Antimalarials in mosquitoes overcome *Anopheles* and *Plasmodium* resistance to malaria control strategies

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Abstract

The spread of insecticide resistance in *Anopheles* mosquitoes and drug resistance in *Plasmodium* parasites is contributing to a global resurgence of malaria, making the generation of control tools that can overcome these issues an urgent public health priority. We recently showed that the transmission of *Plasmodium falciparum* parasites can be efficiently blocked when exposing *Anopheles gambiae* females to antimalarials deposited on a treated surface, with no negative consequences on mosquito fitness. Here, we demonstrate this approach can overcome the hurdles of insecticide resistance in mosquitoes and drug resistant in parasites. We show that the transmission-blocking efficacy of mosquito-targeted antimalarials is maintained when field-derived, insecticide resistant *Anopheles* are exposed to the potent cytochrome b inhibitor atovaquone, demonstrating that this drug escapes insecticide resistance mechanisms that could potentially interfere with its function. Moreover, this approach prevents transmission of field-derived, artemisinin resistant *P. falciparum* parasites (*Kelch13* C580Y mutant), proving that this strategy could be used to prevent the spread of parasite mutations that induce resistance to front-line antimalarials. Atovaquone is also highly effective at limiting parasite development when ingested by mosquitoes in sugar solutions, including in ongoing infections. These data support the use of mosquito-targeted antimalarials as a promising tool to complement and extend the efficacy of current malaria control interventions.

Significance Statement

Effective control of malaria is hampered by resistance to vector-targeted insecticides and parasite-targeted drugs. This situation is exacerbated by a critical lack of chemical diversity in both interventions and, as such, new interventions are badly needed. Recent laboratory studies have shown that an alternative approach based on treating *Anopheles* mosquitoes directly with antimalarial compounds can render the vector incapable of transmitting the *Plasmodium* parasites that cause malaria. While promising, showing that mosquito-targeted antimalarials remain effective against wild parasites and mosquitoes, including drug- and insecticide-resistant populations, respectively, is crucial to the future viability of this approach. In this study, carried out in the US and Burkina Faso, we show that antimalarial exposure is highly effective, even against extremely resistant mosquitoes, and can block transmission of drug-resistant parasites. By combining lab, and field-based studies in this way we have demonstrated that this novel approach can be effective in areas where conventional control measures are no longer as effective.

Introduction

Human malaria, a parasitic disease caused by unicellular eukaryotic *Plasmodium* parasites and spread through the bite of *Anopheles* mosquitoes, remains a substantial cause of global morbidity and mortality (1). Malaria control programs rely on preventative measures focused on mosquito control and on therapeutic measures based on the use of antimalarial drugs. Mosquito-targeted interventions are the most effective tools at reducing the transmission of *Plasmodium* parasites, with long-lasting insecticide-impregnated nets (LLINs) and indoor residuals spraying (IRS) as primary methods for malaria prevention. LLINs alone are predicted to have contributed to 68% of malaria cases averted between 2000 and 2015 (2). Alongside these preventative interventions, artemisinin combination therapies (ACT) have been the cornerstone of human malaria treatment since their widespread

55 introduction at the beginning of this century (3, 4) and have contributed substantially to the reduction
56 in malaria mortality and morbidity observed since then (2).

57 Despite sizeable investment, malaria control and elimination efforts are, however, faltering due to
58 reduced operational effectiveness of these key control tools, largely caused by mosquito resistance to
59 insecticides and parasite resistance to drugs (5-8). In the malaria hyperendemic regions of southern
60 Mali and southwest Burkina Faso, for example, resistance to pyrethroids is extreme (9), driven by
61 multifactorial and synergistic resistance mechanisms including enhanced metabolic detoxification
62 through upregulated cytochrome P450s (metabolic resistance), and reduced tarsal uptake through
63 cuticular thickening (cuticular resistance) (10-13). Similarly, the emergence and spread of artemisinin
64 resistance to sub-Saharan Africa — a region where as many as 93% of annual malaria deaths occur (1)
65 — is a major concern. Until recently, resistance to these first line antimalarials was limited
66 geographically to the Greater Mekong Subregion (GMS), however *de novo* mutations in *PfKelch13*
67 associated with *in vitro* resistance have now been detected in Uganda, Tanzania, and Rwanda (8, 14-
68 19). Of further concern is the recent invasion and spread of the Asian vector *Anopheles stephensi* to
69 the horn of Africa (20), as this mosquito species is highly competent for the transmission of *P.*
70 *falciparum* parasites endemic to the GMS and therefore, invasive populations may facilitate the spread
71 of parasites harboring artemisinin resistance mutations from Asia to Africa. Besides insecticide
72 resistance, an additional hurdle to malaria elimination is represented by residual malaria. Defined as
73 malaria transmission in the presence of universal effective LLIN coverage, residual malaria is driven
74 by mosquitoes that exhibit outdoor or daytime biting preferences and is a considerable hurdle to
75 malaria eradication efforts (21). Increased focus on residual malaria has stimulated interest in the use
76 of attractive toxic, or targeted (22), sugar baits (ATSBs) to attract and kill adult mosquitoes irrespective
77 of blood-feeding behavior, which in field trials have shown some promise as a tool for suppressing
78 vector populations (23, 24).

79 Thus, a control strategy that prevents insecticide resistant *Anopheles* populations from transmitting
80 malaria parasites, including parasite strains carrying drug resistance mutations, regardless of mosquito
81 feeding behavior and without imposing strong selective pressure on mosquitoes could overcome the
82 limitations of current mosquito-targeted interventions. In an effort to generate such a strategy, we
83 recently demonstrated that transmission of *P. falciparum* parasites can be prevented when *Anopheles*
84 *gambiae* are exposed to the antimalarial atovaquone (ATQ) through direct contact — analogous to the
85 mode of insecticide exposure on LLINs or IRS (25). Contact with ATQ-coated surfaces completely
86 abrogated parasite development when it occurred around the time of infection (between 24 hours (h)
87 before and 12 h post feeding on *P. falciparum*-infected blood), preventing onward transmission of the
88 parasite. Importantly, mathematical models based on these results showed that integrating antimalarial
89 ingredients into existing mosquito-targeted interventions could considerably reduce malaria
90 transmission in areas of widespread insecticide resistance, empowering our best malaria prevention
91 tools (25).

92 Here we show that targeting *P. falciparum* with antimalarials during its development in the *Anopheles*
93 female circumvents the hurdles of insecticide and drug resistance, providing a critical addition to the
94 malaria elimination toolkit. Parasite development is substantially reduced when wild, as well as
95 recently lab adapted *Anopheles coluzzii* (a sibling species of *An. gambiae*) that are highly resistant to
96 pyrethroids are exposed to ATQ prior to feeding on blood taken from *P. falciparum*-infected donors
97 in Burkina Faso. ATQ is also fully active against field-derived *P. falciparum* parasites from Cambodia
98 that are resistant to artemisinin. When using distinct drug targets in humans and mosquitoes, this
99 method is therefore capable of both overcoming insecticide resistance mechanisms and stopping
100 transmission of parasite mutations that confer resistance to frontline antimalarials. Finally, we show
101 that delivering ATQ via sugar solutions causes a striking reduction in both parasite numbers and
102 growth, proving that antimalarials could also be incorporated into interventions that target outdoor
103 malaria transmission, such as ATSB. Targeting *Plasmodium* parasites in the mosquito vector is

104 therefore a promising strategy that circumvents key limitations of current malaria control and
105 preventative interventions.

