

1 **Artemisinin-resistant malaria parasites show enhanced** 2 **transmission to mosquitoes under drug pressure**

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21 **SHORT TITLE:** Artemisinin-resistance transmission

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23 **KEYWORDS:** Multidrug resistant malaria; Kelch13; transmission-blocking; artemisinin-combination
24 therapies (ACTs); gametocytes; *Plasmodium falciparum*; *Anopheles stephensi*.

25 **ABSTRACT**

26 **Resistance to artemisinin combination therapy (ACT) in the *Plasmodium falciparum***
27 **parasite is threatening to reverse recent gains in reducing global deaths from malaria.**
28 **Whilst resistance manifests as delayed asexual parasite clearance in patients following**
29 **ACT treatment, the phenotype can only spread geographically via the sexual cycle and**
30 **subsequent transmission through the mosquito. Artemisinin and its derivatives (such as**
31 **dihydroartemisinin, DHA) as well as killing the asexual parasite form are known to sterilize**
32 **male, sexual-stage gametes from activation. Whether resistant parasites overcome this**
33 **artemisinin-dependent sterilizing effect has not, however, been fully tested. Here, we**
34 **analysed five *P. falciparum* clinical isolates from the Greater Mekong Subregion, each of**
35 **which demonstrated delayed clinical clearance and carried known resistance-associated**
36 **polymorphisms in the *Kelch13* gene (*PfK13^{var}*). As well as demonstrating reduced**
37 **sensitivity to artemisinin-derivates in *in vitro* asexual growth assays, certain *PfK13^{var}***
38 **isolates also demonstrated a marked reduction in sensitivity to these drugs in an *in vitro***
39 **male gamete activation assay compared to a sensitive control. Importantly, the same**
40 **reduction in sensitivity to DHA was observed when the most resistant isolate was assayed**
41 **by standard membrane feeding assays using *Anopheles stephensi* mosquitoes. These**
42 **results indicate that ACT use can favour resistant over sensitive parasite transmission. A**
43 **selective advantage for resistant parasite transmission could also favour acquisition of**
44 **further polymorphisms, such as mosquito host-specificity or antimalarial partner–drug**
45 **resistance in mixed infections. Favoured transmission of resistance under ACT coverage**
46 **could have profound implications for the spread of multidrug resistant malaria beyond**
47 **Southeast Asia.**

48

49 **ONE SENTENCE SUMMARY:** Artemisinin-resistant clinical isolates can also demonstrate
50 resistance to the transmission-blocking effects of artemisinin-based drugs, favouring resistance
51 transmission to the mosquito.

52 INTRODUCTION

53 Malaria kills more than 400,000 people each year (1). Whilst there has been a marked reduction in
54 global rates of malaria disease since the new millennium, progress has stalled recently even
55 reversing in some regions (1). A critical factor threatening future gains is the emergence and
56 spread of drug resistance in the most virulent parasite *Plasmodium falciparum* (2). Of most
57 concern is the reported spread of resistance to frontline artemisinin-based drugs in the Greater
58 Mekong Subregion (GMS) of Southeast Asia (3, 4). Artemisinin has revolutionised treatment for
59 severe malaria. The drug acts rapidly to clear the clinical symptoms of malaria by killing the
60 asexual parasite in host red blood cells. Although a precise mechanism of action is contested, it is
61 thought that iron-mediated activation of artemisinin arising from parasite metabolism of
62 haemoglobin causes the drug to be both highly reactive and consumed rapidly in the process of its
63 action(5). Consequently, use of artemisinin or its derivatives requires coformulation with longer-
64 lasting partner drugs as artemisinin-based combination therapies (ACTs). In recent years,
65 however, resistance to both artemisinin and partner drugs, including piperazine and mefloquine,
66 has increased in prevalence throughout Southeast Asia (4, 6-8). The spread of such multidrug
67 resistant parasites beyond the GMS region could prove catastrophic for global malaria control.

68

69 Resistance to artemisinin is strongly associated with non-synonymous single nucleotide
70 polymorphisms (SNPs) in the propeller domain of *P. falciparum* Kelch 13 (PfK13) (9) a protein with
71 multiple likely functions in the parasite cell (5). Based on the SNP analysis, several PfK13 variants
72 (PfK13^{var}) have been defined displaying different degrees of delayed parasite clearance in patients
73 under ACT treatment. PfK13 variants include mutually exclusive SNPs giving rise to amino acid
74 changes C580Y, R539T, I543T and Y493H (4, 7, 10, 11). Whilst the precise mechanism by which
75 PfK13^{var} determines resistance remains ill-defined (5), PfK13^{var} parasites show an upregulation in
76 the unfolded protein cell stress response (12). Given the importance of this pathway to general cell
77 viability, PfK13^{var} parasites may be better able to deal with stresses arising from drug damage on
78 cell function (12). Persistence of parasites in the blood of infected individuals will lead to their
79 delayed clearance and ultimately treatment failure. Among PfK13 polymorphisms, the PfK13^{C580Y}
80 genotype is the most widely spread variant currently circulating in eastern Southeast Asia (7).

81

82 Drug resistance and its spread is traditionally seen through the prism of disease, in the case of
83 malaria the asexual replicative stages of the life cycle carried in blood circulation. However,
84 resistance can only spread with passage of the parasite through the mosquito, a fundamental step
85 in the *Plasmodium* lifecycle (13). Transmission of malaria parasites is solely mediated by non-
86 pathogenic sexual stages called gametocytes. These gametocytes mature over the course of 10-
87 12 days and are the only stages infectious to mosquitoes (13). During a mosquito blood feed, male
88 and female gametocytes are taken up and activate in the mosquito midgut into male and female
89 gametes. These activated gametes then fertilise and form a motile zygote (ookinete) that infects
90 the midgut epithelium forming an oocyst on the gut lining (14). The oocyst eventually bursts
91 releasing sporozoites that can be transmitted back into humans during a subsequent bite from an
92 infected mosquito.

93

94 Whilst the activity of artemisinin derivatives on asexual-stage parasites is well known, one
95 overlooked property of these drugs is their ability to target sexual stages, specifically their ability to
96 block the activation of male gametes (exflagellation), which underpins transmission (15, 16). This
97 raises the question as to whether artemisinin-resistant parasites are also resistant to this sterilizing
98 effect in the context of transmission to the mosquito. Here, we sought to test how clinical isolates
99 with demonstrated tolerance or treatment delay against artemisinin (i.e. asexual stage growth) fair
100 in their transmissibility through the mosquito under artemisinin coverage. We show that PfK13^{var}
101 isolates can exhibit transmission resistance, which manifests through an increased ability to
102 activate gametes and infect mosquitoes under artemisinin treatment compared with sensitive
103 controls. These findings have important implications for modelling the spread of resistance across
104 geographical regions. Artemisinin-resistant transmission emphasizes the need for future
105 combination therapies that include a transmission-blocking component if we are to stem the spread
106 of resistance beyond the Greater Mekong Subregion.

107

108 RESULTS

109 Selection and adaptation of Southeast Asian *P. falciparum* clinical isolates for *in vitro* study

110 *P. falciparum* clinical isolates that successfully adapted to long-term culture (Chotivanich,
111 unpublished data) were derived from a previous, multi-centre, open-label, randomised trial
112 collecting samples from patients with acute, uncomplicated malaria (10). Among isolates, five were
113 followed further based on their ability to form functional mature gametocytes *in vitro*. These were
114 compared to a standard laboratory control parasite NF54. Each was validated by PCR, confirming
115 the five clinical isolates as having variant polymorphisms in the gene, Pfk13^{var} (Table 1). Each *P.*
116 *falciparum* isolate was then tested *ex vivo* for sensitivity to the artemisinin derivative artesunate
117 using the 24-hour trophozoite maturation inhibition assay (TMI)(17). While Pfk13^{var} isolates
118 presented a wide range of IC₅₀ values, they all showed increased resistance to artesunate
119 compared to NF54 and an additional Pfk13^{WT} culture-adapted Thai laboratory strain, TM267
120 (Table 1).

121

122 In addition to Pfk13 genotype, the genetic background of each parasite isolate was investigated to
123 explore whether additional mutations might be present such as those associated with other drug
124 resistance phenotypes. Whole genome sequencing analysis was completed for each, confirming
125 different Pfk13 genotypes (Table 2). In addition, multiple previously reported mutations in genes
126 associated with various drug sensitivities were found among Pfk13^{var} isolates (as reviewed in(2))
127 (Table 2). Mutations were found in the chloroquine resistance transporter (PfCRT) agreeing with
128 reported mutations found in some parasites following ACT treatment(18). None of the isolates,
129 however, carried mutations in PfCRT loci recently associated with increased DHA-piperaquine
130 treatment failure (6, 7, 19).

131

132 Increased copy number for the multidrug resistance transporter Pfmdr1 and enzymes plasmepsin
133 II/plasmepsin III are known to be associated with enhanced survival of parasites exposed to
134 mefloquine or piperaquine respectively(20-23). To test if any of the field isolates harboured copy
135 number variations, we created sashimi plots of the next generation sequencing coverage (Figure
136 S1) and found that plasmepsin II and plasmepsin III are duplicated for isolate APL4G (Table 2).

137 APS3G and APL5G both had copy number variants of *mdr1* (**Table 2**). This suggests that APS3G
138 and APL5G will likely show resistance to mefloquine, whilst APL4G will likely show resistance to
139 piperazine (21, 24).

