeutics and Antimicrobial Resistance, Division of Infectious Diseases, Department of Medicine, Columbia University Irving Medical Center, New York, NY, USA
[7] Institut Pasteur, Université Paris Cité, Malaria Parasite Biology and Vaccines Unit, Paris, France; Malaria Genetics and Resistance Team (MEGATEAM), UR 3073 - Pathogens Host Arthropods Vectors Interactions, Université de Strasbourg, F-67000 Strasbourg, France; CHU Strasbourg, Laboratory of Parasitology and Medical Mycology, Strasbourg, France; Institut Universitaire de France (IUF), F-75231, Paris, France

Abstract

The emergence of *Plasmodium falciparum* parasites partially resistant to artemisinins (ART-R) poses a significant threat to recent gains in malaria control. ART-R has been associated with PfKelch13 (K13) mutations, which differ in fitness costs. This study investigates the gametocyte production and transmission fitness of African and Asian *P. falciparum* isolates with different K13 genotypes across multiple mosquito species. We tested three ART-sensitive (ART-S) isolates (NF54, NF135, NF180) and three ART-R isolates (ARN1G, 3815, PAT-023) for sexual conversion and transmission to *Anopheles stephensi, An. gambiae* and *An. coluzzii*. ART-R levels were quantified *in vitro* using the Ring-stage Survival Assay (RSA), and the transmission-reducing effects of dihydroartemisinin (DHA) on mature gametocytes were assessed. Results showed that ART-S parasite lines consistently produced gametocytes and transmitted effectively in all three mosquito species. ART-R isolates showed variability: ARN1G maintained high transmission levels, whereas 3815 showed limited transmission potential despite higher sporozoite loads in *An. coluzzii*. The African ART-R isolate PAT-023 demonstrated low gametocyte commitment but was transmitted efficiently in both *An. gambiae* and *An. coluzzii*. DHA exposure reduced mosquito infectivity for all isolates, regardless of K13 genotype. These findings, based on a limited number of field isolates, suggest that ART-R parasites remain transmissible across different *Anopheles* species. However, ART-R does not appear to confer a direct transmission advantage. This study highlights the complexity of ART-R dynamics and underscores the need for further research to inform malaria control strategies in regions where ART-R parasites are circulating.

Key words

Malaria, *P. falciparum*, Mosquito Transmission, Artemisinin Resistance, K13 genotype

48 ## Introduction

49 Despite global efforts to reduce malaria burden, progress has plateaued in the last decade and in some
50 areas malaria is again increasing [1]. The recent emergence in Sub-Saharan Africa of *P. falciparum*
51 parasites with partial resistance to artemisinins (ART-R) adds to the concerns about how sustainable
52 malaria control may be. ART-R is characterised by prolonged parasite clearance times (half-life >5
53 hours) or persistence of parasitemia on day 3 following artemisinin monotherapy or artemisinin-based
54 combination therapy (ACT) [2-6]. ART-R is predominantly associated with specific mutations in the
55 essential PfKelch13 (K13) protein's β-propeller domain [7-10]. These mutations reduce K13 protein
56 levels, disrupting haemoglobin import into the parasite's digestive vacuole, a critical step for ART
57 activation [8, 11-13]. While there are validated K13 mutations that associate with ART-R, their impacts
58 on resistance and parasite fitness are variable and highly dependent on the mutation and parasite
59 background [13-16]; as such it is difficult to predict which mutations are of particular concern.

60 Clinically relevant mutations in K13 first were observed in isolates collected in 2002 in western
61 Cambodia [17]. While partial resistance subsequently spread across the Greater Mekong Subregion [7,
62 18], the anticipated migration to the African continent has not occurred. It was hypothesised that
63 lower drug pressures combined with high parasite diversity in many settings in Sub-Saharan Africa may
64 have allowed wild-type parasites to outcompete less-fit K13 mutant parasites, making *de novo*
65 emergence of ART-R less likely and limiting the spread of the K13 mutants upon introduction. The
66 recent independent emergence of K13 mutations associated with ART-R including in East Africa
67 (Uganda, Rwanda, and Tanzania) and the Horn of Africa (Eritrea, Ethiopia and Sudan) [19-23] raises
68 questions about their fitness, resistance, and transmission potential. Transmission to mosquitoes
69 depends on the formation of viable male and female gametocytes, with considerable variation in
70 gametocyte production between parasite isolates [24, 25]. The transmission potential of parasites with
71 K13 mutations is particularly relevant with changes in vector populations and variation in *P. falciparum*
72 vector competence [26]. The recent invasion of the competent Asian vector *Anopheles stephensi* in
73 urban African settings [27, 28] raises concerns about the spread of ART-R parasites of African and Asian
74 origin in settings previously not endemic for malaria.

75 Understanding variations in vector competence and determining whether *K13* mutant parasites
76 possess a transmission advantage, both in the presence and absence of artemisinin derivatives, are
77 crucial for predicting the potential spread of ART-R on the African continent. In this study, we
78 investigate the gametocyte production and transmission fitness of ART-S and ART-R field isolates. We
79 compared ART-S isolates [29, 30] with ART-R isolates carrying two common Southeast Asian K13
80 mutations G449A and C580Y [18, 31, 32], as well as a recently isolated ART-R K13 independent parasite

line from Uganda, where the local emergence of ART-R has been documented including through K13 mutations.

Material and methods

Parasite cultures

All parasite lines – including the ART-S NF54 (Africa), NF135 (Cambodia) and NF180 (Uganda), as well as the ART-R *P. falciparum* field isolates ARN1G (Thailand), 3815 (Cambodia), and PAT-023 (Uganda) – were cultured in an automated culture system using RPMI media supplemented with 10% human serum, with gametocyte cultures for mosquito feeding set up at a uniform 1% parasitemia and allowed to mature for 14 days prior to feeding [33]. Asexual parasite cultures were maintained for no more than 25 cycles in a synchronised state through magnet separation or sorbitol lysis. Gametocyte induction was performed using either minimal fatty acid media or 0.5% Albumax; gametocytes were allowed to mature in RPMI media supplemented with 10% human serum [25]. Parasite DNA was extracted with QIAGEN Blood DNA kit, sequenced on the Illumina Novaseq 6000 platform and analysed using the malaria profiler tool [34].

Ring-stage Survival Assay (RSA)

The Ring-stage Survival Assay (RSA) was performed as previously published [16, 35] with minor modifications. To avoid the negative impact of sorbitol synchronisation on conversion rates within the same cycle [36, 37], we opted for a gentler approach using double magnet purification. Highly synchronised parasites were first passed through a magnetic column (MACS) to collect segmented schizonts. These schizonts were put back into culture for 3-4 hours to allow bursting and reinvasion of new red blood cells (RBCs). The culture was subsequently passed through the same magnetic column, isolating the flow-through, which contained only newly invaded ring-stage parasites. Parasites were split into 2 plates: one for the sexual conversion assay and one for the RSA. For the RSA, parasites were diluted to 1-2% parasitemia in 2% hematocrit. Each line was tested in 4 wells: two for DMSO controls and two for DHA. Washes were performed in separate tubes following DHA treatment before being added to a fresh plate. Survival rates were determined using both Giemsa smears and flow cytometry using MitoTracker Red as a live cell stain from the same well for all replicates.

Sexual conversion assay

110 Sexual conversion rates were performed as previously published [25], with minor modifications. After
111 double synchronisation, parasites were returned to culture for 24 hours prior to initiating the
112 conversion assay. The assay was performed in a plate-based format with each parasite isolate split
113 over 3 wells (one for each media type) at 1% parasitemia in 5% hematocrit. Conversion rates were
114 calculated by Giemsa-stained smears [25].

115 **Mosquito infections**

116 Laboratory colonies of *An. stephensi* (Nijmegen Sind-Kasur strain)[38], *An. coluzzii* (N'gousso strain)
117 [39] and *An. gambiae* s.s (Kisumu strain) [40], were maintained under controlled conditions: 26°C, 70-
118 80% humidity and a 12-hour reverse day/night cycle. Gametocyte cultures were checked for
119 exflagellation to confirm maturation and gametocytaemia reached a minimum of 0.1% before feeding.
120 Culture material was not further normalized for gametocyte density prior to feeding. Groups of 100
121 female *Anopheles stephensi*, *An. gambiae*, and *An. coluzzii* mosquitoes, aged 1–5 days, were blood-fed
122 for 15 minutes using glass membrane mini-feeders (15 mm diameter, convex bottom) connected to a
123 heated circulating water bath. Fully fed mosquitoes were selected, and maintained at 30°C with access
124 to 5-10% glucose.

125 **Ookinetes count**

126 Mosquito midguts were examined 18-24 hours post-infection to identify the presence of round forms,
127 retort forms, and mature ookinetes. Five midguts from each infection group were dissected and
128 incubated with a 1:50 diluted Anti-25KD-FITC conjugate in Evans blue solution. Midguts were gently
129 disrupted using a pipette tip to release the blood meal. Following incubation in the dark for 30 minutes
130 at room temperature, the solution was washed with 1.4 mL of phosphate-buffered saline (PBS),
131 vortexed to dissolve the pellet, and centrifuged for 2 minutes at 10,000 rpm. After removal of the
132 supernatant, the pellet was resuspended in 25 µL of PBS and 5 µL of the suspension was loaded into a
133 Bürker-Turk counting chamber. Round forms, retort forms and mature ookinetes were counted using
134 an incident light fluorescence microscope with a GFP filter at 400× magnification.