106 **Results**

107 **Exposure to ATQ substantially reduces infection with field *P. falciparum* isolates in insecticide**
108 **resistant *An. coluzzii***

109 To determine whether antimalarial exposure can maintain efficacy in insecticide-resistant mosquitoes,
110 we took *An. coluzzii* collected as pupae from larval breeding sites in Bama, Burkina Faso and reared
111 them to adults at the IRSS, Burkina Faso. Adult mosquitoes were infected using *P. falciparum*
112 gametocyte positive blood obtained from a malaria infected human donor on the day of infection. The
113 *An. coluzzii* mosquitoes endemic to this part of Burkina Faso – hereafter named AcVK5 – are highly
114 resistant to pyrethroids (9, 11, 26). AcVK5 mosquitoes were exposed to ATQ for 6 minutes (min) at
115 two concentrations (100 µmol- or 1 mmol/m^2) or a mock-treated blank control surface prior to feeding
116 on infectious blood samples. Infection outcomes were assayed at 7 days (d) post infectious blood meal
117 (pIBM) by dissection of the mosquito midgut to determine the prevalence and intensity of parasite
118 oocysts (Fig. 1 a). Control-exposed AcVK5 females were robustly infected with 81.3% harboring at
119 least one *P. falciparum* oocyst, and median infection intensity in infected females of 19 oocysts per
120 midgut. In contrast, AcVK5 mosquitoes exposed to either dose of ATQ had significantly reduced *P.*
121 *falciparum* infection both in terms of prevalence and intensity (Fig 1b). At the highest concentration,
122 we observed a 99% overall reduction in infection relative to the control (84.6% reduction in prevalence
123 and 94.8% reduction in median intensity), while at the lower dose inhibition of infection reached 96%
124 overall (65.9% reduction in prevalence and 89.5% reduction in median intensity). These results
125 demonstrate that direct tarsal antimalarial exposure, for instance incorporating antimalarials in LLINs
126 and IRS, can effectively block transmission of circulating west African *P. falciparum* parasites in
127 highly insecticide resistant endemic *Anopheles* mosquitoes. Although our previous findings had shown

128 that both ATQ doses tested above are capable of complete inhibition of parasite transmission using the
129 combination of standard *P. falciparum* (NF54) and insecticide-susceptible *Anopheles* (G3) populations
130 (25), our results confirm that parasite development can be considerably impaired when exposing
131 mosquitoes to antimalarials.

132 The observation of few parasites surviving exposure may indicate that parasite or mosquito factors in
133 our Burkinabe populations could be reducing the efficacy of ATQ in this assay, potentially including
134 interference from extant insecticide resistance mechanisms in AcVK5 mosquitoes, or reduced ATQ
135 drug sensitivity in *P. falciparum* in this region. To test these possibilities, we initially assayed the
136 efficacy of ATQ against insecticide resistant, Burkina Faso-derived *An. coluzzii* (hereafter Bama-R)
137 with the lab standard *P. falciparum* strain NF54. Reared under pyrethroid selective pressure under
138 otherwise standard laboratory conditions, Bama-R have maintained the parental trait of 100%
139 resistance to permethrin at the WHO discriminating concentration (DC, 696 μmol/m^2) and exhibit
140 appreciable acute survival at five times this dose (SFig. 1b). Bama-R mosquitoes are segregating for
141 the *kdr* mutation in *para* conferring target site resistance (SFig. 1c), but constitutively overexpress
142 CytochromeP450 genes associated with both metabolic resistance through enhanced small molecule
143 detoxification (SFig. 1d(27)), and cuticular thickening (26) Bama-R females were exposed to the
144 maximal effective concentration for tarsal ATQ (EC_{99}, 100 μmol/m^2, 6 min (25)) or to a vehicle control
145 immediately preceding infection, and parasite prevalence and intensity were determined at 7 d pIBM.
146 While control females were highly infected, with a median of 12 oocysts per infected midgut and
147 81.25% overall prevalence of infection, no oocysts were observed in females exposed to ATQ,
148 suggesting that insecticide-resistance mechanisms found in highly resistant, natural *Anopheles*
149 populations do not interfere with the transmission blocking activity of ATQ (Fig. 1c).

150 Next, we established an *in vitro P. falciparum* culture from a polyclonal isolate (P5) collected from a
151 gametocytemic donor from Burkina Faso (28)) and infected the laboratory standard, insecticide
152 susceptible mosquito strain *An. gambiae* (G3). P5 development was 100% suppressed in females

153 treated with the EC_{99} of ATQ (Fig. 1d) such that zero oocysts were observed in midguts, compared to
154 heavy infections—both in terms of infection intensity (median 59.5 oocysts per midgut) and infection
155 prevalence (95.7%)—in controls. Delivery of ATQ to mosquitoes is therefore fully effective against
156 field-derived *P. falciparum* isolates currently circulating in West Africa.

157 **ATQ prevents the transmission of an artemisinin-resistant *P. falciparum* isolate from the GMS**

158 Given the results obtained with field-derived parasites from Africa, we next tested the ability of ATQ
159 to kill artemisinin resistant parasites from the GMS, where mutations conferring artemisinin resistance
160 occur in a high proportion of *P. falciparum* isolates, constituting a major public health threat. We
161 reasoned that these experiments would also allow us to test the concept of directly targeting drug
162 resistant *P. falciparum* during mosquito development, removing resistance mutations from the parasite
163 population, and thereby "rescuing" ACT efficacy in human treatment. To this end, we used a
164 Cambodian *P. falciparum* patient clone (KH001_029 (5), hereafter ART29) carrying the C580Y
165 mutation in *PfK13* conferring resistance to artemisinin (Fig. 2a). We used the major Asian malaria
166 vector *An. stephensi* for these experiments as initial tests with *An. gambiae* did not produce appreciable
167 infections (SFig. 2). ART29 generated robust infections in control, mock-exposed *An. stephensi*,
168 (median 16 oocysts per midgut, 100% prevalence of infection). Conversely, no oocysts were detected
169 in females exposed to ATQ prior to infection (100 $\mu mol/m^2$, 6 min) (Fig. 2b). These data show that
170 mosquito exposure to antimalarials, such as by incorporation in bed nets, indoor residual sprays (or
171 other contact methods such as eaves tubes (29), could be an effective strategy for reducing the spread
172 of artemisinin resistance both within and between malaria endemic areas, including sub-Saharan
173 Africa.