140

141 **Variation in PfK13 results in a growth defect in asexual blood stages but not in mosquito** 142 **stages**

143 Polymorphisms in the *PfKelch13* gene (*PfK13^{var}*) that are associated with artemisinin resistance
144 are known to also show reduced asexual blood stage growth(25, 26). To validate this in selected
145 isolates, parasites were set up in synchronised ring stage cultures, at a starting parasitaemia of
146 2%, and followed over the course of eight days. Parasitaemia was analysed every second day by
147 flow cytometry and cultures re-diluted to 2%. NF54 parasites showed a cumulative parasitaemia as
148 expected under standard laboratory conditions (**Figure 1A**). Parasites with a *PfK13^{var}* showed a
149 significantly reduced replication rate in *in vitro* compared to NF54 (**Figure 1A**) agreeing with
150 previous studies (25, 26). To explore the underlying mechanism of slowed growth, we measured
151 the number of merozoites per schizont, which will directly determine potential growth rates(27).
152 Late synchronised schizonts were blocked from merozoite egress using the protein-kinase G
153 (PKG) inhibitor, compound 2(28). Thin smears, 12 hours later, were then made of each culture and
154 stained with the nuclear stain 4',6-diamidino-2-phenylindole (DAPI) to count nuclei per schizont.
155 *PfK13^{var}* isolates displayed fewer nuclei per schizont than the *PfK13^{WT}* isolate and NF54 control,
156 suggesting that the observed reduced growth rate may at least be partly explained by a reduction
157 in the number of progeny (**Figure 1B**).

158

159 To investigate the transmission capability of each *P. falciparum* field isolate, we induced
160 gametocytes at a starting parasitaemia of 2% (29) and, 14 days post induction, fed cultures to
161 *Anopheles stephensi* mosquitoes by standard membrane feeding assay (SMFA)(30). No significant
162 differences were noted in the stage V (mature) gametocytaemia for isolates (in terms of relative
163 numbers of gametocytes to asexual parasites). However, upon activation, male exflagellation rates
164 were reduced in *PfK13^{var}* isolates compared with *PfK13^{WT}* (**Figure S2**). Ten days post-feeding,
165 mosquito midguts were dissected, and oocysts numbers recorded. All field isolates were found to

166 be capable of infecting mosquito midguts at varied intensity levels, i.e. oocyst counts per midgut,
167 as reported previously for Cambodian field isolates (31). To test if Pfk13^{var} led to a reduced
168 replication rate in mosquito stage growth (following the reduced merozoite count), we measured
169 the diameter of each oocyst in these infections as a proxy for replication. Oocyst size showed no
170 consistent pattern of variation when compared to controls other than a Pfk13^{R539T} variant which
171 displayed significantly larger oocysts than the Pfk13^{WT} control (**Figure S2**). This shows that whilst
172 variations in Pfk13 may reduce parasite multiplication rate in the asexual blood stage it does not
173 appear to directly influence transmission and growth in the mosquito-stage.

174

175 **Exflagellation sensitivity to different antimalarials among isolates with varied Pfk13** 176 **genotypes**

177 It has previously been shown that artemisinin and its derivatives have an inhibitory effect on male
178 gamete exflagellation, irreversibly sterilizing male gametocytes from activation (15, 16). To explore
179 whether Pfk13^{var} isolates were resistant to this sterilizing effect we tested mature gametocyte
180 culture capacity to activate in the dual gamete formation assay (PfdGFA) (15, 16). 24-hour
181 incubation of cultures with the artemisinin derivative dihydroartemisinin (DHA) was found to be
182 insufficient to elicit a complete inhibition of exflagellation for Pfk13^{WT} NF54 parasites (**Figure S3**),
183 likely as a result of the rapid instability of the drug (32). To improve activity and allow for a
184 comparative analysis between isolates, gametocytes were exposed to a second compound dose
185 24 hours after the first, resulting in a double-dose regimen with a readout after 48 hours. Double
186 exposure consistently gave complete inhibition of male activation with DHA at the highest
187 concentration tested (**Figure S3**). Female gamete activation was unaffected as found previously
188 (15). In parallel, two other artemisinin derivatives and four other antimalarial drugs were tested by
189 PfdGFA (**Figure S4** and **Figure S5**). Exflagellation rates for Pfk13^{var} isolates varied in the
190 presence of drug, with two Pfk13^{R539T} and Pfk13^{C580Y} isolates consistently showing tolerance to
191 artemisinin-derivatives (**Figure 2A**). The lack of a consistent pattern of reduced sensitivity to
192 artemisinin-based drugs across Pfk13^{var} isolates suggests that K13 polymorphisms alone likely do
193 not completely explain sensitivity of sexual stages to artemisinin treatment. This observation is
194 corroborated by similar findings with piperaquine resistance, which is mostly, but not always,

195 explained by copy number variants in plasmepsin II and plasmepsin III (21). However, the
196 presence of even a single isolate with both reduced sensitivity to artemisinin-based drugs in
197 asexual and sexual stages, suggests that there is the potential that a resistant strain at treatment
198 might be favoured for transmission to the mosquito.

199

200 **Transmission of field isolates with different PfK13 genotypes under DHA drug selection**

201 To explore the hypothesis that PfK13^{var}-associated resistance might allow resistant parasites to
202 more efficiently infect mosquitoes under drug coverage, we selected the PfK13^{C580Y} isolate APL5G.
203 This isolate showed a comparable level of mosquito infection to NF54 (**Figure S2**) and also
204 showed a high level of male gamete activation resistance to DHA (**Figure 2**). Gametocyte cultures
205 of both parasites were exposed to different concentrations of DHA for 48 hours using our double-
206 dosing regimen, before feeding to *An. stephensi* mosquitoes by SMFA. At day 10 post-feed,
207 mosquitos were dissected, and midguts examined for oocyst load (**Figure S6**). Generalised linear
208 mixed effects models were used to analyse infection intensity (number of oocysts per midgut) and
209 infection prevalence (proportion of midguts with oocysts) in response to treatment with DHA, in
210 order to incorporate data from 18 individual SMFA experiments within the same modelling
211 framework. A decrease in both intensity and prevalence of infected mosquitoes was observed for
212 both parasite isolates with increasing DHA concentration (**Figure 3A** and **Figure 3B**). A significant
213 decrease in both the oocyst intensity (ratio of oocyst intensity = 0.73, 95% CI: 0.66-0.80) and
214 prevalence (odds ratio = 0.46, 95% CI: 0.41-0.52) of mosquito infection was observed for NF54
215 with increasing drug concentration. In contrast, APL5G parasites (C580Y) showed no evidence for
216 a significant decrease in oocyst intensity with increasing DHA (ratio of oocyst intensity = 0.84, 95%
217 CI: 0.65-1.07). Increasing concentrations of DHA did still reduce the oocyst prevalence for APL5G
218 (odds ratio = 0.72, 95% CI: 0.56-0.96), however, this effect was significantly less than the effect
219 seen for WT parasites (**Table 3**).

220

221 To position these findings in the context of likely transmission events, we further explored the
222 impact of DHA on transmission at a single concentration (2 μ M) of DHA in comparison with DMSO.
223 2 μ M reflects the likely peak of DHA serum concentration when following the recommended WHO

224 dose for the ACT, DHA-piperaquine (33-35). In the absence of DHA, NF54 parasites were
225 consistently observed to have an increased infection prevalence (55.1%, 95% CI: 50.5% - 57.8%)
226 compared to APL5G parasites (19.2%, 95% CI: 13.3% - 26.0%) i.e. all things considered, NF54
227 transmits better in the absence of drug (**Figure 3C**). This suggests that a fitness cost is associated
228 with the APL5G genotype, which causes a sizeable reduction in the onward probability of infection
229 relative to WT parasites in the absence of DHA. However, this changes significantly in the
230 presence of drug. With 2 μ M DHA, no significant difference was observed between NF54 parasites
231 (20.6%, 95% CI: 27.7% - 14.3%) and APL5G parasites (12.1%, 95% CI: 4.1% - 25.4%) (**Figure**
232 **3C**) demonstrating a profound impact on NF54 but not APL5G parasites. The decreased impact of
233 DHA on the ability of the K13^{var} isolate to infect mosquitoes indicates that the resistance
234 polymorphism significantly increases the transmission potential of the parasite in the presence of
235 DHA. This observed transmission resistance phenotype may offset any fitness costs (such as
236 growth) observed in the absence of drug.

237 **DISCUSSION**

238 The threat of spreading artemisinin resistance to the treatment of malaria disease has focussed
239 global attention on the mechanisms underlying resistance in the parasite, *Plasmodium falciparum*.
240 However, only limited focus has been placed on how resistant parasites transmit through the
241 *Anopheles* mosquito vector. In this paper, we have shown clear evidence that certain clinical
242 isolates, with defined artemisinin resistance based on the known PfKelch13 marker (in terms of
243 clinical delayed clearance and reduced asexual growth sensitivity in the presence of artemisinin-
244 based drugs) are also able to transmit better to mosquitoes under drug coverage compared to
245 artemisinin-sensitive controls. The molecular basis of this transmission-resistance phenotype is
246 likely complex and is clearly not defined simplistically by PfKelch13 alone. Ongoing studies using
247 CRISP/Cas9 gene editing(26) may be able to address the polygenic nature of this phenotype.
248 However, the disconnect between PfK13 and transmission-resistance is clear from the observation
249 that of the five clinically resistant isolates tested, whilst each showed clear resistance to
250 artemisinin-based drugs in asexual growth, there was varied sensitivity in transmission stages.
251 However, the transmission-resistance phenotype is nonetheless robust for certain isolates.
252
253 As with previous studies, we first started with investigation of the asexual growth rate, confirming a
254 consistent reduced rate in resistant isolates. The asexual growth rate reduction seen in PfK13^{var}
255 isolates likely acts as both a selective cost for parasite growth (in being out competed in normal
256 infections) but also likely explains how these parasites persist during drug treatment, i.e. explaining
257 delayed clearance(5). Switching our focus to sexual commitment and development, we next
258 explored gametocyte production. With the caveat that different parasite isolates always show
259 marked differences in gametocyte formation capacity, we did not observe any obvious reduced
260 capacity among PfK13^{var} isolates in gametocyte production. All five field isolates produced
261 equivalent numbers of mature gametocytes (stage V gametocytes) after day 14 upon induction.
262 Indeed, the reverse correlation between drug resistance and sexual commitment has been
263 consistently reported. Clinical isolates with demonstrated drug resistance and delayed clearance
264 have been consistently reported to produce a higher gametocytaemia, suggesting a potentially
265 elevated potential for transmission(10).