135 **Oocysts count**

136 Seven days post-infection, 20 mosquito midguts per group were dissected, stained with 1%
137 mercurochrome, and examined under an optical microscope at 100× magnification to detect and
138 quantify oocysts.

139 **Sporozoites count**

140 Mosquitoes used to examine sporozoite development received a second (uninfected) bloodmeal on
141 day 4-6 post infection to synchronise oocyst development Fifteen days post-infection, mosquito
142 salivary glands were dissected in PBS and transferred to oocyst lysis buffer (NaCl 0.1M: EDTA 25mM:

143 TRIS-HCl 10mM). Following overnight incubation at 56°C with Proteinase K, DNA was extracted with
144 the automated MagNA Pure LC instrument using the MagNA Pure LC DNA Isolation Kit – High
145 performance. Sporozoite density was analysed using COX-I qPCR [41].

146 **Statistical analysis**

147 Statistical analyses were conducted using R software (v 3.1.12) [42]. Mean counts of oocysts,
148 sporozoites, and ookinetes (with 95% confidence intervals) were estimated using a mixed-effects
149 negative binomial regression model, incorporating random intercepts for biological replicates and
150 fixed effects for parasite line, mosquito species, and their interactions. Mean survival and commitment
151 rates (with 95% confidence intervals) were calculated using a mixed-effects beta regression model.
152 Transmission-reducing activity (TRA) and transmission-blocking activity (TBA)—reflecting reductions in
153 oocyst density and the proportion of infected mosquitoes, respectively—were assessed for 700 nM
154 and 7000 nM DHA using a Bayesian Poisson regression model.

155

156 ## Results

157 **Parasite Backgrounds**

158 We used well-characterised *P. falciparum* reference lines from Africa (NF54), Southeast Asia (NF135,
159 Cambodia) [29], and a new isolate from East Africa (NF180, Uganda). These lines were complemented
160 with previously published ART-R K13 mutant parasite lines from Thailand (ARN1G, K13-449A) [32],
161 from Cambodia (3815, K13-580Y) [31, 35] and a recently collected isolate from Uganda with *in vitro*
162 ART-R that is independent of K13 mutations (PAT-023). Genotyping revealed that some isolates
163 harboured mutations associated with resistance to chloroquine and sulfadoxine-pyrimethamine (Table
164 1).

165 **Artemisinin resistance**

166 ART-R lines ARN1G and 3815 showed survival rates in the RSA that were higher than the ART-S NF54,
167 as previously published ([16, 32, 35];Figure 1a, Sup Table 1). The ART-S NF135 line showed variable
168 survival rates, which were lower than those observed for known ART-R lines but higher than those
169 observed for ART-S NF54 and NF180 lines. The PAT-023 parasite line exhibited a survival rate of 9.1%
170 (95% CI: 6.6% - 12.5%), higher than the ART-S lines.

171 **Sexual conversion**

172 We assessed the ability of ART-R and ART-S lines to convert under two different conditions that mimic
173 the natural sensing system [25, 43, 44]. All conversion assays were performed on a single asexual cycle.
174 The highest rates of gametocyte conversion were typically observed using minimal fatty acid media

175 with intermediate conversion rates for media containing Albumax only, and lowest conversion in non-
176 inducing serum media (Figure 1b, Sup Table 2) [25]. The conversion rates observed for K13 mutants
177 were comparable to those recorded for ART-S lines, except for the Cambodian line 3815, which
178 exhibited highly variable conversion rates (Figure 1b). The Ugandan ART-R line PAT-023 exhibited
179 minimal sexual conversion with little difference between media types (Figure 1b, Sup Table 2).

180 **Transmission to mosquitoes**

181 Transmission was examined using three mosquito vector species. We first assessed whether
182 fertilisation occurred in mosquitoes for each parasite isolate by quantifying parasite developmental
183 stages in the mosquito midgut 20-24 hours post-infectious blood meal. We distinguished between
184 mature and immature stages following Pfs25 antibody labelling. Immature stages included
185 underdeveloped ookinetes and rounded forms classified as either zygotes or unfertilised females. The
186 ART-S parasite isolates NF180 and NF135 showed no clear difference in the numbers of immature or
187 mature ookinetes across the mosquito species tested (Figure 2a). For NF54, there was a tendency for
188 fewer mature ookinetes in *An. gambiae* compared to *An. stephensi* and *An. coluzzii*, though this was
189 not statistically significant (Figure 2a). A similar trend was observed with the ART-R ARN1G parasite
190 line. For the Ugandan ART-R isolate PAT-023, significantly fewer mature ookinetes were observed
191 in *An. stephensi* (Figure 2a) compared to *An. coluzzii* ($p = 0.019$). However, this significance level should
192 be interpreted cautiously due to the number of comparisons made. The ART-R isolate 3815
193 consistently produced low numbers of mature ookinetes in all *Anopheles* species tested (Figure 2a).

194 To confirm that mature ookinetes can establish mosquito infections, we quantified oocyst prevalence
195 (Sup Figure 1) and density (Figure 2b) on day 7 post-bloodmeal. African parasite lines (NF54, NF180,
196 and PAT-023) exhibited higher oocyst densities in traditional African mosquitoes (*An. gambiae s.s., An.*
197 *coluzzii*) compared to *An. stephensi,* particularly for PAT-023 ($p < 0.0001$; Sup Table 3). In contrast,
198 NF135 showed consistent infection intensities across all tested mosquito species. For the Asian K13
199 mutant lines, oocysts densities in *An. stephensi* were slightly higher compared to *An. coluzzii* and *An.*
200 *gambiae*. Specifically, ARN1G had higher densities in *An. stephensi* relative to *An. coluzzii* ($p = 0.009$)
201 and *An. gambiae* ($p < 0.0001$), while 3815 showed similar trends ($p = 0.007$ compared to *An. coluzzii*
202 and $p<0.0001$ compared to *An. gambiae*; Sup Table 3). The 3815 isolate exhibited low and highly
203 variable infection levels, consistent with its reduced mature ookinete counts (Figures 2a and 2b).
204 Interestingly, a trend towards higher sporozoite production was observed in the two K13 mutant lines
205 (ARN1G and 3815), which persisted even after normalising for oocyst density within the same batch of
206 mosquitoes. However, transmission results for the 3815 isolate remained highly variable (Sup Figure
207 2).

208 **Transmission-blocking effect of DHA**

209 With all parasite lines being able to infect mosquitoes in the absence of drug pressure, we determined
210 the transmission-reducing effect of DHA on mosquito infection intensity and prevalence. Mature
211 gametocytes were exposed to both the physiologically relevant concentration of 700nM and a tenfold
212 higher concentration of 7000nM for 48 hours before mosquito feeding with the drug not removed
213 before mosquito feeding [32, 45]. Given the labour-intensive nature of these assays and the known
214 permissiveness of *An. coluzzii* for the parasites tested herein, experiments were performed exclusively
215 with this mosquito species. All isolates showed a consistent decrease in mosquito infection intensity
216 and prevalence as DHA concentrations increased, independent of the K13 genotype and the parasite
217 background (Figure 3, Sup Figure 3). At 7000nM DHA, sporadic infections were observed for the African
218 isolates NF54, NF180, and PAT-023, but none of the Asian isolates infected mosquitoes at this
219 concentration. When relative reductions in oocyst intensity were calculated, DHA exposure at 7000nM
220 reduced oocyst intensities by >93% for all parasite isolates. There were no indications of reduced
221 transmission-blocking efficacy of DHA for ART-R isolates.

222 **Correlating resistance and transmission stages**

223 Although the number of parasite isolates examined was modest, our data on asexual parasite survival
224 under DHA pressure, combined with findings on sexual conversion and mosquito transmission, provide
225 an opportunity to investigate whether ART-R confers a transmission advantage. We initially
226 hypothesised that parasites with high levels of *in vitro* resistance to ART at the ring stage would show
227 enhanced transmission to mosquitoes under drug pressure. However, no correlation was observed
228 between parasite survival in the RSA and the reduction in oocyst density under DHA pressure (Figure
229 4a). In contrast with our initial hypothesis, we observed a weak negative correlation between parasite
230 survival rates in the RSA and gametocyte conversion rates upon induction (Figure 4b).

231

232 Discussion

233 Understanding the intricacies of transmission fitness of parasites that survive ART-based treatment is
234 important for developing strategies that aim to prevent or slow down the spread of resistance. Some
235 reports have suggested that K13 mutant parasites may have higher intrinsic gametocyte production
236 [4, 46], yet some *in vivo* evidence suggests following ART treatment in a Vietnam cohort, the
237 conversion marker *ap2g* decreases – suggesting a decrease in gametocyte production linked to K13
238 mutations [47]. In contrast, an *in vitro* study observed increased survival of specifically male
239 gametocytes of K13 mutants under DHA exposure [32].

240 We selected three parasite isolates from Southeast Asia and three from Sub-Saharan Africa, each with
241 distinct K13 genotypes and genetic backgrounds. The Asian ART-R lines ARN1G and 3815 exhibited
242 higher parasite survival in the RSA, consistent with previous studies [16, 32, 35]. PAT-023, a newly
243 characterised Ugandan ART-R line that is wild-type for K13, demonstrated a high level of survival in the
244 *in vitro* RSA. Interestingly, we also observed that NF135, a line previously associated with treatment
245 failure following artemether-lumefantrine therapy *in vivo* [48], showed erratic and occasionally
246 increased DHA survival rates.