174 **ATQ exposure during an ongoing infection delays oocyst growth and decreases sporozoite**
175 **prevalence**

176 In the field, mosquitoes that contact an antimalarial compound through mosquito-targeted
177 interventions may harbor parasites from a previous blood meal that have already traversed the midgut
178 lumen and formed oocysts. We therefore investigated the effects of ATQ on parasites in which oocyst
179 development is already underway, exposing G3 mosquitoes 6d pIBM (NF54, Fig. 3a). In contrast to
180 females exposed before infection, ATQ had no effect on the prevalence or intensity of infection, as
181 measured at 10d pIBM, suggesting ATQ acts differently on oocysts compared to zygote and ookinetes
182 (Fig. 3b). However, when we measured the size of the developing oocysts, we observed a significant,
183 45% decrease in the mean oocyst cross-sectional area (Fig. 3c). Oocyst size is a good proxy for rate of
184 growth (30) and as such, when we sampled mosquitoes at a later time point when sporozoite invasion
185 of salivary glands has already occurred (14 d pIBM), we observed a 33% reduction in the prevalence
186 of sporozoites in the salivary glands of ATQ-treated females (Fig. 3d). Similar results were obtained
187 when ATQ exposure instead occurred at 3 d pIBM (SFig. 3). Taken together, these results suggest that
188 ATQ exposure after oocyst formation has a partial cytostatic effect on *P. falciparum*.

189 **Ingestion of an ATQ-glucose solution blocks the establishment of *P. falciparum* infection**

190 To determine whether anti-parasitic compounds in sugar could suppress *Plasmodium* development in
191 the mosquito, we began by testing the efficacy of mosquito ATQ-glucose ingestion against the
192 transmission of *P. falciparum* parasites isolated from gametocytemic donor blood samples collected
193 from gametocyte carriers in Nasso, near Bobo Dioulasso, Burkina Faso. Adult female *An. gambiae,*
194 collected as pupae from larval breeding sites, were denied sugar for 24 h, then given access to an ATQ-
195 treated sugar solution (100 µM ATQ/0.5% v/v DMSO/10% w/v glucose) *ad libitum* for the 24 h
196 preceding infection (Fig. 4a). We observed a striking, 85% reduction in the prevalence of wild *P.*
197 *falciparum* infection in female mosquitoes that had access to ATQ-glucose prior to infection (Fig. 4b).
198 Importantly, median mosquito survival between control and ATQ treatment groups was not
199 significantly different (SFig. 4), confirming previous findings that atovaquone is not toxic to

200 mosquitoes at parasiticidal concentrations (25). This implies that other *Plasmodium*-specific inhibitors
201 would therefore not impose selective pressures leading to resistance mechanisms in the mosquito.

202 Using the same conditions with *in vitro* cultured *P. falciparum* (NF54) and lab-adapted *An. gambiae*
203 (G3) resulted in a remarkably similar infection outcome, with a 92.5% reduction in oocyst prevalence
204 in female *An. gambiae* given access to ATQ-glucose solution relative to controls (Fig. 4c). When ATQ
205 concentration was reduced to 10- and 100-fold ATQ dilutions, we observed progressively reduced,
206 dose dependent effects on prevalence (Fig. 4d).

207 **ATQ ingestion during an ongoing *P. falciparum* infection impairs sporogony**

208 As mosquitoes may often visit a sugar bait after acquiring an infectious blood meal, we also
209 investigated the impact of ATQ ingestion on ongoing *P. falciparum* (NF54) infections, providing
210 ATQ-treated sugar to G3 females from 2 d pIBM, when ookinetes have escaped the midgut lumen and
211 formed oocysts on the midgut basal lamina (Fig. 5a). This time, as based on our previous results (Fig.
212 3) we expected a possible cytostatic effect on oocyst growth, we performed a sampling time course to
213 capture oocyst development through mid- to late sporogony (7d, 10d and 14 d pIBM). We also counted
214 salivary gland sporozoites, the end point of parasite development in the mosquito, at 14 d pIBM. In
215 agreement with our previous results, we observed no change in oocyst prevalence and intensity because
216 of ATQ ingestion (SFig. 5). However, we observed an 80.8% decrease in oocyst cross-sectional area
217 relative to controls at 7 d pIBM, which persisted at later time points (89% and 76.3% decreases at 10-
218 and 14 d pIBM, respectively) (Fig. 5b). By 14 d pIBM, ATQ-exposed oocysts had a similar size to 7
219 d pIBM control oocysts, suggesting a remarkable suppression of growth. Inspection of DAPI-stained
220 infected midguts revealed a stark decrease in the number of nuclear foci, with a single, diffuse DNA
221 signal compared to many condensed foci in oocysts in controls (Fig. 5c). Strikingly, we detected no
222 sporozoites in the salivary glands of mosquitoes given access to ATQ-glucose 14 d pIBM, despit robust
223 infection in controls (Fig. 5d). Combined, these data point to a strong suppression of oocyst growth,

224 DNA replication and sporozoite differentiation after ATQ ingestion, suggesting that besides
225 preventing new mosquito infection, this delivery method would be extremely effective at curbing
226 ongoing infections and transmission.

227 **Discussion**

228 In this study we demonstrate the strong potential of incorporating antimalarials into LLINs, IRS and
229 ATSBs to stop transmission of endemic parasites by insecticide-resistant *Anopheles*. Our proof of
230 principle compound ATQ was very effective at killing a polyclonal, field-derived parasite isolate from
231 sub-Saharan Africa, showing that an antimalarial-based LLIN or IRS could suppress transmission even
232 in areas of malaria hyperendemicity, where vector pyrethroid resistance is exceptionally high, and
233 multiple parasite haplotypes coexists (31, 32).

234 Tarsal ATQ exposure against wild *An. coluzzii* (AcVK5) collected as pupae from breeding sites was
235 able to strongly suppress the transmission of *P. falciparum* isolates circulating in children.
236 Interestingly, in these infections, carried out in Burkina Faso, we observed a small number of
237 "breakthrough" oocysts at ATQ doses that are non-permissive in tests using, respectively, lab-adapted
238 and susceptible NF54 parasites and G3 mosquitoes (25). We reasoned that this marginal reduction in
239 efficacy could be due to extant insecticide resistance circulating in wild *Anopheles gambiae* s.l
240 populations in Burkina Faso, including the wild AcVK5 mosquitoes used for these experiments. While
241 clearly target-site resistance mechanisms are unlikely to impact the activity of antimalarials like ATQ,
242 both cuticular and metabolic resistance could potentially interfere with function by limiting compound
243 uptake and stability, respectively. The CYP450 monooxygenases associated with pyrethroid metabolic
244 detoxification in *An. gambiae* s.l. have been shown to confer some degree of resistance to a structurally
245 and functionally diverse array of compounds including the insecticide DDT, the juvenile hormone
246 agonist pyriproxyfen (33), and several arthropod mitochondrial complex I inhibitors including the
247 otherwise promising insecticide fenpyroximate (34). Similarly, cuticular resistance, where the waxy

248 exocuticle of the mosquito has thickened as an adaptation to insecticide pressure (11), could slow or
249 eliminate uptake of other small molecules. However, tarsal ATQ (at the EC_{99}, 100 μmol/m^2 for 6 min)
250 completely abrogated parasite infection in Bama-R mosquitoes, which are derived from the parental
251 AcVK5 population and exhibit a similar, high level of insecticide resistance combining the additive
252 effects of metabolic and cuticular resistance components (SFig. 1, (26)). This observation suggests that
253 mosquito pyrethroid resistance status, or other vector factors, did not affect tarsal antimalarial efficacy
254 in our experiments.