266 Having confirmed capacity to form gametocytes, we assessed the capacity to generate
267 exflagellation centres, a marker of male gamete activation capacity in the mosquito. We found no
268 direct correlation between gametocytaemia and exflagellation count. This lack of correlation may
269 be due to differences in the sex ratio between parasite isolates (reduced males mean less
270 exflagellation centres though gametocytaemia may be the same). Whilst commitment of
271 gametocytes to either male or female is poorly understood, it is entirely conceivable that
272 gametocytes mature or differentiate into either male or female at differing rates in each isolate.
273 Unfortunately, sex ratios were untested here due to a paucity of markers for male and female
274 gametocytes and challenges with definitive differentiation of the sexes using Giemsa stain.
275 Irrespective, gametocyte conversion rates have been shown to be sensitive to asexual stage
276 replication, which itself is affected by drugs. This suggests that there is the potential for a trade-off
277 between asexual stages and sexual stages in ensuring the spread of the artemisinin resistant
278 parasites(36).

279

280 With sexual commitment and exflagellation *in vitro* seemingly uncompromised in resistant isolates
281 we next sought to explore transmissibility directly. We saw no obvious defect in either the
282 transmission capacity (number of oocysts) or the transmission replication rate (as measured by
283 oocyst size) among the five parasite field isolates and NF54. This latter point is noteworthy since it
284 is clear that artemisinin-resistant parasite isolates show a lower asexual growth rate and merozoite
285 (progeny) rate (**Figure 1**), however, number of oocysts and sporogony in the mosquito doesn't
286 appear to be affected. Thus, Pfk13^{var} parasites appear able to commit to sexual reproduction,
287 activate and transmit to mosquitoes at levels that don't differ dramatically to those commonly seen
288 in sensitive parasites (i.e. beyond variability usually seen between isolates).

289

290 Shifting our attention to transmission under drug coverage, tests of the viability of gametocytes for
291 gamete activation using the dual gamete formation assay (PfdGFA) with artemisinin derivatives;
292 DHA, Artemether and Artemisone clearly found that certain Pfk13^{C580Y} and Pfk13^{R539T} parasites
293 demonstrated significantly higher resistance compared to sensitive controls. Of note, whilst
294 undertaking this work, a parallel study made similar observations. Testing male exflagellation

295 sensitivity to DHA in unrelated culture-adapted PfK13^{var} Cambodian field isolates, Lozano et al
296 found that PfK13^{var} isolates showed a reduced sensitivity of exflagellation rates to DHA treatment,
297 though onward mosquito infectivity was not tested(37). Extending this observation to transmission
298 directly we took the most competent transmissible field isolate representing the resistant
299 phenotypes (APL5G, C580Y) compared to the laboratory reference strain NF54, and tested
300 whether transmission resistance plays out in terms of capacity to infect mosquitoes over a range of
301 drug concentrations. Controlling for gametocytaemia, number of cells and haematocrit level for
302 each, infection prevalence in mosquitoes could then be tested and compared between lines. Of
303 note, the infection intensity (number of oocysts found in each mosquito) was consistently different
304 between NF54 and APL5G, as it is for each different culture-adapted parasite strain (see (31)).
305 These differences make direct measures of mixed infections challenging. Nonetheless, we found
306 that artemisinin resistant, PfK13^{C580Y} (APL5G) was consistently more likely to transmit malaria
307 under drug pressure compared to its DMSO treated controls than the control NF54 parasite
308 (**Figure 3C**). This was due to the greater impact of DHA on oocyst infection exhibited by the wild-
309 type isolate, which served to offset the decreased transmission potential for APL5G in the absence
310 of artemisinin. This demonstrates that the artemisinin resistant phenotype of APL5G impacts both
311 on asexual blood stages and during transmission to the mosquito in the presence of artemisinin
312 drug.

313

314 Although the numbers are small here, the implications are that in the context of a mixed infection, a
315 resistant parasite may be more likely to survive ACT treatment and its gametocytes may be more
316 likely to transmit to the mosquito. Thus, ACT coverage in the field may be favouring, even driving,
317 artemisinin-resistant parasite persistence and transmission. This could explain an important part of
318 the selection of PfKelch mutants observed in the field. For instance, the F446I PfKelch mutation
319 results in only a slight prolongation in parasite clearance half-life and is not associated with ACT
320 treatment failure (10). Yet, there is clear selection of this genotype in Myanmar, which could be
321 explained by preferential transmission under artemisinin drug pressure. The effect on outcrossing
322 is also worth considering. Because the sterilizing effects of artemisinin-based drugs appears to be
323 biased towards the male gametocyte (15, 16), there is the very real potential that ACT usage in the

324 context of a mixed infection might favour acquisition of other selectively advantageous mutations
325 during transmission. Since the female gametocytes remain unaffected, successful transmission
326 under ACT coverage would likely favour either resistant parasite selfing or mating between
327 resistant males and sensitive females. It is clear from our own usage of a central Asian mosquito
328 vector (*An. stephensi*) and the work of others using the major African vector, *An. coluzzii* (31) that
329 artemisinin-resistant parasites can infect non-native mosquitoes. Thus, in a mixed infection where
330 local parasites show a degree of geographical vector adaptation (38) an invasive resistant
331 parasite, otherwise at a disadvantage (reduced vector adaptation and slower asexual growth), may
332 acquire a key advantage under ACT coverage in terms of its ability to both transmit and acquire
333 necessary adaptive mutations via recombination with sensitive females. Importantly, this may play
334 out even without a decline in cure rates if transmissibility of the treated infection is increased, such
335 as in high intensity transmission areas at the early stages of resistance invasion before partner
336 drug resistance has emerged. Mixed infection studies *in vivo* and modelling of drug coverage
337 effects with different rates of transmission intensity are clearly needed to explore the implications
338 of transmission resistance in various invasive settings.

339

340 Ultimately, these data stress the importance of considering transmission in the context of drug
341 resistance spread and argue strongly for the inclusion of a parasite transmission-blocking
342 component in future antimalarial combination therapies or control strategies.

343

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- 450

451 **ACKNOWLEDGEMENTS**

452 **Funding.** This work was supported by a joint Medical Research Council (MRC) UK Newton and
453 National Science and Technology Development Agency (NSTDA), Thailand award (MR/N012275/1
454 to JB, SP, NJW and KC). Further support came from the Medicines for Malaria Venture (MMV)
455 (MMV08/2800 to JB). JB is supported by an Investigator Award from Wellcome (100993/Z/13/Z).
456 NJW is supported by Wellcome with a Principal Research Fellowship (107886/Z/15/Z). The
457 Mahidol University Oxford Tropical Medicine Research Programme is funded by Wellcome (AMD
458 106698/Z/14/A). The Wellcome Sanger Institute is funded by Wellcome (206194/Z/17/Z), which
459 supports MKNL. OJW would like to acknowledge funding from a Wellcome Trust PhD Studentship
460 (109312/Z/15/Z). We thank Olivo Miotto (MORU) for sharing genome sequences of the parasite
461 isolates and for helping in the analysis of SNP calling. We also thank the gametocyte team at
462 Imperial College London for ongoing provision of gametocytes, in particular Alisje Churchyard,
463 Irene García Barbazán, Josh Blight and Eliana Real and staff of Sequencing facility at the
464 Wellcome Sanger Institute for their contribution. We also thank Mark Tunnicliff for ongoing
465 provision of *An. stephensi* mosquitoes.

466 **Author contributions.** MJD, AR, KC and JB conceptualised the study; KW, FAD, MJD and AR
467 designed experiments; experiments were undertaken by KW, FAD, MJD, SY, US, SC, and BS;
468 RDP, VMH, MKNL, KW and AR generated and curated the genome data; modelling components
469 were designed and executed by OJW and LO. SP, NJW, AD, KC supervised collection of clinical
470 isolates used in the study. KW, FD and JB wrote the manuscript. All authors contributed to overall
471 editing and manuscript approval.

472 **Competing interests.** The authors declare no conflict of interest.

473 **Data availability.** Raw experimental data are available on request, genome data is publicly
474 available at the European Nucleotide Archive (<https://www.ebi.ac.uk/ena>).