247 This study focuses on the transmission potential of *P. falciparum* isolates, beginning with their
248 commitment to sexual stages. Fatty acids play a key role in signalling the parasite to commit to sexual
249 conversion [36, 37]; we used two methods targeting this signalling pathway to robustly compare sexual
250 conversion rates. From all media conditions tested, minimal fatty acid media consistently produced
251 the highest sexual conversion rates across all lines. Among the two ART-S parasite isolates, NF54 and
252 NF135 consistently exhibited high conversion levels. The newly introduced NF180 line, which has not
253 been characterised in detail previously, demonstrated high conversion rates, comparable to NF54 and
254 higher than NF135. Among the ART-R parasite lines, PAT-023 displayed the lowest commitment to
255 gametocyte production, while ARN1G showed commitment levels similar to NF135. The Cambodian
256 3815 line exhibited an inconsistent commitment to gametocyte production, with occasional high levels
257 that decreased with prolonged culture, potentially reflecting epigenetic silencing during *in vitro*
258 cultivation.

259 We observed no evidence of increased sexual conversion in the ART-R isolates in the absence of
260 treatment. Of note, we did not explore the possible impact of DHA on conversion rates in these ART-R
261 lines. An earlier study reported that the ART-S NF54 line was associated with higher sexual conversion
262 when trophozoites were treated with DHA [49]. Future studies that investigate whether DHA-treated
263 ART-R lines may give rise to an elevated conversion rate can be of value. Such studies would allow us
264 to disentangle the contributions to the overall transmission competence; if this is linked to the
265 increased conversion to or survival of the sexual stages after ART treatment or an inherent increased
266 conversion rate in the K13 mutant lines.

267 While sexual conversion is a critical step for transmission, high commitment rates do not always
268 correlate with high mosquito infectivity [25, 29]. The presence of mature ookinetes is a direct indicator
269 of fertilisation in the mosquito environment. In our study, we fed the same gametocyte material to the
270 three mosquito species. We did not normalize for gametocyte density prior to feeding; instead we had
271 a uniform starting parasite concentration prior to gametocyte induction. We are thus able to compare
272 the composite product of gametocyte production and infectivity between lines. When examining the

273 impact of individual mutations on transmission competence in matched genetic backgrounds, such
274 normalization at different points in the transmission cycle may be beneficial. We reproducibly
275 observed low transmission potential of the 3815 line, which consistently formed very few ookinetes in
276 all species and exhibited low and sporadic oocyst infections. We also observed a tendency for African
277 parasites to fare better in the African mosquitoes, illustrated by both the numbers of mature ookinetes
278 and oocysts densities. Although our sample size is too small for a comprehensive analysis of the
279 underlying biological mechanisms, one could speculate that genes influencing the parasite's ability to
280 evade the mosquito immune system may play a role. Pfs47 is essential for *P. falciparum* infection in
281 *An. gambiae* but not for *An. stephensi* [50, 51]. Contrary to this observation for African parasite lines,
282 all three Southeast Asian parasite lines were able to infect Asian and African mosquito vectors.
283 Interestingly ARN1G achieved slightly higher ookinete numbers in *An. coluzzii* but this did not translate
284 into higher oocyst densities. Similar to our observations on sexual conversion rates, we found no
285 significant transmission advantage for ART-R parasite lines with reduced ART susceptibility in the RSA.

286 To confirm the ability of the parasites to complete sporogony, we quantified sporozoite numbers in
287 mosquito salivary glands. Previous studies have suggested that K13 mutant lines might produce larger
288 oocysts [32], potentially indicating increased sporozoite production. While we did not observe a clear
289 increase in oocyst size (data not shown) whilst sporozoite production varied between parasite lines.
290 The two Asian ART-R lines, each with a unique K13 mutation, exhibited significantly higher sporozoite
291 loads in at least one African mosquito species. This effect was most pronounced for ARN1G that
292 showed significantly higher sporozoite numbers in *An. coluzzii*, even after adjusting for oocyst density
293 within the same mosquito batch. Further studies are needed to confirm these findings and to explore
294 the underlying biological mechanisms. However, our results are consistent with the suggestion that
295 the K13 mutation may lead to larger oocysts [32], which could lead to increased sporozoite production.

296 Lastly, we examined the impact of DHA exposure on transmission efficiency, hypothesising that
297 parasite lines with increased survival rate of asexual parasites under DHA exposure may show similar
298 survival advantages when gametocytes are exposed. Previous work has suggested that male
299 gametocytes with K13 mutations may have partial protection against DHA [32, 52]. Using *An. coluzzii,*
300 we tested two DHA concentrations and found no evidence that DHA was less effective against mature
301 gametocytes and subsequently reducing transmission in ART-R parasites. Immature gametocytes are
302 known to be more susceptible to artemisinins than mature gametocytes [32, 53]. Since we did not
303 examine the impact of DHA on immature gametocytes, we cannot rule out preferential survival of
304 immature ART-R gametocytes. Even at the highest DHA concentrations, both ART-S and ART-R African
305 parasite lines occasionally infected mosquitoes. This observation is consistent with previous *ex vivo*

experiments [45] and aligns with clinical studies showing that treatment with DHA-piperaquine fails to fully prevent transmission in the first weeks after treatment [54].

In this study, we examined a limited number of ART-S and ART-R parasite lines across three mosquito species. We observed no consistent evidence of increased gametocyte production or increased mosquito infectivity. While the transmission-reducing effect of DHA was imperfect, its efficacy was not reduced in gametocyte-producing lines with partial resistance to ARTs. To fully uncover the discrete impacts of individual K13 mutations on gametocyte production, infectivity and gametocyte resistance to ARTs, future studies using genetically engineered lines in controlled isogenic backgrounds are required.

Ethics declarations

Experiments with *in vitro* cultured parasites and *Anopheles* mosquitoes at Radboud University Medical Center were conducted following approval from the Radboud University Experimental Animal Ethical Committee (RUDEC 2009-019, RUDEC 2009-225). PAT-023 was obtained with patient informed consent for sample collection and for its future use. The study protocol that this collection was part of was approved by the Makerere University School of Biomedical Sciences Research and Ethics Committee (SBS-363), the Uganda National Council for Science and Technology, and the University of California, San Francisco Committee on Human Research [55].

Acknowledgements and financial support

We would like to thank Jolanda Klaassen, Laura Pelser-Posthumus, Astrid Pouwelsen and Jacqueline Kuhnen for breeding of mosquitoes and handling of the infected mosquitoes. We are grateful for the support of Marga de Vegte-Bolmer in starting parasite culture with the 3815 line. DAF gratefully acknowledges funding support from the NIH (R01 AI109023). SM acknowledges the support from the Human Frontier Science Program Long-term Fellowship (LT000976/2016-L) and funding from United States National Institutes of Health NIAID grant R01 AI182318 (PI: SM). This work was supported by a fellowship from the Netherlands Organization for Scientific Research (Vici fellowship NWO 09150182210039).