255 Similarly, in the reciprocal experiment, transmission of the culture-adapted Burkinabe *P. falciparum*
256 isolate P5 was blocked in an insecticide susceptible *An. gambiae* lab strain (G3), again indicating that
257 parasite factors are likely not responsible for the observed reduction in efficacy. Clinically-induced
258 resistance to ATQ in *P. falciparum* is associated with mutation at position 268 of the mitochondrial
259 gene *cytochrome B* (35), while *in vitro* selection typically results in mutation elsewhere in the gene
260 (36-38). ATQ is not in clinical use in sub-Saharan Africa, so it is unlikely that ATQ resistance-
261 conferring mutations are circulating in Burkina Faso at any appreciable frequency. However, while
262 certain ATQ-resistance mutations induce a transmission defect in both *P. berghei* and *P. falciparum* -
263 resulting in failure to establish infection in mosquitoes (37) there are contradictory findings in the
264 literature (38), and it has been shown that naturally occurring ATQ resistance-conferring mutations
265 can persist at ultra-low frequency in parasite populations through mitochondrial heteroplasmy (39).
266 Thus while the Burkinabe isolate (P5) used in our lab-based studies was susceptible to ATQ *in vitro*,
267 and Sanger sequencing of *Cytochrome B* from both P5 asexual blood stages and oocysts showed that
268 these parasites are wild-type, we cannot rule out the possibility that either cryptic parasite factors, lost
269 during the short P5 laboratory adaptation period, or specific vector-parasite interactions in wild
270 populations are responsible for the observed reduction in ATQ efficacy in our Burkina-based
271 experiments. The observed decrease in *P. falciparum* numbers remains extremely significant (above
272 96% total inhibition at 100 μmol/m^2). Taken together, these data demonstrate that mosquito-targeted

273 antimalarial exposure can bypass currently circulating insecticide resistance mechanisms, maintaining
274 activity even in areas where conventional insecticides have ceased to be effective — an essential trait
275 of any new mosquito-targeted tool.

276 ATQ exposure also killed artemisinin-resistant *P. falciparum* parasite ART29 in *An. stephensi*. *De*
277 *novo PfK13* mutations associated with *in vitro* resistance, already highly prevalent in Cambodia and
278 other part of the GMS, have now been detected in Uganda, Tanzania and Rwanda (14-19) and the
279 recent invasion and spread of *An. stephensi* to the horn of Africa (20) may facilitate the spread of these
280 parasites. Widespread artemisinin resistance in Africa, a region where malaria prevention is already
281 challenging due to insecticide resistance, would be a major public health concern. Although our results
282 were somewhat expected given ART29 parasites are ATQ-sensitive *in vitro* during asexual
283 development (40), they are an important proof of concept, both that drug-based mosquito-targeted
284 interventions could be developed to specifically contain and eliminate parasite haplotypes conferring
285 resistance to human antimalarial therapeutics, and that tarsal uptake of antimalarials function similarly
286 in *An. stephensi*. Antimalarial pressure directed at different drug targets in the human and mosquito
287 life stages could effectively suppress the spread of resistance mutations selected in either host and to
288 either target, allowing the possibility for close integration of human- and mosquito-targeted
289 antimalarial interventions in the future. Importantly, mosquito-targeted antimalarials attack the
290 parasite during an extreme population bottleneck and in a non-cyclical stage in its life cycle (41-43),
291 reducing the probability of selection of both extant and *de novo* resistance mutations during mosquito-
292 stage drug challenge compared to treatment during the human asexual cycle. Nevertheless, even the
293 possibility for selection of resistance during parasite sporogonic development means, as a fundamental
294 principle, any mosquito-targeted antimalarial compound integrated into LLIN or IRS must not share a
295 mode of action with current human antimalarial therapeutics. Thus, despite its efficacy in our studies,
296 ATQ could not be responsibly incorporated into a mosquito-targeted intervention due to its use as a

297 human prophylactic and therapeutic drug (44), making identification of additional active compounds
298 in diverse mode of action classes a priority.

299 Ingestion of sugar solutions containing ATQ before *P. falciparum* infection blocked transmission in
300 both field-derived and *in vitro* cultured *P. falciparum*. Such close agreement between these
301 experiments, carried out at different sites with different parasites and mosquitoes, is a testament to the
302 promise of this strategy, demonstrating its high effectiveness in spite of the inherent variability of
303 mosquito sugar feeding behavior (45). Furthermore, continued mosquito access to ATQ-sugar post
304 infection reduced oocyst growth resulting in absence of salivary gland sporozoites. These results
305 support the use of antimalarials in ATSB-like strategies to reduce residual malaria transmission carried
306 out by mosquitoes that predominantly rest and feed outside and thereby avoid both LLINs and IRS.
307 While current proposed ATSB designs rely on insecticidal ingredients (23, 24, 46), the use of
308 antimalarials in sugar baits could act as a more specific and environmentally benign paradigm for this
309 promising intervention should suitable antimalarial ingredients be identified (47-49). ATQ uptake via
310 tarsal contact at 3- or 6-days post infection also significantly reduced oocyst growth, resulting in an
311 appreciable reduction in the proportion of salivary gland sporozoite-positive mosquitoes. *P.*
312 *falciparum* is therefore also vulnerable to inhibition through tarsal exposure during the oocyst stage,
313 which is by far the longest developmental stage in the mosquito, taking between 7-10 days depending
314 on the frequency of blood feeding (30), and thus is the parasite life stage most likely to encounter a
315 mosquito-targeted intervention. As such, the ability to stall or kill oocysts and sporozoites is a highly
316 desirable quality for mosquito-targeted antimalarials.

317 ATQ targets the ubiquinol oxidation (Qo) site of cytochrome b (50), a key element of the
318 mitochondrial electron transport chain (mtETC) which has dual roles in both ATP generation through
319 oxidative phosphorylation and DNA replication through ubiquinone-mediated redox of dihydroorotate
320 dehydrogenase (51). Thus, inhibition of either DNA replication or ATP production, or both, could
321 explain the cytostatic effect observed here. Consistent with our findings, previous studies have shown

322 that disruption of ATP production through knock-out of components of the tricarboxylic acid cycle in
323 *P. falciparum* (52) and mtETC in *P. berghei* (53-55) can cause oocyst arrest. Moreover, chemical
324 inhibition of *P. vivax* DNA replication during sporogony was also sporontocidal (56), suggesting
325 mitochondrial inhibitors could also be utilized to prevent transmission of these widespread human
326 malaria parasites. Identifying the specific mechanism by which ATQ and other mtETC inhibitors affect
327 sporogonic development in *Plasmodium* is an interesting area for further study.

328 Although at an early stage, mosquito-targeted antimalarials have the potential to be an effective
329 element to drive malaria incidence down. To this end, identifying more compounds with strong
330 antiparasitic activity during the mosquito stages of *P. falciparum* development – and in particular
331 compounds with sporogony-specific activity – will be a crucial next step, and one that should leverage
332 the extensive libraries of known antimalarials. Indeed, one of the key strengths of this approach is the
333 potential to exploit and repurpose compounds that are otherwise unsuitable for human therapeutic use,
334 whether due to toxicity, poor bioavailability, poor kinetics, or other limiting factors. By integrating
335 human and mosquito-based interventions, this strategy will extend and protect the efficacy of human
336 therapeutics and vector control strategies, giving malaria control efforts renewed vigor.