475

476 **SUPPLEMENTARY MATERIALS:**

477 Materials and Methods

478 Figure S1-S6

479 Supplementary Data Tables (Separate Excel Spreadsheets)

480 **TABLES AND FIGURES**

481 **Tables**

Clinical isolate code	Year	Origin	Parasite clearance half-life (hours)	Mean (SD) artesunate IC₅₀ (ng/ml)	PfK13 genotype (PCR)
TM267	1995	Thailand	ND	0.7 (0.5–0.9)	WT (17)
ARN1G	May 2011- April 2013	Ranong, Thailand	7.1	6.8 (0.1)	G449A
APS2G	May 2011- April 2013	Srisaket, Thailand	6.1	9.3 (1.6)	R539T
APS3G	May 2011- April 2013	Srisaket, Thailand	6.2	12.9 (0.4)	R539T
APL4G	May 2011- April 2013	Pailin, West Cambodia	5.1	6.6 (1.0)	C580Y
APL5G	May 2011- April 2013	Pailin, West Cambodia	7.7	5.2 (2.4)	C580Y

482

483 **Table 1. Characteristics of the *P. falciparum* field isolates presented in this study.** Clinical

484 isolate codes are shown. IC₅₀ values of field isolates assayed with three independent biological

485 replicates using the trophozoite maturation inhibition assay (TMI) (17). Standard deviations are

486 indicated in brackets (SD). *P. falciparum* TM267 isolate originate from a previous study (17) with

487 others from (10).

488

gene ID	product	amino acid change	Isolate				
			ARN1G	APS2G	APS3G	APL5G	APL4G
PF3D7_134 3700	kelch protein K13	G449A	x	-	-	-	-
		R539T	-	x	x	-	-
		C580Y	-	-	-	x	X
PF3D7_011 2200	multidrug resistance-associated protein 1	H191Y	x	x	x	x	x
		K202E	-	-	-	x	-
		N325S	-	x	x	-	-
		S437A	x	x	x	x	x
		I876V	x	-	-	x	x
		F1390I	x	-	-	x	x
		<i>amplification</i>	-	-	x	x	-
PF3D7_041 7200	bifunctional dihydrofolate reductase-thymidylate synthase	N51I	x	x	x	x	x
		C59R	x	x	x	x	x
		S108N	x	x	x	x	x
		I164L	-	-	-	-	x

PF3D7_052 3000	multidrug resistance protein 1	Y184F	-	X	X	X	X
		K76T	X	X	X	X	X
PF3D7_070 9000	chloroquine resistance transporter	T93S	-	-	-	-	-
		H97Y	-	-	-	-	-
		F145I	-	-	-	-	-
		Q271E	X	X	X	X	X
		I218F	-	-	-	-	-
		M343I	-	-	-	-	-
		G353V	-	-	-	-	-
		I356T	X	X	X	X	X
		R371I	X	X	X	X	X
		PF3D7_081 0800	Hydroxy- methyldihydropterin pyrophosphokinase- dihydropteroate synthase	S436A	-	X	X
K540E	X			X	X	-	-
K540N	-			-	-	X	X
A581G	X			-	-	X	X
PF3D7_130 3500	sodium/hydrogen exchanger	N894K	X	-	-	-	-
		V950G	X	X	X	X	X
		H1375Y	X	-	X	-	-
		H1379Y	-	X	-	X	-

		F1557S	x	x	x	x	x
PF3D7_140 800 PF3D7_140 8100	plasmepsin II plasmepsin III	<i>amplification</i>	-	-	-	-	x

489

490 **Table 2. Summary molecular markers associated with antimalarial drug resistance for**

491 **isolates used in this study.** Presence (x) or absence (-) of the polymorphism is indicated for each

492 protein. All amino acid changes in italics have recently been associated with ACT treatment failure

493 within the eastern Greater Mekong subregion.

494

495

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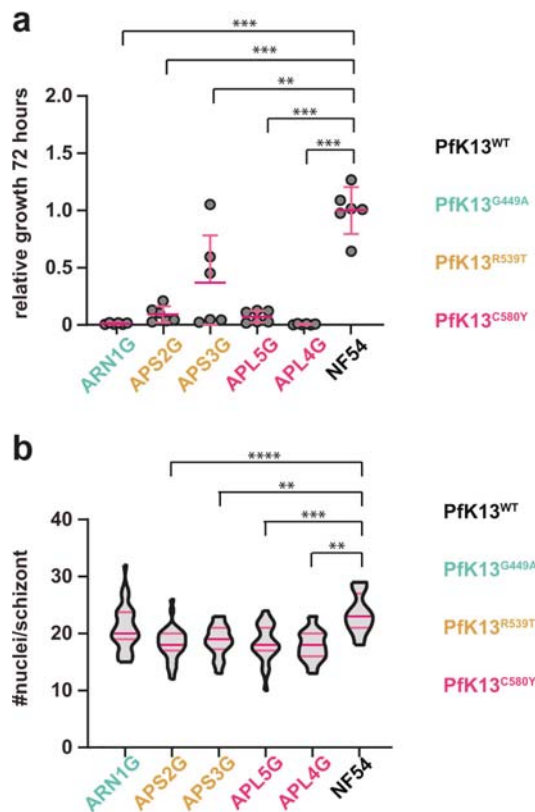
	Prevalence of Infection			Oocyst Intensity*		
	<i>Odds Ratio</i>	<i>95% CI</i>	<i>p</i>	<i>Ratio of oocyst intensity</i>	<i>95% CI</i>	<i>p</i>
K13 ^{var} in the absence of DHA	0.20	0.16 – 0.25	<0.001	0.17	0.13 – 0.23	<0.001
DHA (μM): WT line	0.46	0.41 – 0.52	<0.001	0.73	0.66 – 0.80	<0.001
DHA (μM): K13 ^{var}	0.72	0.56 – 0.96	<0.001	0.84	0.65 – 1.07	0.069

* Negative binomial overdispersion parameter: 0.837

497

498 **Table 3. Effects of DHA treatment on oocysts prevalence and intensity.** Analysis was done
 499 using generalised linear mixed effects models to incorporate 18 SMFA experiments into a single
 500 analysis.

501 **Figures**



502

503 **Figure 1. Characterisation of *P. falciparum* clinical isolates. A.** Relative cumulative growth of

504 *P. falciparum* clinical isolates compared to NF54. Parasitaemia was measured by flow cytometry

505 every other day for eight consecutive days (four replication cycles). Six biological replicates from

506 two parallel experiments are shown. Clinical isolates with K13^{var} grow significantly slower than

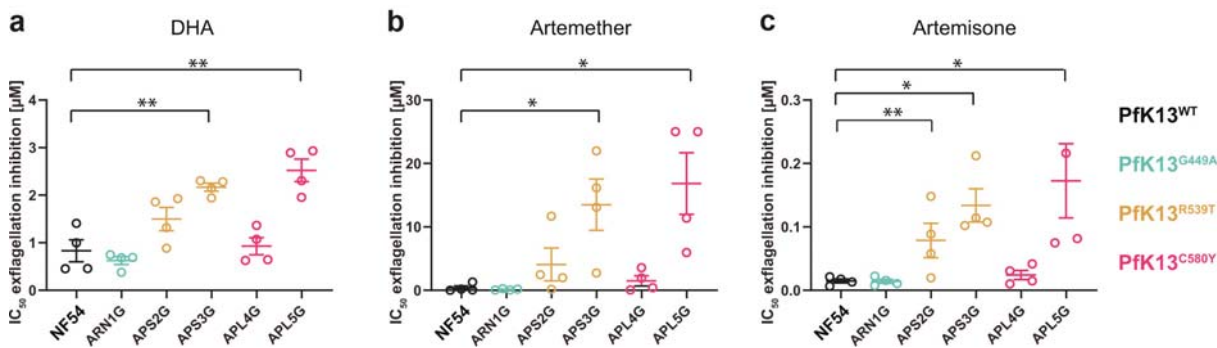
507 NF54 (K13^{WT}, unpaired t-test, ** p<0.01; ***p<0.0001). **B.** K13^{var} parasites have less nuclei per

508 schizont than K13^{WT} parasites with the exception of isolate ARN1G (unpaired t-test ** p<0.01;

509 ***p<0.0001).