References

1. Organization, W.H., *World malaria report.* 2024
2. Noedl, H., Y. Se, K. Schaecher, B.L. Smith, D. Socheat, M.M. Fukuda, and C. Artemisinin Resistance in Cambodia 1 Study, *Evidence of artemisinin-resistant malaria in western Cambodia.* N Engl J Med, 2008. **359**(24): p. 2619-20.10.1056/NEJMc0805011.
3. Dondorp, A.M., F. Nosten, P. Yi, D. Das, A.P. Phyo, J. Tarning, et al., *Artemisinin resistance in Plasmodium falciparum malaria.* N Engl J Med, 2009. **361**(5): p. 455-67.10.1056/NEJMoa0808859.
4. Amaratunga, C., P. Lim, S. Suon, S. Sreng, S. Mao, C. Sopha, et al., *Dihydroartemisinin-piperaquine resistance in Plasmodium falciparum malaria in Cambodia: a multisite prospective cohort study.* Lancet Infect Dis, 2016. **16**(3): p. 357-65.10.1016/S1473-3099(15)00487-9.
5. Mairet-Khedim, M., R. Leang, C. Marmai, N. Khim, S. Kim, S. Ke, et al., *Clinical and In Vitro Resistance of Plasmodium falciparum to Artesunate-Amodiaquine in Cambodia.* Clin Infect Dis, 2021. **73**(3): p. 406-413.10.1093/cid/ciaa628.
6. Phyo, A.P., E.A. Ashley, T.J.C. Anderson, Z. Bozdech, V.I. Carrara, K. Sriprawat, et al., *Declining Efficacy of Artemisinin Combination Therapy Against P. Falciparum Malaria on the Thai-Myanmar Border (2003-2013): The Role of Parasite Genetic Factors.* Clin Infect Dis, 2016. **63**(6): p. 784-791.10.1093/cid/ciw388.
7. Ashley, E.A., M. Dhorda, R.M. Fairhurst, C. Amaratunga, P. Lim, S. Suon, et al., *Spread of artemisinin resistance in Plasmodium falciparum malaria.* N Engl J Med, 2014. **371**(5): p. 411-23.10.1056/NEJMoa1314981.
8. Birnbaum, J., S. Scharf, S. Schmidt, E. Jonscher, W.A.M. Hoeijmakers, S. Flemming, et al., *A Kelch13-defined endocytosis pathway mediates artemisinin resistance in malaria parasites.* Science, 2020. **367**(6473): p. 51-59.10.1126/science.aax4735.
9. Miotto, O., R. Amato, E.A. Ashley, B. MacInnis, J. Almagro-Garcia, C. Amaratunga, et al., *Genetic architecture of artemisinin-resistant Plasmodium falciparum.* Nat Genet, 2015. **47**(3): p. 226-34.10.1038/ng.3189.
10. Straimer, J., N.F. Gnadig, B. Witkowski, C. Amaratunga, V. Duru, A.P. Ramadani, et al., *K13-propeller mutations confer artemisinin resistance in Plasmodium falciparum clinical isolates.* Science, 2015. **347**(6220): p. 428-31.10.1126/science.1260867.
11. Bridgford, J.L., S.C. Xie, S.A. Cobbold, C.F.A. Pasaje, S. Herrmann, T. Yang, et al., *Artemisinin kills malaria parasites by damaging proteins and inhibiting the proteasome.* Nat Commun, 2018. **9**(1): p. 3801.10.1038/s41467-018-06221-1.
12. Duffy, S. and V.M. Avery, *Naturally Acquired Kelch13 Mutations in Plasmodium falciparum Strains Modulate In Vitro Ring-Stage Artemisinin-Based Drug Tolerance and Parasite Survival in Response to Hyperoxia.* Microbiol Spectr, 2022. **10**(5): p. e0128221.10.1128/spectrum.01282-21.
13. Behrens, H.M., S. Schmidt, I.G. Henshall, P. Lopez-Barona, D. Peigney, R. Sabitzki, et al., *Impact of different mutations on Kelch13 protein levels, ART resistance, and fitness cost in Plasmodium falciparum parasites.* mBio, 2024. **15**(6): p. e0198123.10.1128/mbio.01981-23.
14. Nair, S., X. Li, G.A. Arya, M. McDew-White, M. Ferrari, F. Nosten, and T.J.C. Anderson, *Fitness Costs and the Rapid Spread of kelch13-C580Y Substitutions Conferring Artemisinin Resistance.* Antimicrob Agents Chemother, 2018. **62**(9).10.1128/AAC.00605-18.
15. Mok, S., B.H. Stokes, N.F. Gnadig, L.S. Ross, T. Yeo, C. Amaratunga, et al., *Artemisinin-resistant K13 mutations rewire Plasmodium falciparum's intra-erythrocytic metabolic program to enhance survival.* Nat Commun, 2021. **12**(1): p. 530.10.1038/s41467-020-20805-w.
16. Stokes, B.H., S.K. Dhingra, K. Rubiano, S. Mok, J. Straimer, N.F. Gnadig, et al., *Plasmodium falciparum K13 mutations in Africa and Asia impact artemisinin resistance and parasite fitness.* Elife, 2021. **10**.10.7554/eLife.66277.

17. Ariey, F., B. Witkowski, C. Amaratunga, J. Beghain, A.C. Langlois, N. Khim, et al., *A molecular marker of artemisinin-resistant Plasmodium falciparum malaria.* Nature, 2014. **505**(7481): p. 50-5.10.1038/nature12876.
18. Menard, D., N. Khim, J. Beghain, A.A. Adegnika, M. Shafiul-Alam, O. Amodu, et al., *A Worldwide Map of Plasmodium falciparum K13-Propeller Polymorphisms.* N Engl J Med, 2016. **374**(25): p. 2453-64.10.1056/NEJMoa1513137.
19. Conrad, M.D., V. Asua, S. Garg, D. Giesbrecht, K. Niare, S. Smith, et al., *Evolution of Partial Resistance to Artemisinins in Malaria Parasites in Uganda.* N Engl J Med, 2023. **389**(8): p. 722-732.10.1056/NEJMoa2211803.
20. Grossman, T., J. Vainer, Y. Paran, L. Studentsky, U. Manor, R. Dzikowski, and E. Schwartz, *Emergence of artemisinin-based combination treatment failure in patients returning from sub-Saharan Africa with P. falciparum malaria.* J Travel Med, 2023. **30**(8).10.1093/jtm/taad114.
21. Uwimana, A., E. Legrand, B.H. Stokes, J.M. Ndikumana, M. Warsame, N. Umulisa, et al., *Emergence and clonal expansion of in vitro artemisinin-resistant Plasmodium falciparum kelch13 R561H mutant parasites in Rwanda.* Nat Med, 2020. **26**(10): p. 1602-1608.10.1038/s41591-020-1005-2.
22. Rosenthal, P.J., V. Asua, J.A. Bailey, M.D. Conrad, D.S. Ishengoma, M.R. Kamya, et al., *The emergence of artemisinin partial resistance in Africa: how do we respond?* Lancet Infect Dis, 2024. **24**(9): p. e591-e600.10.1016/S1473-3099(24)00141-5.
23. Rosenthal, P.J., V. Asua, and M.D. Conrad, *Emergence, transmission dynamics and mechanisms of artemisinin partial resistance in malaria parasites in Africa.* Nat Rev Microbiol, 2024. **22**(6): p. 373-384.10.1038/s41579-024-01008-2.
24. Stewart, L.B., A. Freville, T.S. Voss, D.A. Baker, G.A. Awandare, and D.J. Conway, *Plasmodium falciparum Sexual Commitment Rate Variation among Clinical Isolates and Diverse Laboratory-Adapted Lines.* Microbiol Spectr, 2022. **10**(6): p. e0223422.10.1128/spectrum.02234-22.
25. Graumans, W., A. van der Starre, R. Stoter, G.J. van Gemert, C. Andolina, J. Ramjith, et al., *AlbuMAX supplemented media induces the formation of transmission-competent P. falciparum gametocytes.* Mol Biochem Parasitol, 2024. **259**: p. 111634.10.1016/j.molbiopara.2024.111634.
26. Cohuet, A., C. Harris, V. Robert, and D. Fontenille, *Evolutionary forces on Anopheles: what makes a malaria vector?* Trends Parasitol, 2010. **26**(3): p. 130-6.10.1016/j.pt.2009.12.001.
27. Ashine, T., A. Eyasu, Y. Asmamaw, E. Simma, E. Zemene, A. Epstein, et al., *Spatiotemporal distribution and bionomics of Anopheles stephensi in different eco-epidemiological settings in Ethiopia.* Parasit Vectors, 2024. **17**(1): p. 166.10.1186/s13071-024-06243-3.
28. Emiru, T., D. Getachew, M. Murphy, L. Sedda, L.A. Ejigu, M.G. Bulto, et al., *Evidence for a role of Anopheles stephensi in the spread of drug- and diagnosis-resistant malaria in Africa.* Nat Med, 2023. **29**(12): p. 3203-3211.10.1038/s41591-023-02641-9.
29. van de Vegte-Bolmer, M., W. Graumans, R. Stoter, G.J. van Gemert, R. Sauerwein, K.A. Collins, and T. Bousema, *A portfolio of geographically distinct laboratory-adapted Plasmodium falciparum clones with consistent infection rates in Anopheles mosquitoes.* Malar J, 2021. **20**(1): p. 381.10.1186/s12936-021-03912-x.
30. Teirlinck, A.C., M. Roestenberg, M. van de Vegte-Bolmer, A. Scholzen, M.J. Heinrichs, R. Siebelink-Stoter, et al., *NF135.C10: a new Plasmodium falciparum clone for controlled human malaria infections.* J Infect Dis, 2013. **207**(4): p. 656-60.10.1093/infdis/jis725.
31. Imwong, M., K. Suwannasin, C. Kunasol, K. Sutawong, M. Mayxay, H. Rekol, et al., *The spread of artemisinin-resistant Plasmodium falciparum in the Greater Mekong subregion: a molecular epidemiology observational study.* Lancet Infect Dis, 2017. **17**(5): p. 491-497.10.1016/S1473-3099(17)30048-8.
32. Witmer, K., F.A. Dahalan, M.J. Delves, S. Yahiya, O.J. Watson, U. Straschil, et al., *Transmission of Artemisinin-Resistant Malaria Parasites to Mosquitoes under Antimalarial Drug Pressure.* Antimicrob Agents Chemother, 2020. **65**(1).10.1128/AAC.00898-20.