Materials and Methods

Mosquito lines, insecticide resistance selection and husbandry

Anopheles spp. mosquito populations used in this study were: 1) wild *An. coluzzii* captured as pupae from breeding sites, described below. 2) Laboratory-reared *An. gambiae* obtained from an outbred colony established in 2019 and repeatedly replenished with F1 from wild-caught females collected in Soumousso (11°23′14″N, 4°24′42″W).3) *An. gambiae* G3 ("G3"), a highly lab-adapted, insecticide-susceptible strain competent for *P. falciparum* of African origin. 4) *An. stephensi* (Anst-S), a similarly lab-adapted, insecticide-susceptible strain competent for *P. falciparum* of both African and Asian origin received as a gift from The Institute of Molecular Medicine, University of Lisbon, Portugal. 5) *An. coluzzii.* Bama-R ("Bama-R") a colony established through hybridization of the F1 progeny of female *An. coluzzii* collected from Vallee du Kou, Burkina Faso, with our G3 colony. Since establishment, Bama-R has been kept under frequent permethrin selection pressure and exhibits a consistent pyrethroid resistance phenotype. At the time of this study, (F17-20) Bama-R females where highly resistant to pyrethroids, exhibiting 97% survival in standard WHO insecticide resistance assays — briefly, 1 h exposure to the WHO discriminating concentration (DC, 275 mg/m^2) of permethrin-impregnated papers with mortality scored at 24 h post-exposure — and 43% survival at 5x the DC. Except for selection of resistance in Bama-R, all mosquito colonies were maintained identically at 26 ºC ± 2 ºC and 80% ± 10%. relative humidity (RH). Larvae were cultured in 2-liter (l) catering pans in 500 ml distilled water (dH_2O) under an optimized density and feeding regimen. At the onset of pupation, pupae were separated from larvae using a vacuum aspirator, collected in dH_2O, and placed in a 30x30x30 cm cage (Bugdorm, Megaview Science Co, Ltd, Thailand). After emergence, adult mosquitoes had access to separate sources of 10% glucose (Sigma Aldrich, US) and dH_2O *ad libitum*. For colony maintenance, 5–7-day-old adults were provided a blood meal of donated human blood using an artificial membrane feeding system (Hemotek, UK). For mosquito colony 2) females were maintained on rabbit blood by direct feeding (protocol approved by the national committee of Burkina

362 Faso; IRB registration #00004738 and FWA 00007038) and adult males and females fed with a 5%
363 glucose solution. Larvae were reared at a density of about 300 first-instar larvae in 700 ml of water in
364 plastic trays and fed with Tetramin Baby Fish Food (Tetrawerke, Melle, Germany).

365 *P. falciparum strains and culture*

366 *P. falciparum* strains used in this study were: 1) *P. falciparum* NF54. NF54 is the drug-susceptible
367 standard strain for mosquito transmission studies, obtained from BEI Resources in 2014. This parasite
368 culture was received through a material transfer agreement (MTA) with the laboratory of Dr. Carolina
369 Barillas-Mury. 2) *P. falciparum* P5. P5 is polyclonal (n=3, KMMM, KMKM, RMMM), as determined
370 by MSP1 PCR genotyping following standard procedures (28), and has been culture-adapted from a
371 blood sample contributed by a gametocytemic malaria donor in Burkina Faso in 2017. 3) *P. falciparum*
372 KH001_029 "ART29". ART29 is a *P. falciparum* monogenomic parasite isolate obtained from an
373 infected human patient in Pursat, Cambodia (KH1 clade) between 2011 and 2013 as part of the TRAC
374 I initiative (5). This parasite carries the *PfK13* mutation C580Y associated with resistance to
375 artemisinin. ART29 has clear phenotypic artemisinin resistance, both as determined by *in vivo*
376 clearance time (11.8 h) and *in vitro* ring-stage survival (22.6%) (57), alongside resistance to other
377 antimalarial drugs, including mefloquine and chloroquine, but is not resistant to piperaquine or ATQ
378 (40). These parasites generate robust infections in *An. stephensi* mosquitoes, but not *An. gambiae*
379 (SFig. 2).

380 For mosquito infection with gametocytemic donor blood samples, *P. falciparum* samples were
381 collected and prepared for infection as described previously (58). Briefly, *P. falciparum* gametocyte-
382 positive whole blood samples were collected from 5–13-year-old donors from the villages surrounding
383 Bobo Dioulasso, Burkina Faso as part of a separate study. Red blood cells were isolated by
384 centrifugation of a 4 ml aliquot of donor blood followed by resuspension in *Plasmodium* naïve human
385 AB serum. Blood samples were then provided to mosquitoes using a custom blown, water heated glass
386 feeder. For mosquito infection with *in vitro* cultured parasites, females were transferred to a secure

387 malaria infection facility and provided a 14-21 d post-induction stage V *P. falciparum* gametocyte
388 culture using a custom blown, water heated glass feeder. For all infection experiments, within 24 h of
389 infection, partially engorged or unfed mosquitoes were collected by vacuum aspiration and discarded.
390 To determine oocyst burden, between 7 and 14 d pIBM, infected mosquitoes were collected by vacuum
391 aspiration, and dissected to isolate the midgut. The oocyst burden was determined after staining
392 midguts with 0.2% w/v mercurochrome and examination under a 40x air objective inverted compound
393 light microscope (Olympus, US). For sporozoites, at 14 d pIBM infected mosquitoes were collected
394 by vacuum aspiration and beheaded. The mosquito salivary glands were extracted into RPMI media
395 by applying pressure to the lateral thorax. *P. falciparum* sporozoites were isolated by homogenization
396 and centrifugation of salivary gland material, followed by resuspension in a known volume of RPMI.
397 Sporozoites were counted using a disposable haemocytometer under a 20x air objective inverted
398 compound light microscope (Olympus, US). All *P. falciparum* strains were cultured and induced to
399 form gametocytes using standard protocols (59, 60). All strains have been confirmed to be *P.*
400 *falciparum* by PCR followed by DNA sequencing of the amplified products (61) and have been
401 confirmed free of mycoplasma infection.

402 *Pre- and post-infection tarsal contact infection assays*

403 Tarsal exposure plates were prepared as described previously (25). Briefly, for 100 μmol/m^2 plate
404 concentrations, 0.1 ml of this solution of a 0.1% w/v solution of ATQ in acetone was diluted with 1
405 ml additional acetone and spread onto a 6 cm diameter (0.002628 m^2) glass petri dish. For 1 mmol/m^2,
406 1 ml of 0.1% w/v ATQ/acetone solution was added directly to the plate. Plates dried for a minimum
407 of 4 h, with agitation on a lateral shaker at room temperature. For pre-infection exposure, 30 min prior
408 to infection, 3-5 d old virgin female mosquitoes were incubated on either ATQ-coated plates, or an
409 acetone treated control, for 6 min. To prevent crowding and agitation, a maximum of 25 mosquitoes
410 were exposed per plate, with all exposures occurring in parallel. For post-infection exposure, due to

411 biosafety considerations, compound exposures were carried out in serial, with a maximum of 10
412 infected mosquitoes per plate.