510

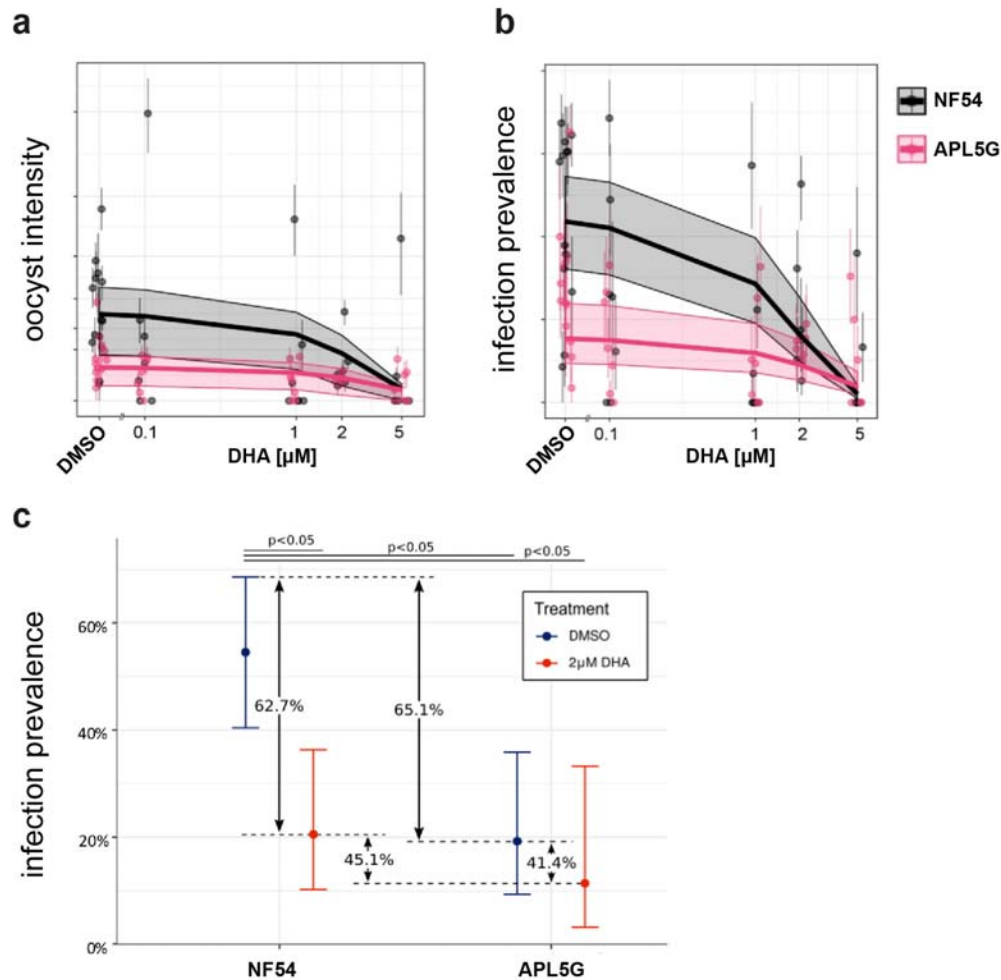
511



512

513 **Figure 2. Exflagellation inhibition reported as IC₅₀ values of clinical isolates.** Two parasite
514 isolates (APS3G and APL5G) show consistent resistance to sterilizing effects of three different
515 artemisinin-related drugs, dihydroartemisinin (DHA) (A), Artemether (B) and Artemisone (C) on
516 exflagellation compared to the NF54 PfK13^{WT} control. One additional isolate (APLS2G) shows
517 increased resistance to Artemisone. IC₅₀ values were compared to NF54 (unpaired t-test * p<0.05;
518 ** p<0.01)

519



520

521 **Figure 3. The impact of DHA on the Pfk13^{WT} and Pfk13^{C580Y} transmission potential.** Graphs
522 show the overall results of oocysts count from 18 individual SMFA experiments oocysts infection
523 intensity (A) and infection prevalence (B) of the sensitive isolate Pfk13^{WT} (NF54) versus the
524 artemisinin resistant Pfk13^{C580Y} isolate (APL5G) after incubation with either DMSO (no DHA) and
525 DHA at specified concentrations. Points and whiskers on each plot show mean and bootstrapped
526 95% CI for each replicate, with the predicted relationship and 95% CI shown with the trend line and
527 shaded region. In the absence of DHA (DMSO), APL5G is predicted to produce significantly fewer
528 oocysts and infections, whereas in the presence of DHA concentrations greater than 2 µM DHA,
529 the transmission potential of Pfk13^{C580Y} is comparable to NF54/Pfk13^{WT}. **C.** Fitness costs
530 associated with DHA resistance. The relative reduction in infection prevalence due to DHA
531 treatment in NF54 is greater (62.7%) than APL5G (41.4%), which suggests that APL5G is
532 significantly more likely to infect mosquitoes under drug treatment (p<0.05) compared to the
533 absence of drug.

534

Supplementary Materials for

535

536 **Artemisinin-resistant malaria parasites show enhanced transmission to** 537 **mosquitoes under drug pressure**

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540 Richard D. Pearson⁵, Nicholas J. White^{4,6}, Arjen Dondorp^{6,7}, Lucy C. Okell³, Andrea Ruecker^{4,6},
541 Kesinee Chotivanich^{6,7} and Jake Baum¹

542

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544

545 **This PDF file includes:**

546 Materials and Methods

547 Supplementary Figures Fig. S1-S6

548 List of Supplementary Data Sheets (Excel Spreadsheets – separate files)

549

550

551 **MATERIAL AND METHODS**

552

553 ***P. falciparum* asexual blood stage and gametocyte maintenance**

554 Asexual blood stage and gametocytes were cultured as previously described (29) with the
555 following modifications: Asexual blood stage cultures were maintained in asexual culture medium
556 (RPMI 1640 with 25 mM HEPES (Life Technologies), 50 µg L⁻¹ hypoxanthine (Sigma), 5% A+
557 human serum (Interstate Blood-Bank) and 5% AlbuMAX II (Life Technologies)). Gametocyte
558 cultures were maintained in gametocyte culture medium (RPMI 1640 with 25 mM HEPES (Life
559 Technologies), 50 µg L⁻¹ hypoxanthine (Sigma), 2 g L⁻¹ sodium bicarbonate (Sigma), 5% A+ human
560 serum (Interstate Blood-Bank) and 5% AlbuMAX II (Life Technologies)).

561

562 **Mosquito rearing**

563 *Anopheles stephensi* mosquitoes were reared under standard conditions (26°C-28°C, 65%-80%
564 relative humidity, 12 hour:12 hour light/darkness photoperiod). Adults were maintained on 10%
565 fructose.

566

567 **Whole genome sequencing**

568 Genomic DNA isolate and whole genome sequencing, calling of single nucleotide variants and
569 gene amplification copy number variants were undertaken essentially as recently described(39).

570

571 **Flow cytometry**

572 For the growth assay, asexual parasites were sorbitol-synchronised at least twice 16 hours apart to
573 create an 8-hour growth window. Starting parasitaemia was seeded at 1-2% early ring stages in
574 triplicates that were treated separately. The assay was performed twice using 3 replicates each.
575 Every other day, parasites were fixed in 4% formaldehyde and 0.2% Glutaraldehyde for at least 10
576 minutes. After washing with PBS, and DNA was stained with SybrGreen1 (diluted 1:10'000) in the
577 dark for 20 minutes at room temperature. After incubation, cells were washed three times with PBS
578 and resuspended in 80ul PBS. Flow cytometry was performed counting a total of 100'000 cells per
579 condition.

580

581 **Nuclei count**

582 Parasites were synchronised twice using 5% Sorbitol to obtain a 10-hour life cycle window. 10 μ M
583 compound 2 was added to late trophozoite stages for a maximum of 12 hours to block egress of
584 the red blood cells (RBC) (28). Resulting segmented schizonts were thinly smeared, fixed with 4%
585 Formaldehyde and 0.2% Glutaraldehyde for 20 minutes. Smears were then stained with 1 μ g/ml
586 DAPI for 5 minutes and mounted in Vectashield (Vector Laboratories). Z-stacks were taken using a
587 Leica microscope at 100x magnification. Nuclei of arrested segmented schizonts were counted
588 using the plugin tool “Manual counting” on ICY (40). Only singly-invaded RBC were counted.

589

590 **Trophozoite maturation assay (TMI)**

591 The trophozoite maturation assay was performed according to (17). Briefly, *P. falciparum* infected
592 blood was collected into heparin tubes and centrifuged at 800 g at 4°C for 5 minutes to allow the
593 removal of the plasma and buffy coat. This was followed by three washes in RPMI 1640 (without
594 serum supplement) and adjusted to 3% cell suspension in 10% A+ human serum supplemented
595 RPMI 1640. 96-well microtiter plates (Nunc™ MicroWell™ 96-Well Microplate; Thermofisher
596 Scientific) were pre-dosed with Artesunate dissolved in 5% NaHCO₃ (Guilin Pharmaceutical Co.,
597 Ltd., China), ranging from 0.01 to 400 ng/ml final concentration or no drug as negative control. A
598 75 μ l *P. falciparum* ring stage infected RBC cell suspension was added to the test plate and
599 incubated for 24 hour at 37°C in 5% CO₂. All samples were tested in triplicate. Upon completion of
600 drug exposure thick and thin blood smears were prepared of all wells and the number of 24 to 30
601 hour trophozoites (41) was counted per 100 infected red blood cells. To identify the inhibition
602 activity of artesunate the percentage of trophozoite maturation compared to the negative control
603 was assessed. The IC₅₀ (50% inhibitory concentration) was calculated as the drug concentration
604 causing 50% inhibition of *P. falciparum* maturation from ring stage to trophozoite stage and
605 normalised to the negative control wells. All IC₅₀s were determined by sigmoid curve fitting using
606 WinNonlin computer software (version 3.1; Pharsight Corporation, USA). As technical control all ex
607 vivo assays were performed in parallel to the standard laboratory Thai strain, TM267.

608

609 **Dual gamete formation assay (DGFA) double-dose format**

610 The double dose DGFA was adapted from previously described methods (16) to incorporate an
611 additional drug dosage, accounting for the low compound half-lives of artemisinin and its
612 derivatives. Briefly, compounds were prepared in 10 mM DMSO stocks, and dispensed in serial
613 dilutions into multiwell plates using a HP D300 Digital Dispenser. Samples were normalised to
614 0.25% DMSO and contained 0.25% DMSO and 12.5 µM Gentian Violet as negative and positives
615 controls, respectively. Half the maximal DMSO content was plated per plate, accounting for the
616 accumulation of DMSO over two dosages. Mature gametocytes with an exflagellation rate of
617 >0.2% of total cells were diluted in gametocyte culture medium to 25 million RBCs per mL. Mature
618 gametocyte culture was plated in drugged 96-well plates and incubated in a humidified chamber
619 under 92% N₂/5% CO₂/3% O₂ (BOC special gases) at 37°C for 24 h. For the second drug dosage
620 at 24 h, the drugged culture was transferred to a second drugged well plate and incubated for a
621 further 24 h under 92% N₂/5% CO₂/3% O₂ at 37°C in a humidified chamber.