33. Ponnudurai, T., A.H. Lensen, A.D. Leeuwenberg, and J.H. Meuwissen, *Cultivation of fertile Plasmodium falciparum gametocytes in semi-automated systems. 1. Static cultures.* Trans R Soc Trop Med Hyg, 1982. **76**(6): p. 812-8.10.1016/0035-9203(82)90116-x.
34. Phelan, J.E., A. Turkiewicz, E. Manko, J. Thorpe, L.N. Vanheer, M. van de Vegte-Bolmer, et al., *Rapid profiling of Plasmodium parasites from genome sequences to assist malaria control.* Genome Med, 2023. **15**(1): p. 96.10.1186/s13073-023-01247-7.
35. Witkowski, B., N. Khim, P. Chim, S. Kim, S. Ke, N. Kloeung, et al., *Reduced artemisinin susceptibility of Plasmodium falciparum ring stages in western Cambodia.* Antimicrob Agents Chemother, 2013. **57**(2): p. 914-23.10.1128/AAC.01868-12.
36. Harris, C.T., X. Tong, R. Campelo, I.M. Marreiros, L.N. Vanheer, N. Nahiyaan, et al., *Sexual differentiation in human malaria parasites is regulated by competition between phospholipid metabolism and histone methylation.* Nat Microbiol, 2023. **8**(7): p. 1280-1292.10.1038/s41564-023-01396-w.
37. Brancucci, N.M.B., J.P. Gerdt, C. Wang, M. De Niz, N. Philip, S.R. Adapa, et al., *Lysophosphatidylcholine Regulates Sexual Stage Differentiation in the Human Malaria Parasite Plasmodium falciparum.* Cell, 2017. **171**(7): p. 1532-1544 e15.10.1016/j.cell.2017.10.020.
38. Feldmann, A.M. and T. Ponnudurai, *Selection of Anopheles stephensi for refractoriness and susceptibility to Plasmodium falciparum.* Med Vet Entomol, 1989. **3**(1): p. 41-52.10.1111/j.1365-2915.1989.tb00473.x.
39. Meister, S., B. Agianian, F. Turlure, A. Relogio, I. Morlais, F.C. Kafatos, and G.K. Christophides, *Anopheles gambiae PGRPLC-mediated defense against bacteria modulates infections with malaria parasites.* PLoS Pathog, 2009. **5**(8): p. e1000542.10.1371/journal.ppat.1000542.
40. Deletre, E., T. Martin, C. Dumenil, and F. Chandre, *Insecticide resistance modifies mosquito response to DEET and natural repellents.* Parasit Vectors, 2019. **12**(1): p. 89.10.1186/s13071-019-3343-9.
41. Andolina, C., W. Graumans, M. Guelbeogo, G.J. van Gemert, J. Ramijth, S. Harouna, et al., *Quantification of sporozoite expelling by Anopheles mosquitoes infected with laboratory and naturally circulating P. falciparum gametocytes.* Elife, 2024. **12**.10.7554/eLife.90989.
42. RC, T. *The R project for statistical computing Cran.* 2019; Available from: https://www.r-project.org/.
43. Omorou, R., I. Bin Sa'id, M. Delves, C. Severini, Y.I. Kouakou, A.L. Bienvenu, and S. Picot, *Protocols for Plasmodium gametocyte production in vitro: an integrative review and analysis.* Parasit Vectors, 2022. **15**(1): p. 451.10.1186/s13071-022-05566-3.
44. Tanaka, T.Q., S.M. Tokuoka, D. Nakatani, F. Hamano, S.I. Kawazu, T.E. Wellems, et al., *Polyunsaturated fatty acids promote Plasmodium falciparum gametocytogenesis.* Biol Open, 2019. **8**(7).10.1242/bio.042259.
45. Henry, N.B., I. Soulama, S.S. S, J.M. Bolscher, T.G.H. T, S.C. A, et al., *Assessment of the transmission blocking activity of antimalarial compounds by membrane feeding assays using natural Plasmodium falciparum gametocyte isolates from West-Africa.* PLoS One, 2023. **18**(7): p. e0284751.10.1371/journal.pone.0284751.
46. Zhu, L., R.W. van der Pluijm, M. Kucharski, S. Nayak, J. Tripathi, N.J. White, et al., *Artemisinin resistance in the malaria parasite, Plasmodium falciparum, originates from its initial transcriptional response.* Commun Biol, 2022. **5**(1): p. 274.10.1038/s42003-022-03215-0.
47. Portugaliza, H.P., H.M. Natama, P. Guetens, E. Rovira-Vallbona, A.M. Some, A. Millogo, et al., *Plasmodium falciparum sexual conversion rates can be affected by artemisinin-based treatment in naturally infected malaria patients.* EBioMedicine, 2022. **83**: p. 104198.10.1016/j.ebiom.2022.104198.
48. van der Boor, S.C., M. Alkema, G.J. van Gemert, K. Teelen, M. van de Vegte-Bolmer, J. Walk, et al., *Whole sporozoite immunization with Plasmodium falciparum strain NF135 in a randomized trial.* BMC Med, 2023. **21**(1): p. 137.10.1186/s12916-023-02788-9.

484 49. Portugaliza, H.P., S. Miyazaki, F.J. Geurten, C. Pell, A. Rosanas-Urgell, C.J. Janse, and A. Cortes,
485 *Artemisinin exposure at the ring or trophozoite stage impacts Plasmodium falciparum sexual*
486 *conversion differently.* Elife, 2020. **9**.10.7554/eLife.60058.
487 50. Molina-Cruz, A., L.S. Garver, A. Alabaster, L. Bangiolo, A. Haile, J. Winikor, et al., *The human*
488 *malaria parasite Pfs47 gene mediates evasion of the mosquito immune system.* Science, 2013.
489 **340**(6135): p. 984-7.10.1126/science.1235264.
490 51. van Schaijk, B.C., M.R. van Dijk, M. van de Vegte-Bolmer, G.J. van Gemert, M.W. van Dooren,
491 S. Eksi, et al., *Pfs47, paralog of the male fertility factor Pfs48/45, is a female specific surface*
492 *protein in Plasmodium falciparum.* Mol Biochem Parasitol, 2006. **149**(2): p. 216-
493 22.10.1016/j.molbiopara.2006.05.015.
494 52. Lozano, S., P. Gamallo, C. Gonzalez-Cortes, J.L. Presa Matilla, R.M. Fairhurst, E. Herreros, et al.,
495 *Gametocytes from K13 Propeller Mutant Plasmodium falciparum Clinical Isolates Demonstrate*
496 *Reduced Susceptibility to Dihydroartemisinin in the Male Gamete Exflagellation Inhibition*
497 *Assay.* Antimicrob Agents Chemother, 2018. **62**(12).10.1128/AAC.01426-18.
498 53. Adjalley, S.H., G.L. Johnston, T. Li, R.T. Eastman, E.H. Ekland, A.G. Eappen, et al., *Quantitative*
499 *assessment of Plasmodium falciparum sexual development reveals potent transmission-*
500 *blocking activity by methylene blue.* Proc Natl Acad Sci U S A, 2011. **108**(47): p. E1214-
501 23.10.1073/pnas.1112037108.
502 54. Stone, W., A. Mahamar, K. Sanogo, Y. Sinaba, S.M. Niambele, A. Sacko, et al., *Pyronaridine-*
503 *artesunate or dihydroartemisinin-piperaquine combined with single low-dose primaquine to*
504 *prevent Plasmodium falciparum malaria transmission in Ouelessebougou, Mali: a four-arm,*
505 *single-blind, phase 2/3, randomised trial.* Lancet Microbe, 2022. **3**(1): p. e41-
506 e51.10.1016/S2666-5247(21)00192-0.
507 55. Tumwebaze, P.K., M.D. Conrad, M. Okitwi, S. Orena, O. Byaruhanga, T. Katairo, et al.,
508 *Decreased susceptibility of Plasmodium falciparum to both dihydroartemisinin and*
509 *lumefantrine in northern Uganda.* Nat Commun, 2022. **13**(1): p. 6353.10.1038/s41467-022-
510 33873-x.

511

512 # Tables

513 **Table 1. Parasite isolates**

Parasite Isolate	Origin (Year)	Artemisinin resistance phenotype[2]	Drug resistance-conferring mutations				
			Artemisinin	Chloroquine	Mefloquine	Pyrimethamine	Sulfadoxine
NF54	Africa (1979)	S	-	-	-	-	-
NF180	East Africa (Uganda 2016)	S	-	-	-	DHFR (N51I, S108N)	DHPS (A437G, K540E)
PAT-023	East Africa (Uganda 2021)	R	ART-R[1]	PfMDR1 (Y184F)	-	DHFR (C59R, S108N)	DHPS (A437G, K540E)
NF135	Southeast Asia (Cambodia 1993)	S	-	PfCRT (M74I, N75E, K76T, A220S, Q271E, I356T, R371I)	-	DHFR (N51I, C59R, S108N, I164L)	DHPS (S436A, A437G, K540E)
ARN1G	Southeast Asia (Thailand 2011-2013)	R	K13 (G449A)	PfCRT (M74I, N75E, K76T, A220S, Q271E, N326S, I356T, R371I)	-	DHFR (N51I, C59R, S108N, I164L)	DHPS (A437G, K540E, A581G)
3815	Southeast Asia (Cambodia 2010)	R	K13 (C580Y)	PfCRT (M74I, N75E, K76T, A220S, Q271E, I356T, R371I)	-	DHFR (N51I, C59R, S108N, I164L)	DHPS (S436A, A437G, K540E)

514 [1]The ART-R of PAT-023 is not linked to K13 mutation. [2]S denotes the artemisinin sensitive isolates and R those
515 that exhibit partial resistance to artemisinins.

516 ## Figure Legends

517 **Figure 1.** Parasite survival after exposure to dihydroartemisinin (DHA) and gametocyte conversion
518 rates of *P. falciparum* isolates with different K13 genotypes and genetic backgrounds. **A)** Survival rate
519 from the Ring-stage Survival Assay (RSA) of each parasite line following 6hr exposure to 700nM DHA
520 compared to DMSO controls in duplicate wells (4-6 replicates were performed for each parasite line).
521 The dots represent biological replicate survival rates at 72h post invasion from counts either by
522 microscopy or flow cytometry and the error bars represent the 95% confidence intervals. **B)** Sexual
523 conversion in a plate-based assay that quantifies conversion rates from a single asexual round using
524 three different media types (shown different colour gradient of the bars). Conversion rates are
525 calculated by dividing the final gametocytaemia by the starting parasitemia in the same well. Dots
526 represent a single well from independent plates; error bars represent 95% confidence intervals.