413 *Pre- and post-infection sugar feeding infection assays*

414 For experiments involving compound-treated sugar solutions, 20 mM stock solutions of each active
415 ingredient (AI) were prepared in 100% DMSO. Each 20 mM stock solution was diluted 200-fold in
416 10% w/v glucose to achieve the final working concentration of 100 µM AI/0.5% DMSO/10% w/v
417 glucose. For pre-infection sugar feeding experiments, 2-4 d post-emergence females were denied
418 access to glucose or water for 24 h and then provided access to either the test solution, or a control
419 solution of 0.5% v/v DMSO/10% glucose, for 24 h. After this time had elapsed, all mosquitoes were
420 provided with an infectious *P. falciparum* blood meal as described above. Infected mosquitoes were
421 provided with untreated 10% w/v glucose *ad libitum* for the remainder of the experiment. For post-
422 infection sugar-feeding experiments, 3-5 d post-emergence female mosquitoes were infected with *P.*
423 *falciparum* and denied access to glucose or water for 48 h pIBM. After this time, infected mosquitoes
424 were continuously provided either 100 µM ATQ/0.5% DMSO/10% w/v glucose or 0.5% DMSO/10%
425 w/v glucose as control, *ad libitum.* Sugar feeders were replaced every 48 h for the remainder of the
426 experiment, up to 14 d pIBM.

427 *Statistical analyses*

428 Statistical analyses were carried out using GraphPad Prism v8.4.2 for MacOSX (GraphPad Software
429 Inc., USA) and JMP Pro 15 (SAS Corp. US).

430 *Data generated from donor isolated gametocytes*

431 For infections with donor isolated gametocytes (Fig. 1(b), Fig. 4(b)), prevalence and intensity of
432 infection were analyzed using more complex statistics to account for between-replicate effects of
433 different human gametocyte donors. **For prevalence:** we constructed a General Linear Model as
434 follows, independent variable/y "Infected?" (Two-level, categorical (yes/no)), with cofactors

435 “Treatment” (Two-level, categorical (Control/ATQ)) and “Gametocyte Donor” (Two-level,
436 categorical (Donor 1/Donor 2)), we also included the interaction term Treatment*Gametocyte Donor
437 to detect higher level effects. As the output was categorical, the GLM model was run with a binomial
438 distribution and logit link-function. To achieve the best model fit, we iteratively removed cofactors
439 from the model, and selected the model output with the lowest corrected Akaike information criterion
440 (AICc). In all cases, the best model fit included both cofactors, but excluded the interaction term. **For**
441 **intensity:** Independent variable “Oocyst Count” (Continuous, positive integer) with cofactors
442 “Treatment” (Two-level, categorical (Control/ATQ)) and “Gametocyte Donor” (Two-level,
443 categorical (Donor 1/Donor 2)), and the interaction term Treatment*Gametocyte Donor. To account
444 for the overdispersion typical of parasite count data, we again took an iterative approach to model
445 construction. Data were analyzed using both a GLM using a Poisson distribution, (link function: log;
446 overdispersion parameter estimated by Pearson Chi-square/DF) and Generalized Regression with a
447 Negative Binomial distribution. Relative model quality was determined by comparison of AICc for
448 each distribution function (For GLM, with and without the overdispersion correction) and by iterative
449 removal of cofactors. The highest quality model fit was an overdispersion-corrected Poisson/Log GLM
450 with both cofactors but without the interaction term.

451 *Data generated from in vitro experiments*

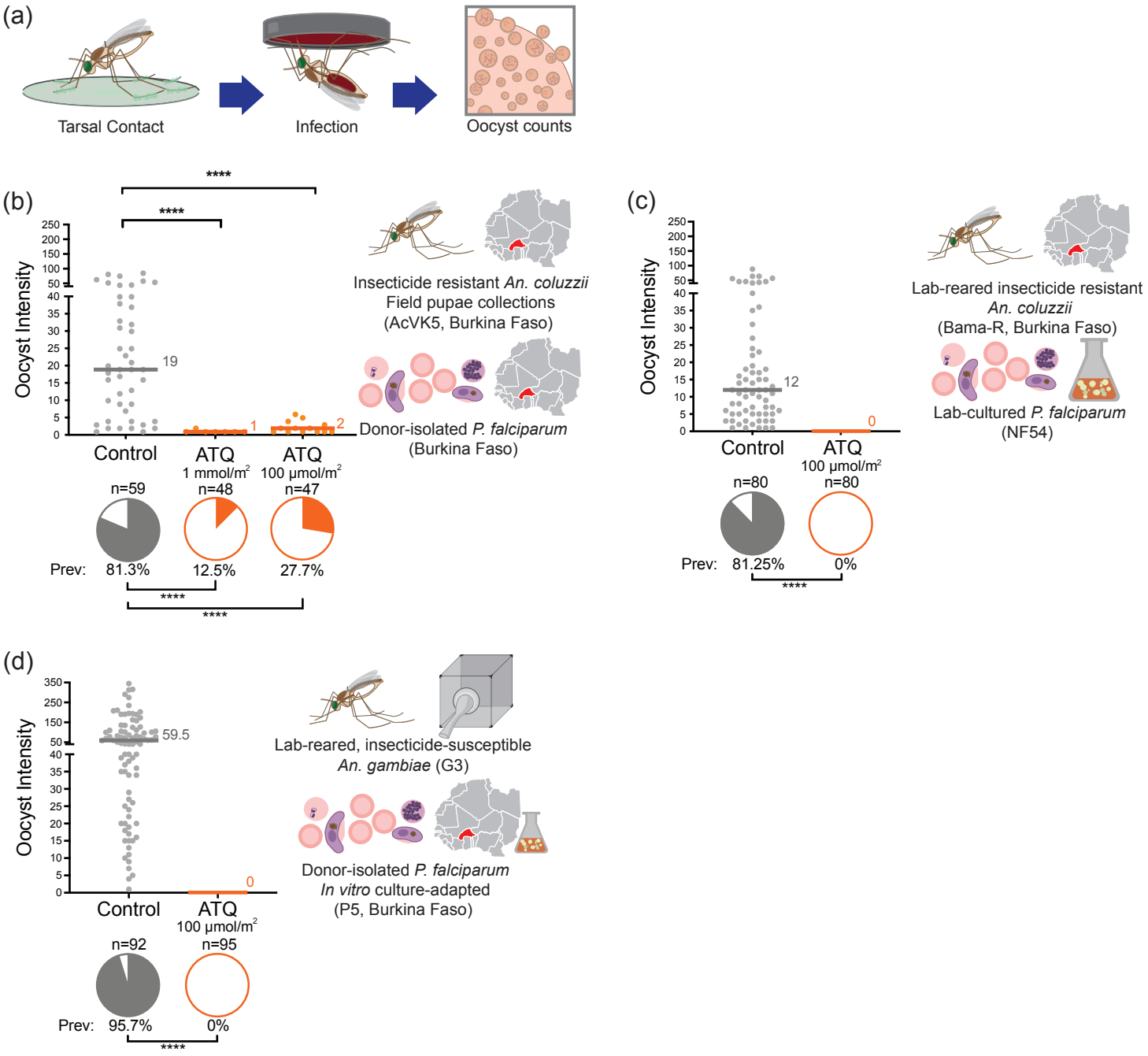
452 For all other infections, differences in prevalence were analyzed by Chi^2. In experiments where both
453 treatment groups had individuals that produced >0 oocysts, differences in median oocyst burden
454 between groups (intensity of infection) was analyzed using a Mann-Whitney Mean Ranks test. For
455 multiple comparisons, differences in prevalence between multiple groups were determined using
456 pairwise Chi^2 corrected for multiple comparisons (Bonferroni). Similarly, multiple comparisons of
457 intensity were carried out using a Kruskal-Wallis test with Dunn’s *post hoc.*