622 At 48 h, gametogenesis was induced with ookinete medium (RPMI 1640 with 25 mM HEPES (Life
623 Technologies), 50 µg L⁻¹ hypoxanthine (Sigma), 2 g L⁻¹ sodium bicarbonate (Sigma) and 100 µM
624 xanthurenic acid) prepared with 0.5 µg ml⁻¹ anti-Pfs25 clone 4B7 (BEI Resources) conjugated to
625 Cy3 (GE Healthcare). Plates were immediately incubated at 4 °C for 4 min and then 28 °C for 5
626 min before transferring to a Nikon Ti-E widefield microscope. Exflagellation events were recorded
627 by automated phase contrast microscopy, in either 384-well or 96-well plates. For 384-well plate
628 assays, 10-frame time lapses (4 frames sec⁻¹) were recorded at x4 objective and 1.5x zoom, and
629 for 96-well plate assays, 20-frame time lapses were recorded at x10 magnification and 1.5x zoom.

630 Plates were then protected from light and incubated at 28 °C for 24 h before the automated
631 capture of female gamete formation at the same magnification, utilising single frame fluorescence
632 microscopy. Exflagellation events and female gamete counts per well were derived using an
633 automated ICY Bioimage Analysis algorithm. Resulting counts were converted to percentage
634 inhibition values, calculated relative to positive (C1) and negative (C2) controls:

$$\% \text{ Inhibition} = 100 - \left(\left(\frac{\text{test compound} - C1}{C2 - C1} \right) \times 100 \right)$$

635 Raw data demonstrated a Z' factor ≥ 0.4 and was derived from $n \geq 2$ and $n \geq 3$ technical and
636 biological replicates, respectively. GraphPad Prism (version 8) was used to calculate IC₅₀s from the
637 dose response data using with the log(inhibitor) vs. response – variable slope (four parameters)
638 function. IC₅₀s were derived from curves demonstrating $R^2 \geq 0.95$.

639

640 **Standard membrane feeding assay**

641 Gametocytes were induced and maintained as mentioned above. At day 14 post induction,
642 gametocytes were spun down at 38°C and resuspended in 5 ml of suspended animation buffer
643 (SA) (42). To ensure that a consistent number of RBCs are used for drug incubation, gametocytes
644 were MACS-purified and resuspended in gametocyte medium with 25E6 fresh RBCs. DHA was
645 added to desired end concentration into a 10 ml gametocyte culture and added again 24 hours
646 later (double-dosing within 48 hours). After 48 hours, the parasite culture was mixed with fresh
647 blood and human serum and fed to adult *An. stephensi* mosquitoes using a 3D printed feeder(30).

648

649 **Oocyst counts and size**

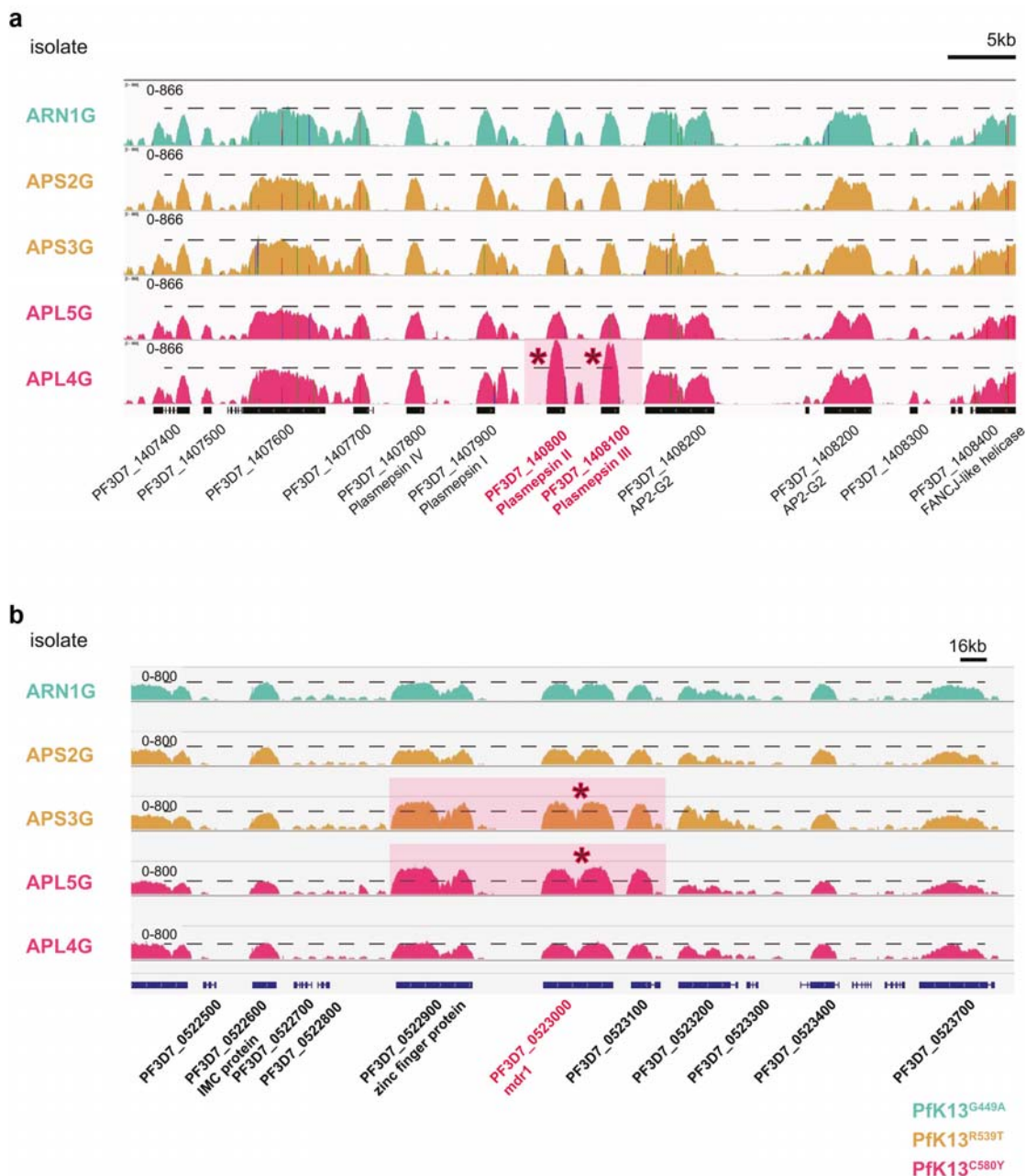
650 At day 10 post feeding, mosquitoes were dissected, and midguts were stained in 0.1%
651 Mercurochrome and inspected using light microscopy with 10x magnification to count oocysts.
652 To measure oocysts size, midguts of *An. stephensi* fed on *P. falciparum*-infected blood were
653 dissected and fixed with 4% formaldehyde, permeabilised with 0.1% Triton X-100 for one hour,
654 blocked with 3% BSA for 30 minutes and stained with 1 µg/ml in DAPI for 3 minutes. Midguts were
655 washed with 1xPBS and mounted in Vectashield. Images were acquired on a Nikon Ti-Eclipse
656 inverted fluorescence microscope. Images of *P. falciparum*-infected midguts were captured using
657 the DAPI channel and Z-stack imaging to obtain greater depth of oocysts. These stacked images
658 were then processed in ND Processing using the Maximum Intensity Projection option which then
659 create an image with brighter intensity of the oocysts in every midgut. Oocysts detection was
660 automated by using the Automated Spot Detection programme based on the intensity of the
661 oocysts compared to midgut cells (NIS-Elements). The size, diameter and intensity of each
662 selected oocysts were generated in an excel file for analysis.

663

664 **Statistical Modelling of Oocyst infection Intensity and Prevalence**

665 To assess the impact of artemisinin on the ability of each parasite line to form oocysts, we used
666 generalised linear mixed effects models in order to incorporate data from different experimental
667 replicates within the same modelling framework. These models have previously been used to
668 model transmission blocking interventions (43). We modelled either oocyst intensity or prevalence
669 as the response with treatment (DHA concentration) included as a fixed effect and 0 μM DHA
670 represented by control groups treated with DMSO. The parasite line treated (PfK13^{WT} or
671 PfK13^{C580Y}) was included as a fixed effect to assess the differential impact of artemisinin on
672 transmission success. The impact of treatment between experimental replicates was allowed to
673 vary at random between replicates. A logistic regression (binomial error structure) was used to
674 model the prevalence of mosquito infection, i.e. the presence or absence of oocysts, and a zero-
675 inflated negative binomial distribution was used to model the intensity of infections, i.e. the
676 numbers of mosquito oocysts (44). 95% confidence interval estimates were generated for the
677 impact of drug concentration by bootstrapping methodology (with 100,000 replicates).