527 **Figure 2.** Infection of three mosquito species with the six different parasite lines. **A)** Ookinete counts
528 20 hours after blood meal. The ookinetes were stained with anti-Pfs25 488 conjugate and counted on
529 a haemocytometer under a fluorescent microscope. Immature forms included rounded zygotes (or
530 unfertilised females) and ookinetes with incomplete maturation (lighter shading bar), while mature
531 forms were only the completely mature ookinetes (dark shaded bar). Dots represent a single count
532 from an independent experiment and the error bars represent 95% confidence intervals. **B)** Average
533 oocysts per midgut were dissected on day 7 post-bloodmeal. A total of 20 mosquitoes per group were
534 dissected and oocysts were counted by mercurochrome staining. Dots represent the counted oocysts
535 from a single midgut; error bars represent 95% confidence intervals. **C)** Salivary glands from individual
536 mosquitoes were dissected and sporozoites quantified by qPCR. The dots represent the sporozoites
537 per salivary gland from a single mosquito and the error bars represent the confidence intervals.

538 **Figure 3.** Transmission reduction in the presence of DHA. **A)** Oocyst densities observed upon exposing
539 mature gametocytes to 700nM and 7000nM DHA prior to being fed to mosquitoes in a blood meal.
540 The dots represent the average oocysts per infected mosquito from a single cage. **B)** Relative
541 reductions in oocysts density compared the no drug control. All error bars represent the confidence
542 intervals.

543 **Figure 4.** Impact of resistance on transmission and commitment. Transmission **A)** and conversion **B)**
544 plotted against survival rates from the RSA for each parasite line. The underlying detailed data on *in*
545 *vitro* parasite resistance in the RSA and gametocyte commitment are presented in Figure 1; data on
546 transmission under DHA exposure are presented in Figure 3.

547

548 # Supplemental Tables

549 **Sup Table 1. RSA comparisons**

Reference parasite line	Parasite line	Ratio (CI)
NF54	NF180	0.99 (0.25, 3.85); p=0.9742
NF54	PAT-023	33.34 (10, 100); p<0.0001
NF54	NF135	6.67 (1.49, 33.34); p=0.0023
NF54	ARN1G	20.00 (6.25, 100); p<0.0001
NF54	3815	33.34 (12.5, 100); p<0.0001
NF135	PAT-023	4.31 (1.20, 15.49); p=0.0057
NF135	ARN1G	3.13 (0.81, 12.5); p=0.0636
NF135	3815	5.26 (1.51, 20); p=0.0014

550

551 **Sup Table 2. Sexual conversion comparisons**

Parasite line	Reference media	Media	Ratio (CI)
NF54	serum	mFa	114.14 (31.57, 412.62); p<0.0001
NF54	serum	Albumax	57.67 (15.77, 210.87); p<0.0001
NF54	mFa	Albumax	0.51 (0.16, 1.60); p=0.1516
NF180	serum	mFa	46.96 (14.23, 154.93); p<0.0001
NF180	serum	Albumax	21.99 (6.47, 74.73); p<0.0001
NF180	mFa	Albumax	0.47 (0.16, 1.39); p=0.0934
PAT-023	serum	mFa	3.95 (1.06, 14.70); p=0.0374
PAT-023	serum	Albumax	2.90 (0.78, 10.79); p=0.1027
PAT-023	mFa	Albumax	0.73 (0.20, 2.74); p=0.5691
NF135	serum	mFa	92.75 (25.51, 337.27); p<0.0001
NF135	serum	Albumax	27.16 (6.95, 106.13); p<0.0001
NF135	mFa	Albumax	0.29 (0.09, 1.01); p=0.0172
ARN1G	serum	mFa	75.74 (22.86, 250.88); p<0.0001
ARN1G	serum	Albumax	30.40 (8.75, 105.61); p<0.0001
ARN1G	mFa	Albumax	0.40 (0.13, 1.24); p=0.0511
3815	serum	mFa	7.27 (1.97, 26.84); p=0.0011
3815	serum	Albumax	4.30 (1.16, 16.00); p=0.0162
3815	mFa	Albumax	0.59 (0.16, 2.19); p=0.3311

552 mFa, minimal fatty acid.

553 **Sup Table 3. Oocyst comparisons**

Parasite line	Reference mosquito species	Mosquito species	Oocyst ratio
NF54	*An. stephensi*	*An. coluzzii*	4.35 (2.5, 7.69); p<0.0001
NF54	*An. stephensi*	*An. gambiae*	1.56 (0.87, 2.78); p=0.0695
NF54	*An. coluzzii*	*An. gambiae*	0.35 (0.20, 0.62); p<0.0001
NF180	*An. stephensi*	*An. coluzzii*	1.66 (1.01, 2.70); p=0.0285
NF180	*An. stephensi*	*An. gambiae*	2.17 (1.33, 3.57); p=0.0005
NF180	*An. coluzzii*	*An. gambiae*	1.31 (0.81, 2.12); p=0.1829
PAT-023	*An. stephensi*	*An. coluzzii*	6.25 (3.45, 11.11); p<0.0001
PAT-023	*An. stephensi*	*An. gambiae*	3.45 (1.96, 5.88); p<0.0001
PAT-023	*An. coluzzii*	*An. gambiae*	0.55 (0.32, 0.94); p=0.0079
NF135	*An. stephensi*	*An. coluzzii*	1.09 (0.66, 1.79); p=0.6706
NF135	*An. stephensi*	*An. gambiae*	0.69 (0.42, 1.12); p=0.1353
NF135	*An. coluzzii*	*An. gambiae*	0.63 (0.38, 1.03); p=0.0732
ARN1G	*An. stephensi*	*An. coluzzii*	0.53 (0.31, 0.9); p=0.0087
ARN1G	*An. stephensi*	*An. gambiae*	0.30 (0.18, 0.53); p<0.0001
ARN1G	*An. coluzzii*	*An. gambiae*	0.57 (0.32, 1.00); p=0.0167
3815	*An. stephensi*	*An. coluzzii*	0.44 (0.22, 0.9); p=0.0066
3815	*An. stephensi*	*An. gambiae*	0.08 (0.03, 0.24); p<0.0001
3815	*An. coluzzii*	*An. gambiae*	0.18 (0.06, 0.56); p=0.0006

554

555 **Sup Table 4. Sporozoite comparisons**

Parasite line	Reference mosquito species	Mosquito species	Ratio (CI)
NF54	*An. stephensi*	*An. coluzzii*	0.28 (0.04, 2.0); p=0.2362
NF54	*An. stephensi*	*An. gambiae*	0.11 (0.01, 0.79); p=0.0236
NF54	*An. coluzzii*	*An. gambiae*	0.38 (0.07, 2.02); p=0.2362
NF180	*An. stephensi*	*An. coluzzii*	1.30 (0.31, 5.26); p=0.6562
NF180	*An. stephensi*	*An. gambiae*	0.28 (0.07, 1.19); p=0.0702
NF180	*An. coluzzii*	*An. gambiae*	0.22 (0.05, 0.92); p=0.0333
PAT-023	*An. stephensi*	*An. coluzzii*	1.69 (0.17, 16.67); p=1.0000
PAT-023	*An. stephensi*	*An. gambiae*	1.59 (0.14, 16.67); p=1.0000
PAT-023	*An. coluzzii*	*An. gambiae*	0.93 (0.08, 10.51); p=1.0000
NF135	*An. stephensi*	*An. coluzzii*	0.09 (0.02, 0.46); p=0.0010
NF135	*An. stephensi*	*An. gambiae*	0.22 (0.04, 1.09); p=0.0488
NF135	*An. coluzzii*	*An. gambiae*	2.20 (0.48, 10.04); p=0.2114
ARN1G	*An. stephensi*	*An. coluzzii*	5.88 (1.35, 25.0); p=0.0084
ARN1G	*An. stephensi*	*An. gambiae*	0.35 (0.06, 1.92); p=0.1388
ARN1G	*An. coluzzii*	*An. gambiae*	0.06 (0.01, 0.29); p<0.0001
3815	*An. stephensi*	*An. coluzzii*	0.02 (0, 0.31); p=0.0011
3815	*An. stephensi*	*An. gambiae*	2.56 (0.2, 33.34); p=0.3758
3815	*An. coluzzii*	*An. gambiae*	106.97 (8.32, 1375.20); p<0.0001