458 **Author Contributions:**

DGP and FC wrote the manuscript. DGP, TL and FC designed the experiments. DGP and TL carried out statistical analysis. DGP, ASP, EM, DFSH, PP, RSY, TL and NS carried out infection experiments. DFSH, RSY, TL, WRS, SKV, and SB collected, established, maintained, and assayed parasite lines. KLA established and reared insecticide-resistant mosquito lines and carried out resistance assays. TL, AD, RKD, DFW, and FC, provided funding and oversight. All authors read and approved the manuscript prior to submission.

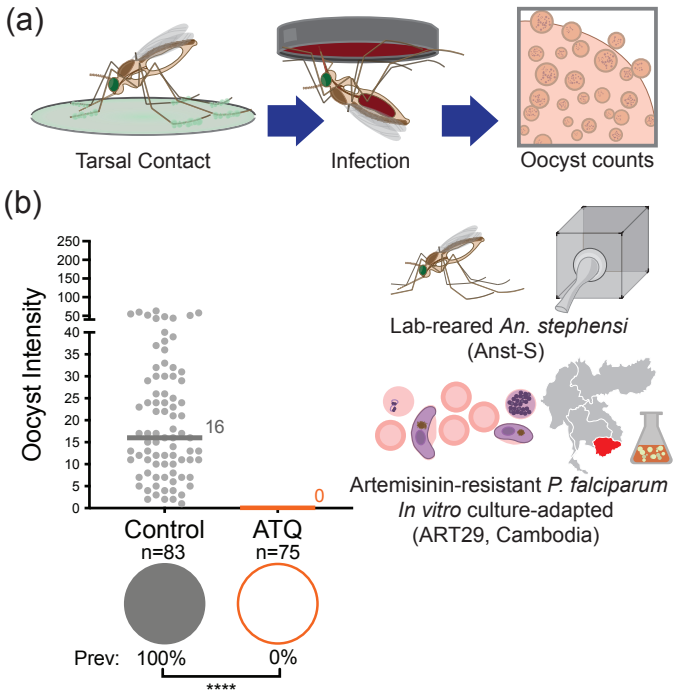
The authors state that they have no conflicting interests.

Figure 1


(a)
Tarsal Contact
Infection
Oocyst counts
(b)
Oocyst Intensity
****
****
19
1
2
Control
ATQ
1 mmol/m²
ATQ
100 µmol/m²
n=59
n=48
n=47
Prev:
81.3%
12.5%
27.7%
****
****
Insecticide resistant An. coluzzii
Field pupae collections
(AcVK5, Burkina Faso)
Donor-isolated P. falciparum
(Burkina Faso)
(c)
Oocyst Intensity
12
0
Control
ATQ
100 µmol/m²
n=80
n=80
Prev:
81.25%
0%
****
Lab-reared insecticide resistant
An. coluzzii
(Bama-R, Burkina Faso)
Lab-cultured P. falciparum
(NF54)
(d)
Oocyst Intensity
59.5
0
Control
ATQ
100 µmol/m²
n=92
n=95
Prev:
95.7%
0%
****
Lab-reared, insecticide-susceptible
An. gambiae (G3)
Donor-isolated P. falciparum
In vitro culture-adapted
(P5, Burkina Faso)

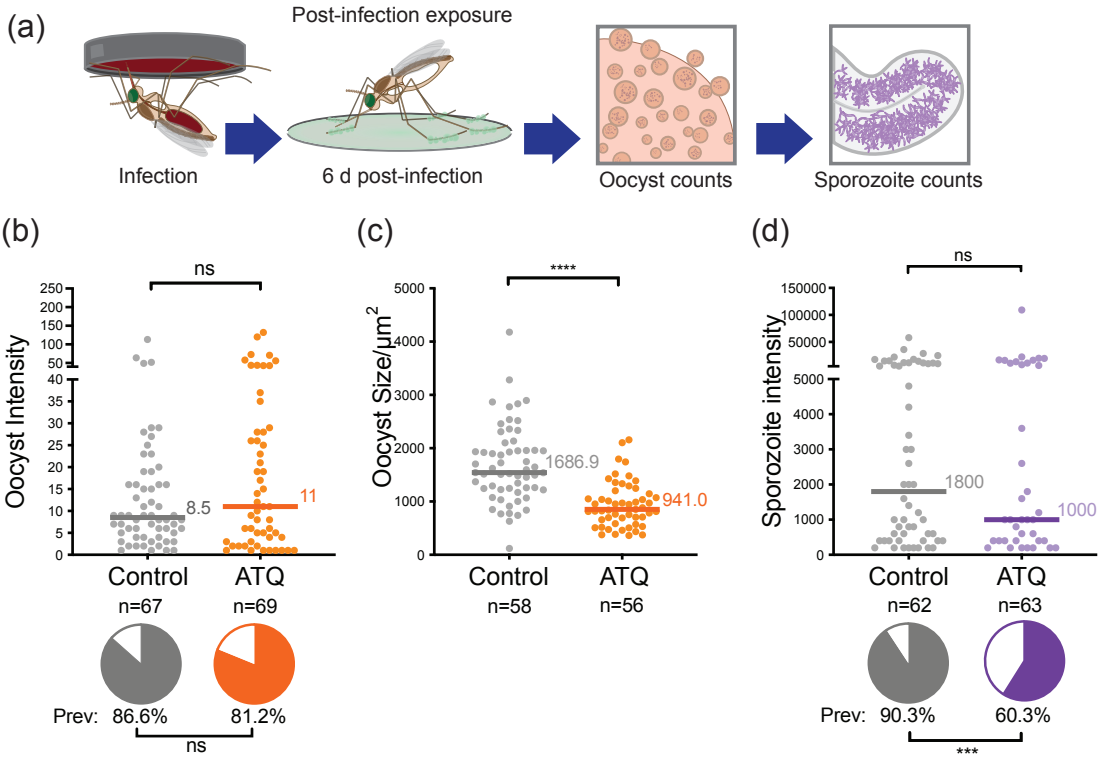
467 **Figure 1: Exposing *An. gambiae* mosquitoes to ATQ suppresses *P. falciparum* development in**
468 **field-derived parasites and insecticide-resistant mosquitoes. (a)** Experimental scheme. **(b)** Female
469 AcVK5 mosquitoes, collected as pupae from breeding sites in southwestern Burkina Faso, were
470 exposed to ATQ by tarsal contact at either the maximal effective concentration (EC_{99}) for insecticide-
471 susceptible mosquitoes (100 µmol/m^2, EC_{99}) or 10x EC_{99} (1 mmol/m^2) for 6 minutes, prior to infection
472 with donor-isolated "wild" *P. falciparum* (Burkina Faso), collected the same day. ATQ treatment
473 resulted in a significant reduction in both infection prevalence (GLM (Binomial;Logit): 100 µmol/m^{2,}
474 n=106, df=1, X^2=31.997, p<0.0001; 1 mmol/m^2, n=107, df=1, X^2=50.240, p<0.0001) and intensity
475 (GLM (Poisson;Log): 100 µmol/m^2: n=53, df=1, X^2=24.687, p<0.0001, 1 mmol/m^2: n=59, df=1,
476 X^2=31.997, p<0.0001) at both doses, as measured by determination of *P. falciparum* oocyst burden at
477 7 d pIBM. **(c)** In contrast, transmission of *in vitro*-cultured *P. falciparum* (NF54) is completely blocked
478 when field-derived, lab-adapted insecticide-resistant *An. gambiae* (Bama-R) are exposed to ATQ for
479 6 min to the maximal effective concentration (EC_{99}) for insecticide-susceptible mosquitoes (100
480 µmol/m^2) prior to infection. In ATQ-exposed mosquitoes, prevalence was zero, in contrast to the robust
481 infection in mock-exposed controls (Chi^2, n=160, df=1, X^2=93.545, p<0.0001). **(d)** Similarly,
482 transmission of a polyclonal *P. falciparum* West African donor isolate (P5 – *in vitro* culture adapted)
483 was blocked (zero evidence of infection at 7 d pIBM) after ATQ exposure prior an infectious blood
484 meal, despite a strong (prevalence 95.7%, median intensity 59.5 oocysts per midgut) infection in
485 controls (Chi^2, n=187, df=1, X^2=167.995, p<0.0001). Median lines and values are indicated, "n"
486 indicates the number of independent samples. To isolate Oocyst Prevalence and Oocyst Intensity,
487 midgut samples with zero oocysts have been excluded from intensity analysis. Statistical significance
488 is indicated where relevant as follows: ns=not significant, * = p<0.05, **=p<0.01, ***=p<0.001,
489 ****=p<0.0001.