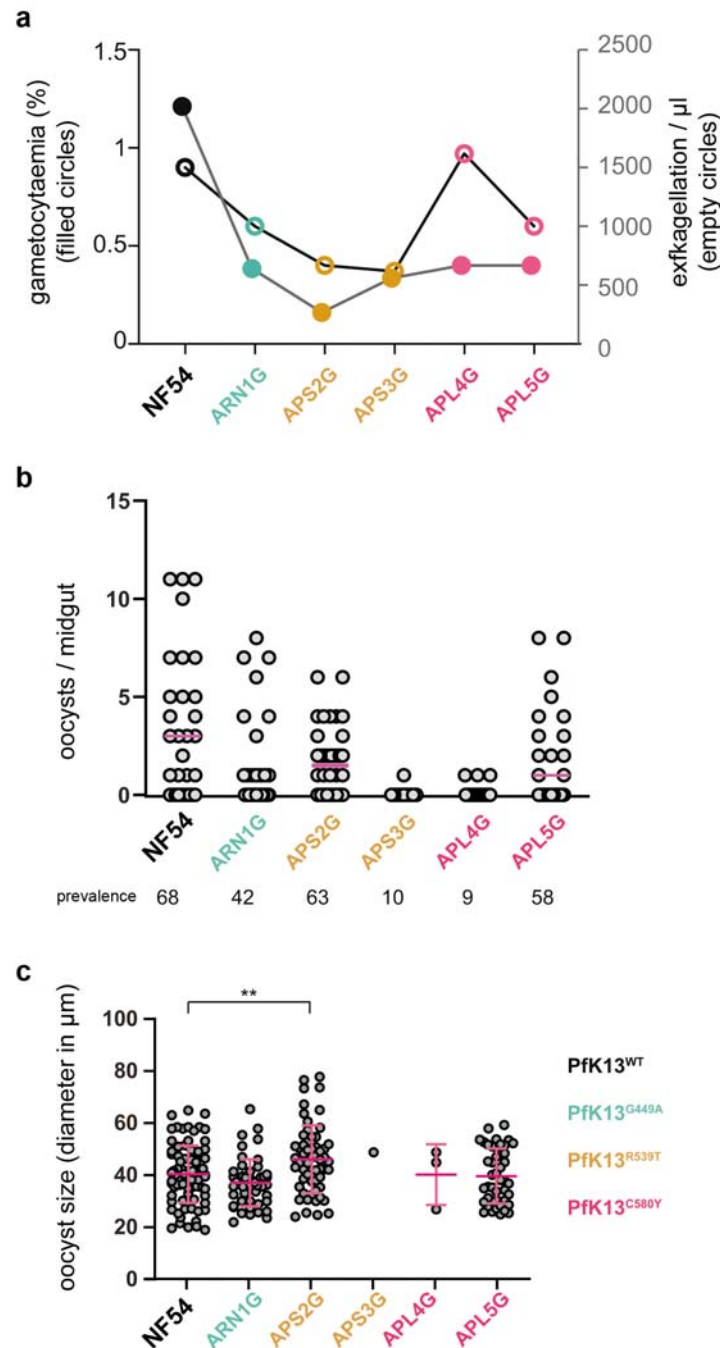
678 **Supplementary Figures**



679

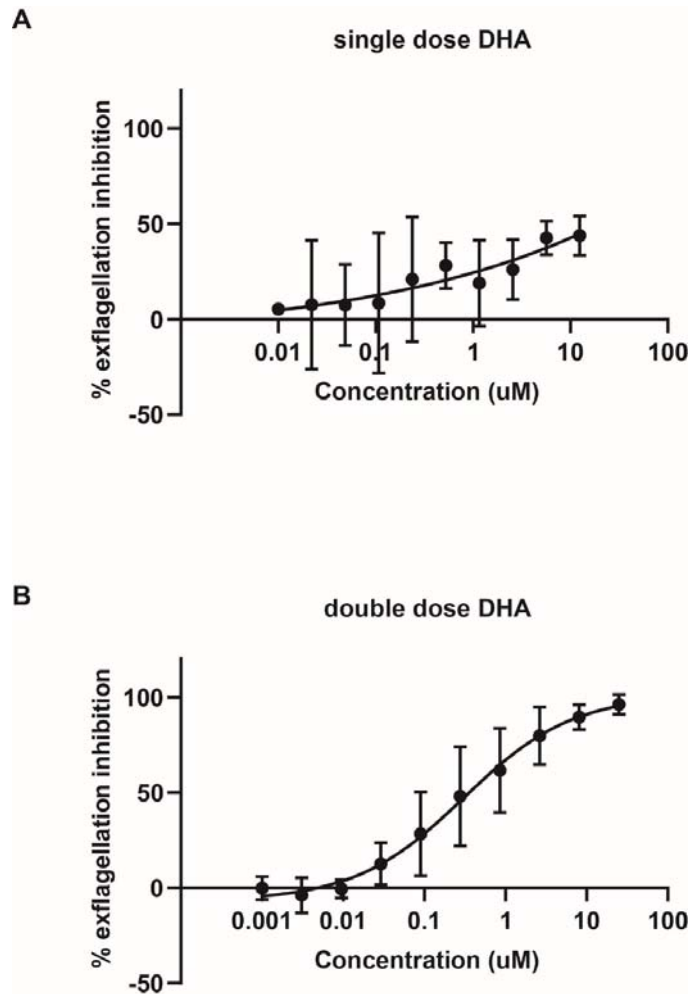
680 **Figure S1.** Sashimi plots of field isolates displaying DNA-sequencing coverage over a genomic
 681 region of chromosome 5 (**A**) and chromosome 14 (**B**). **A.** *Mdr1* is highlighted in pink, and asterisks
 682 indicate a genome duplication event for the gene in isolates APS3G and APL5G. **B.** Plasmepsin II
 683 and plasmepsin III are highlighted in pink, and asterisks indicate a genome duplication event for
 684 these two genes in isolate APL4G. Dashed line indicates raw DNA-seq pileup reads for the
 685 surrounding genes. Parasite isolates and sashimi plots are highlighted according to PfK13 variant.

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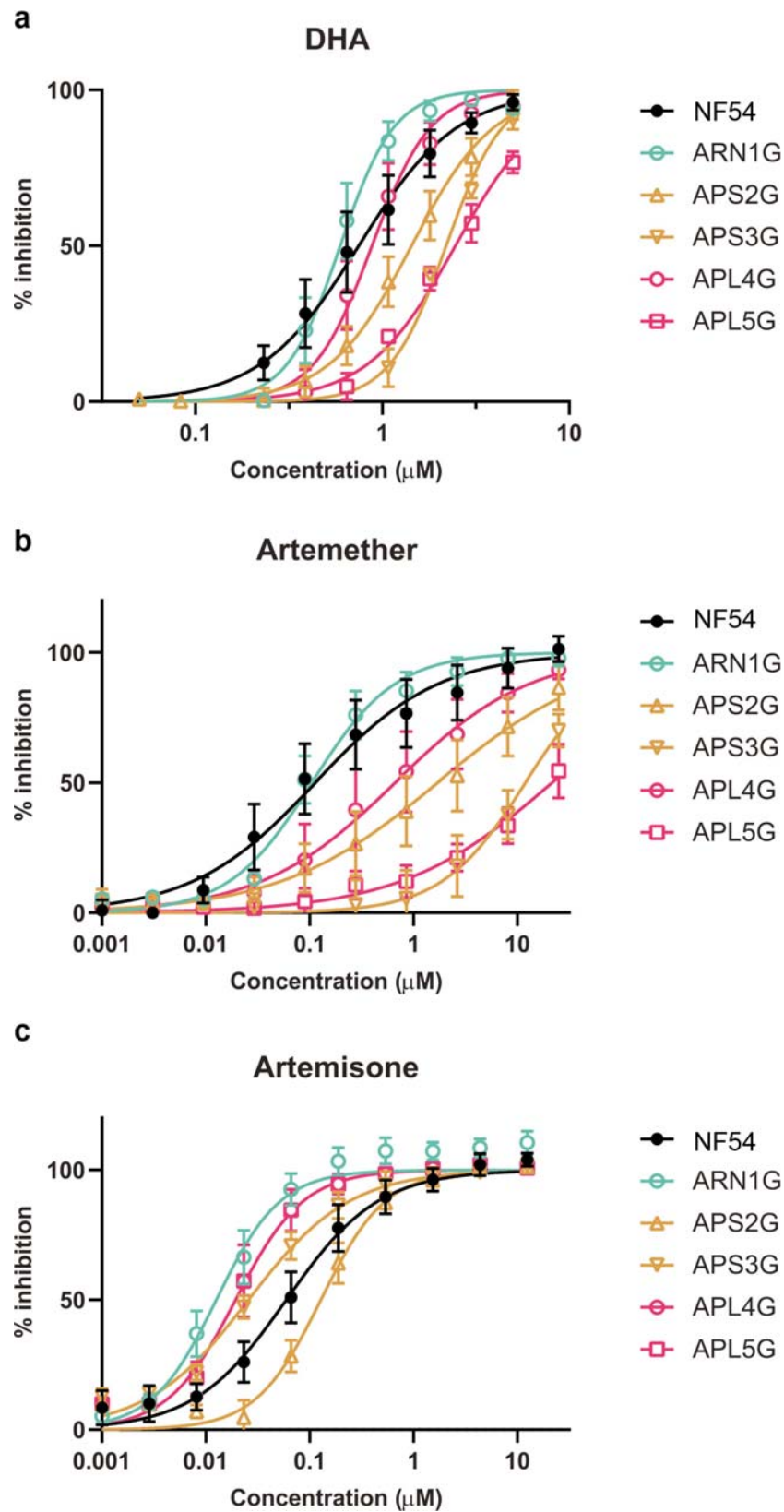
688 **Figure S2. Field isolates form similar-sized oocysts. A.** Gametocytaemia (represented by filled
689 circles) and exflagellation rates (represented by empty circles) of five field isolates. **B.** *An.*
690 *stephensi* mosquitoes were infected with field isolates, and oocyst numbers and prevalence are
691 shown. **C.** The size of day 10 oocysts was measured in (B). Graph shows diameter of each oocyst
692 found. Pfk13 genotype is not related to oocyst size. Isolate APS2G shows significantly bigger
693 oocysts than NF54 (unpaired t-test, ** $p < 0.01$).