556

557

558 **Sup Table 5. Sporozoite per oocyst comparisons**

Parasite line	Reference mosquito species	Mosquito species	Sporozoite per oocyst ratio
NF54	*An. stephensi*	*An. coluzzii*	0.06 (0.01, 0.50); p=0.0044
NF54	*An. stephensi*	*An. gambiae*	0.07 (0.01, 0.56); p=0.0047
NF54	*An. gambiae*	*An. coluzzii*	0.92 (0.16, 5.33); p=0.9092
NF180	*An. stephensi*	*An. coluzzii*	0.78 (0.17, 3.51); p=0.6919
NF180	*An. stephensi*	*An. gambiae*	0.13 (0.03, 0.60); p=0.0042
NF180	*An. gambiae*	*An. coluzzii*	5.89 (1.32, 26.36); p=0.0096
PAT-023	*An. stephensi*	*An. coluzzii*	0.27 (0.03, 2.88); p=0.5525
PAT-023	*An. stephensi*	*An. gambiae*	0.46 (0.04, 5.59); p=0.9104
PAT-023	*An. gambiae*	*An. coluzzii*	0.59 (0.05, 7.06); p=0.9104
NF135	*An. stephensi*	*An. coluzzii*	0.09 (0.02, 0.45); p=0.0012
NF135	*An. stephensi*	*An. gambiae*	0.32 (0.06, 1.74); p=0.1198
NF135	*An. gambiae*	*An. coluzzii*	0.29 (0.06, 1.42); p=0.1198
ARN1G	*An. stephensi*	*An. coluzzii*	11.30 (2.31, 55.28); p=0.0009
ARN1G	*An. stephensi*	*An. gambiae*	1.15 (0.19, 6.97); p=0.8499
ARN1G	*An. gambiae*	*An. coluzzii*	9.81 (1.79, 53.69); p=0.0028
3815	*An. stephensi*	*An. coluzzii*	0.05 (0.00, 0.76); p=0.0084
3815	*An. stephensi*	*An. gambiae*	31.40 (1.95, 504.25); p=0.0062
3815	*An. gambiae*	*An. coluzzii*	0.00 (0.00, 0.03); p<0.0001

559

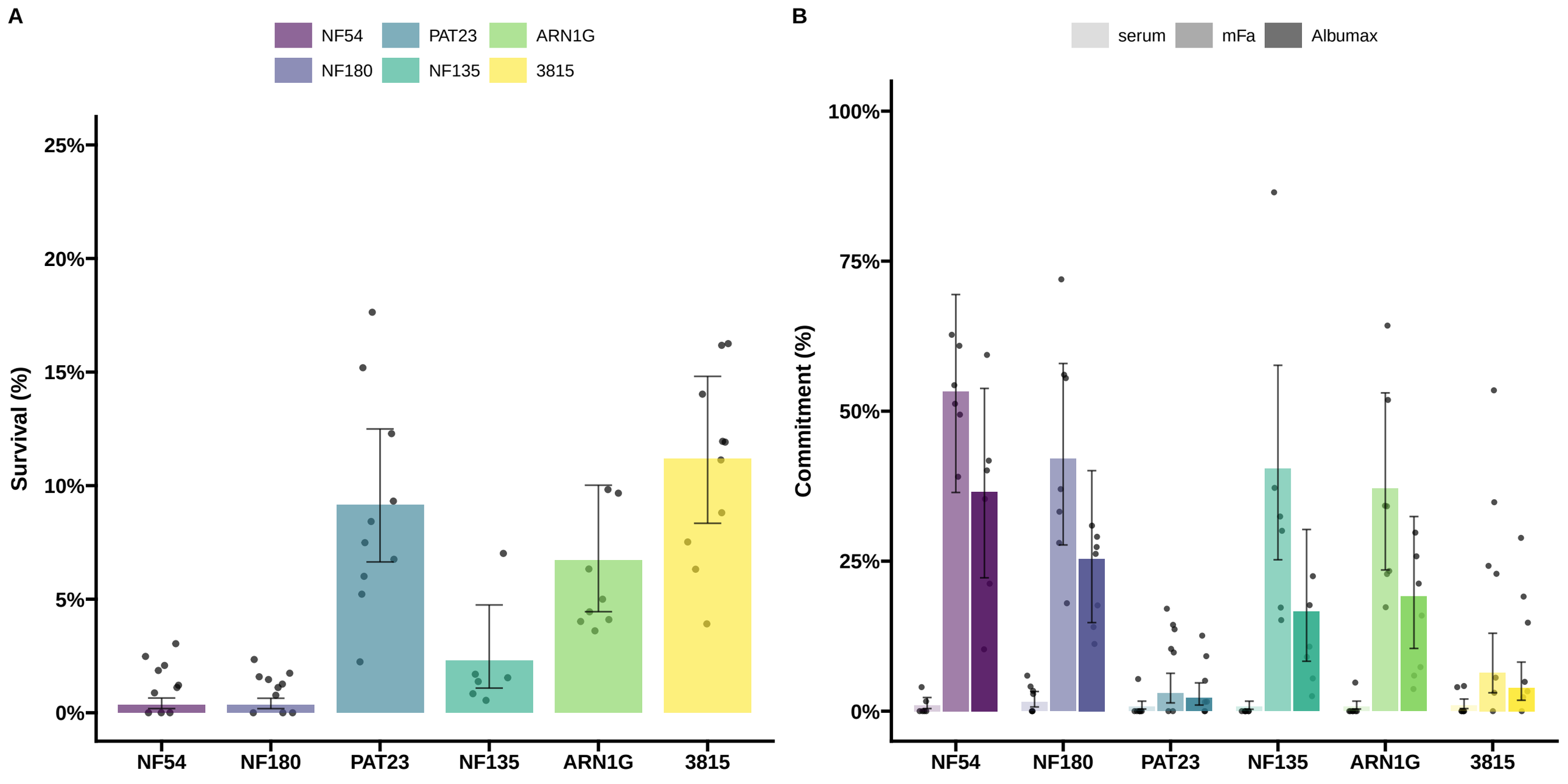
560 ## Supplemental Figure Legends

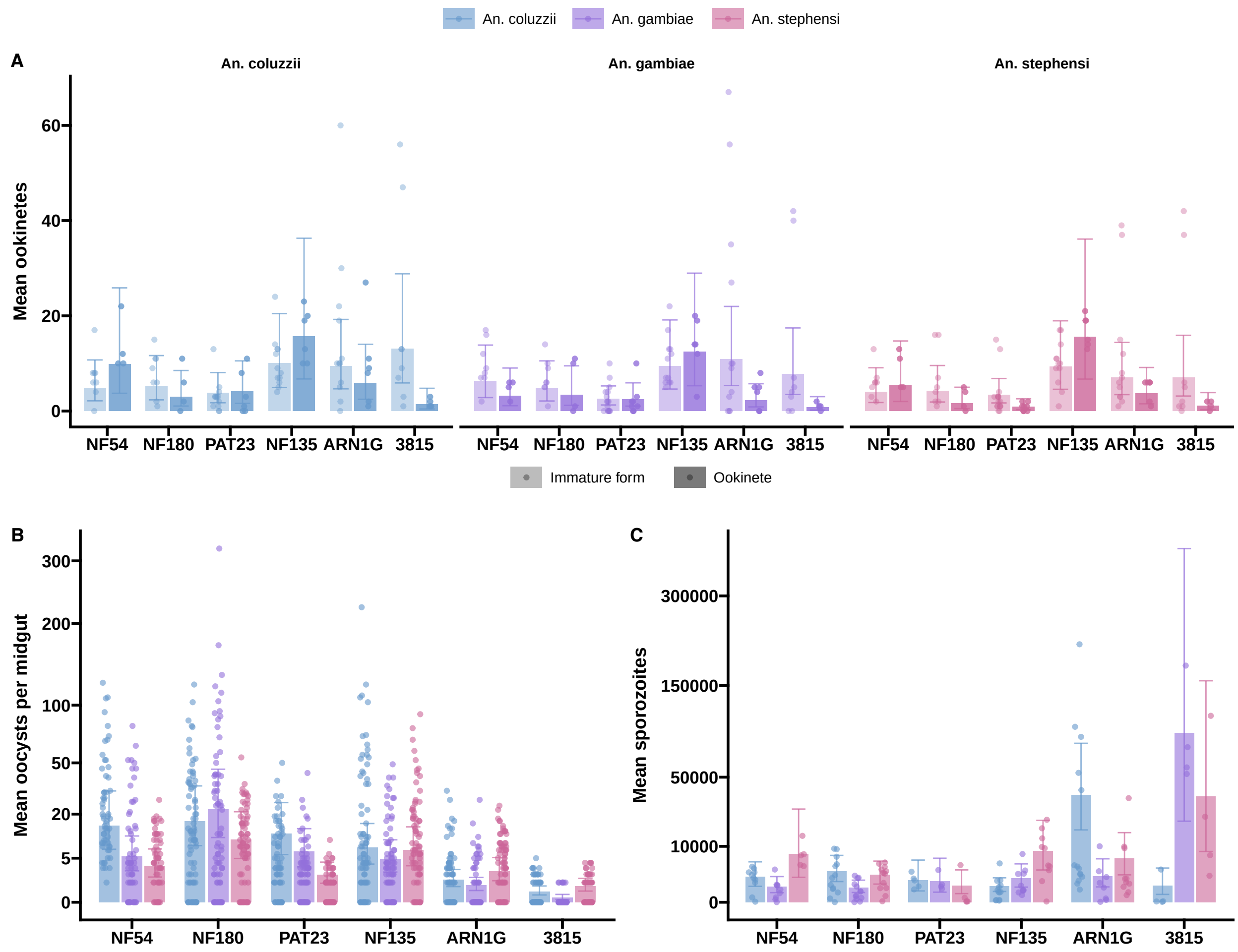
561 **Sup Figure 1.** Mosquito infection rates for the six parasite lines in each mosquito species across three
562 to four independent mosquito feeding experiments.