694

695 **Figure S3.** Double-dose DHA elicits a more stable dose-response curve measuring exflagellation
696 inhibition. **A.** NF54 stage V gametocytes were incubated with increasing concentrations of DHA for
697 24 hours. After incubation, exflagellation centres were counted to give an estimate of %
698 exflagellation inhibition of DHA. Results showed to be very unstable. Three independent biological
699 replicates are shown. **B.** NF54 stage V gametocytes were incubated with increasing
700 concentrations of DHA for 24 hours, after which incubation with the same concentration was
701 repeated for another 24 hours. After 48 hours, exflagellation centres were counted to give an
702 estimate of % exflagellation inhibition of DHA. Results are more stable than with the single dose
703 regimen. Three independent biological replicates are shown

704

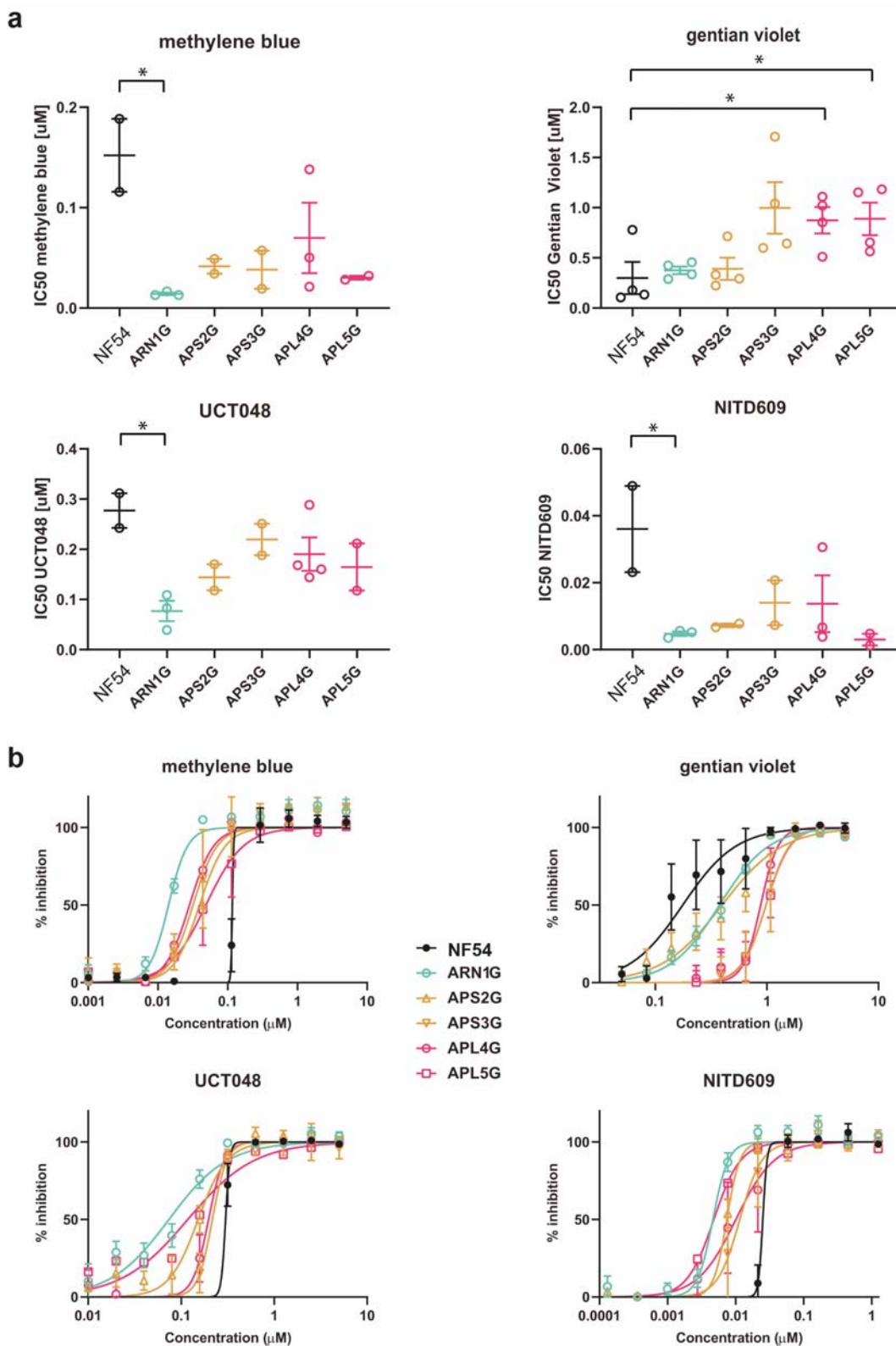


705

706 **Figure S4. Dose-response curves of three artemisinin-derivatives and their effect on**

707 **exflagellation inhibition. A. DHA. B. Artemether. C. Artemisone.**

708



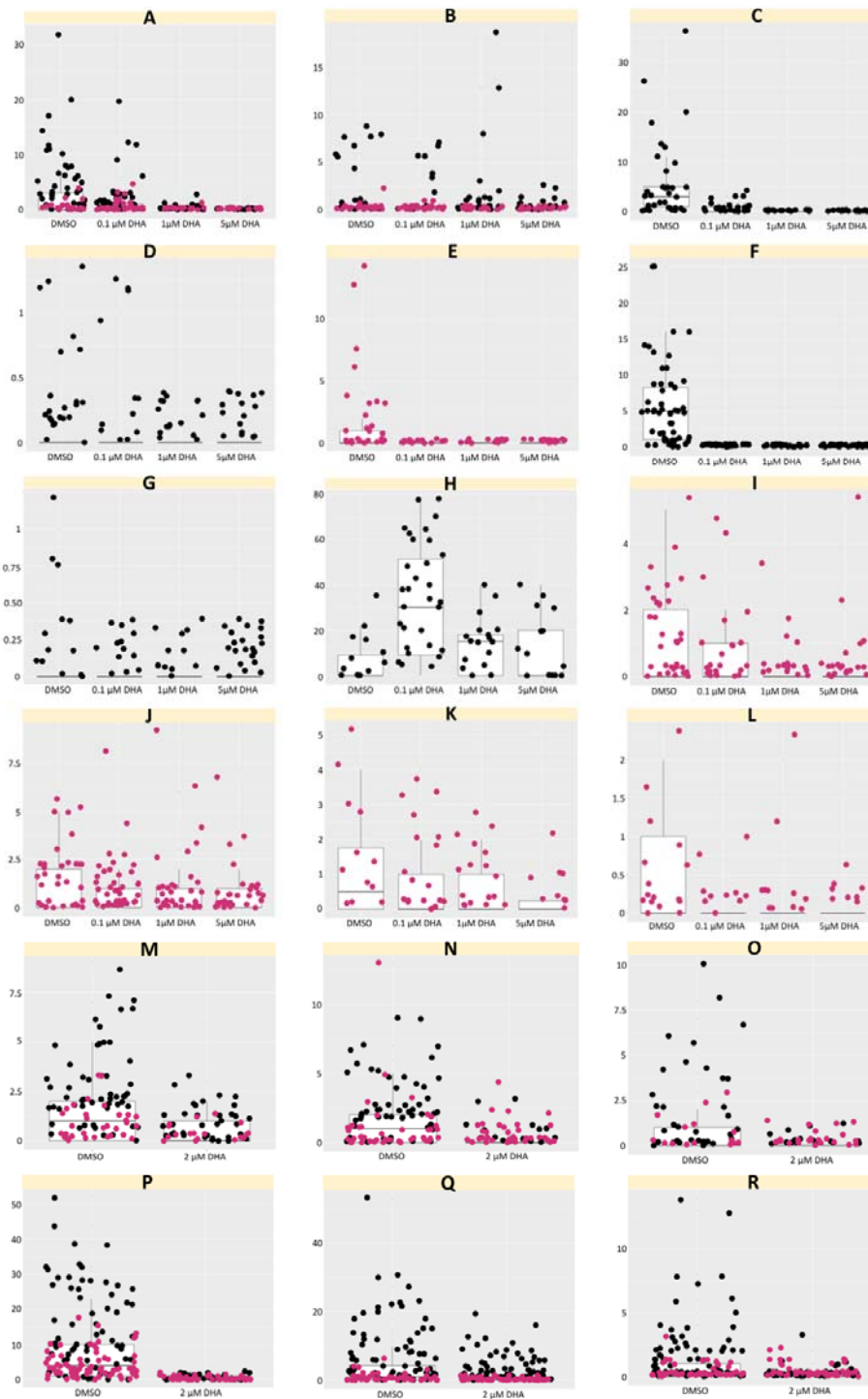
709

710 **Figure S5. Dose-response curves of four antimalarial compounds and their effect on**

711 **exflagellation inhibition. A. IC₅₀ values. B. Drug curves.**

712

713



714

715 **Figure S6.** Overall *P. falciparum* infection intensity in 18 individual SMFA (Feed A-R). Each dot

716 represents a single midgut dissected with the number of oocysts plotted on the graph. **A-L.** DMSO,

717 0.1 μM DHA, 1 μM DHA, and 5 μM DHA were added to **NF54** (blue) and **APL5G** (red). **M-R.**

718 Incubation for 48 hours pre-feed with DMSO and 2 μM DHA.

719

720 **Supplementary Data Sheets (separate files)**

721 Excel spreadsheets of:

722 1. DGFA data – male exflagellation data

723 2. SMFA data – oocyst counts