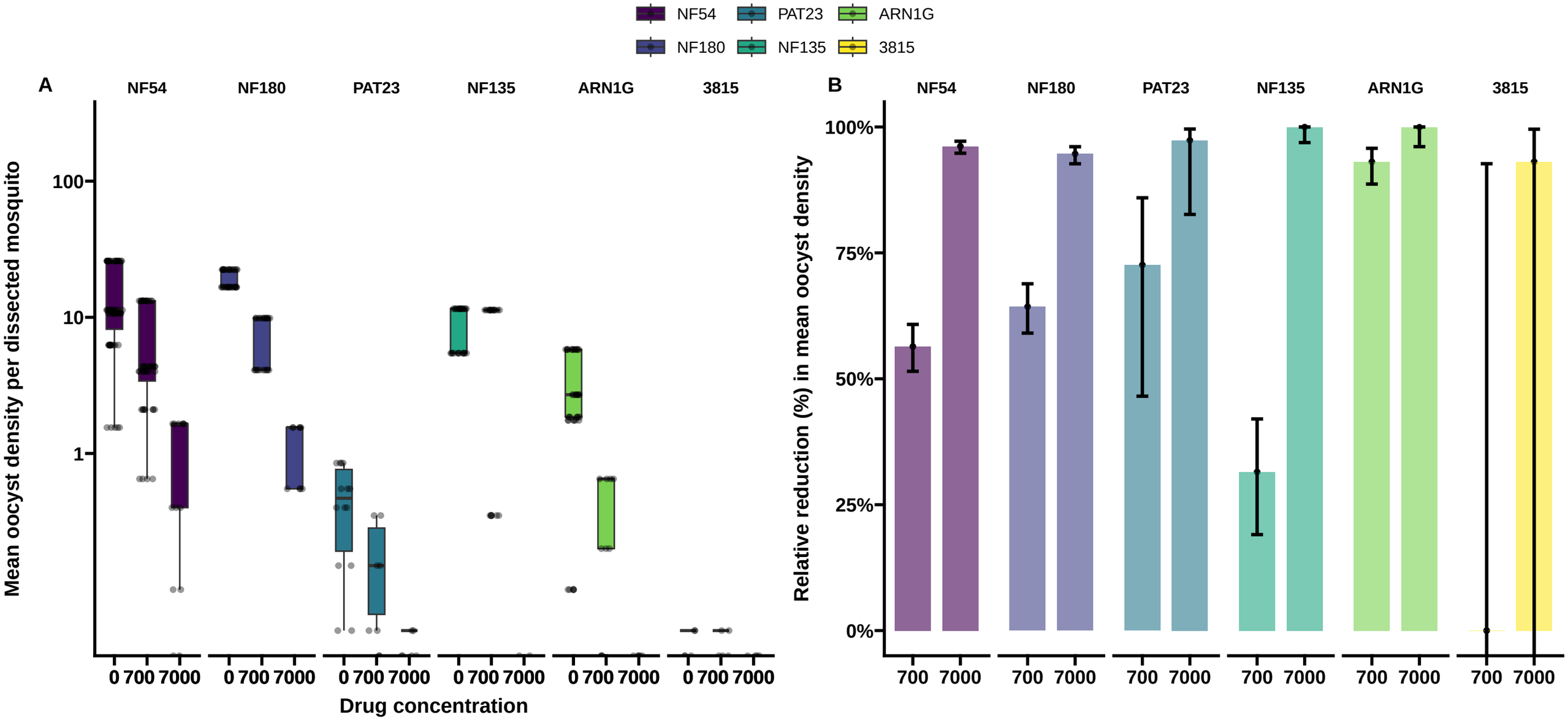
563 **Sup Figure 2.** Calculated sporozoite per oocyst**.** Sporozoites per salivary gland from individual
564 mosquitoes were dissected and processed for qPCR. The sporozoites per oocysts was calculated based
565 on the cage matched oocysts per mosquito average. The error bars represent the confidence intervals.

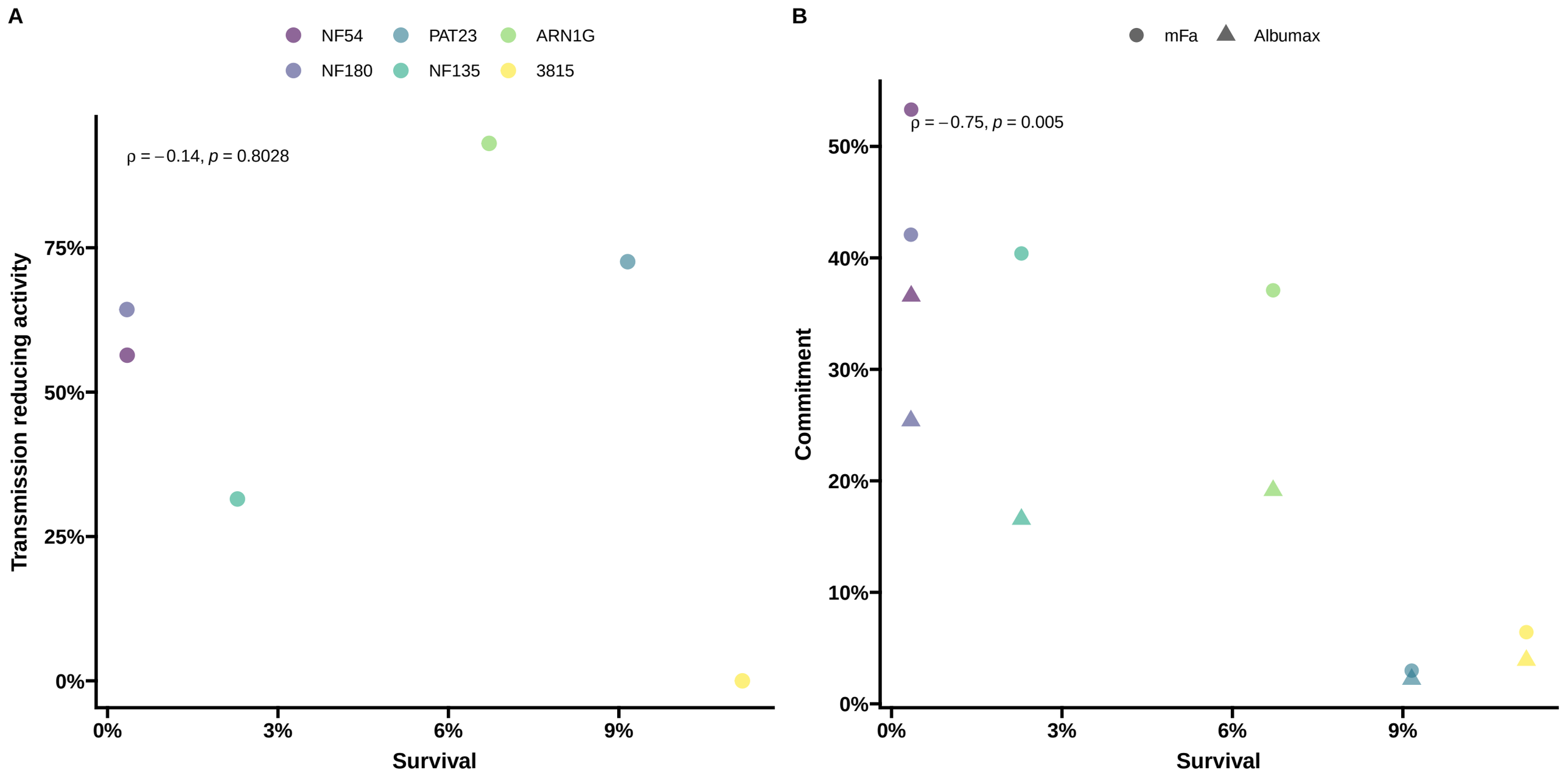
566 **Sup Figure 3.** Reduction in the overall oocyst prevalence of the six parasites lines following exposure
567 to DHA. **A)** Shows the decrease in mosquito infectivity upon exposing mature gametocytes to 700nM
568 and 7000nM DHA prior to being fed to mosquitoes in a blood meal. The dots represent the prevalence
569 from an individual mosquito cage. **B)** Represents the relative reduction in oocysts density compared
570 the no drug control. All error bars represent the confidence intervals.

571


A
NF54
NF180
PAT23
NF135
ARN1G
3815
Survival (%)
25%
20%
15%
10%
5%
0%
NF54
NF180
PAT23
NF135
ARN1G
3815
B
serum
mFa
Albumax
Commitment (%)
100%
75%
50%
25%
0%
NF54
NF180
PAT23
NF135
ARN1G
3815


An. coluzzii
An. gambiae
An. stephensi
A
An. coluzzii
An. gambiae
An. stephensi
Mean ookinetes
0
20
40
60
NF54
NF180
PAT23
NF135
ARN1G
3815
Immature form
Ookinete
B
Mean oocysts per midgut
0
5
20
50
100
200
300
C
Mean sporozoites
0
10000
50000
150000
300000


NF54
NF180
PAT23
NF135
ARN1G
3815
A
B
Mean oocyst density per dissected mosquito
100
10
1
0 700 7000
Drug concentration
Relative reduction (%) in mean oocyst density
100%
75%
50%
25%
0%
700 7000


A
NF54
PAT23
ARN1G
NF180
NF135
3815
ρ = −0.14, p = 0.8028
Transmission reducing activity
75%
50%
25%
0%
0%
3%
6%
9%
Survival
B
mFa
Albumax
ρ = −0.75, p = 0.005
Commitment
50%
40%
30%
20%
10%
0%
0%
3%
6%
9%
Survival