Figure 2


(a)
Tarsal Contact
Infection
Oocyst counts
(b)
Oocyst Intensity
250
200
150
100
50
40
35
30
25
20
15
10
5
0
16
0
Control
n=83
ATQ
n=75
Prev: 100%
0%
****
Lab-reared An. stephensi
(Anst-S)
Artemisinin-resistant P. falciparum
In vitro culture-adapted
(ART29, Cambodia)

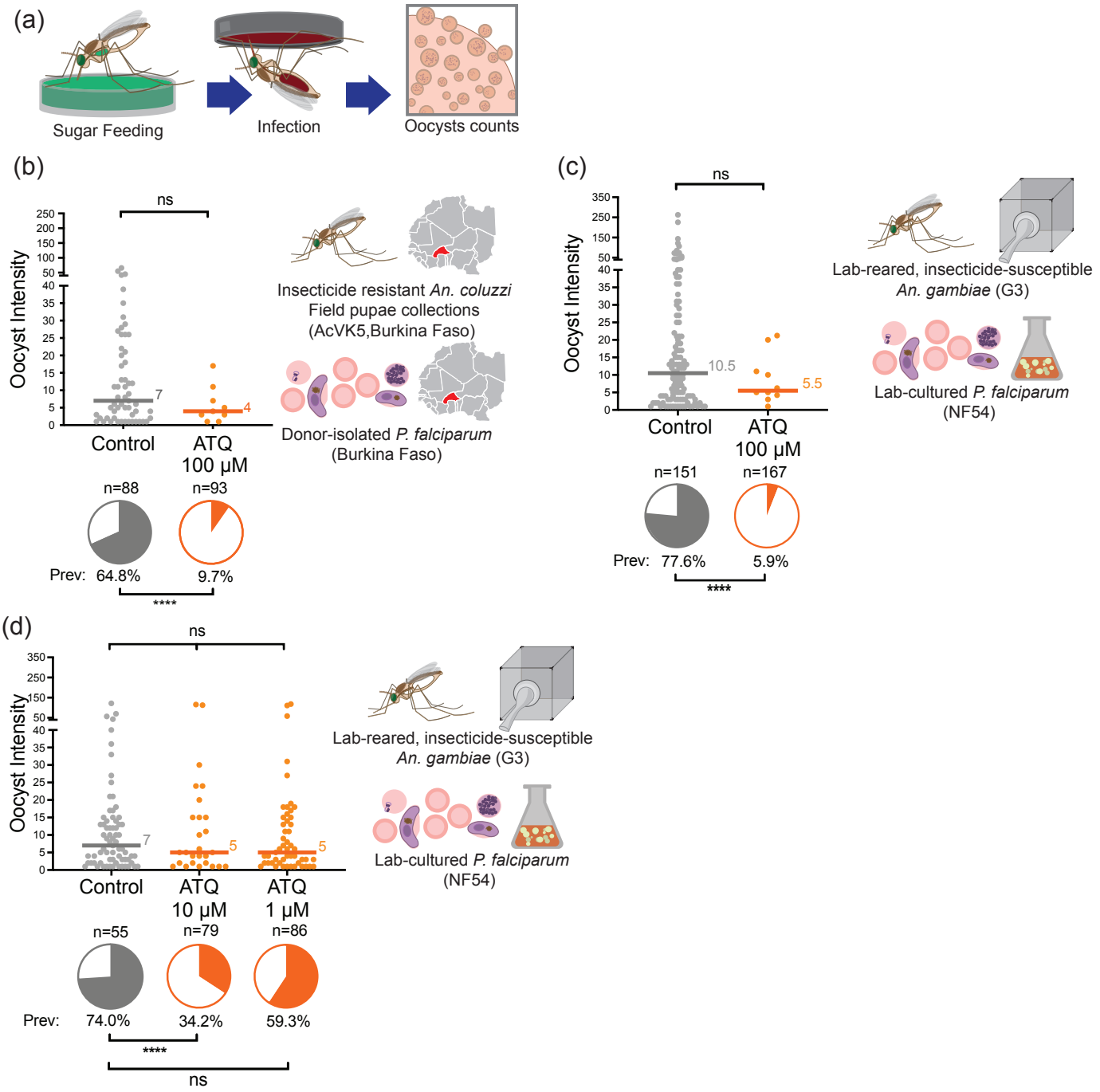
490 **Figure 2: Exposing *An. stephensi* to ATQ blocks an artemisinin-resistant *P. falciparum* patient**
491 **isolate from Cambodia. (a)** Experimental scheme. **(b)** Transmission of artemisinin resistant *P.*
492 *falciparum* (ART29) is completely blocked when *An. stephensi* females (Anst-S) are exposed to ATQ
493 for 6 min to the maximal effective concentration (EC_{99}) for insecticide-susceptible mosquitoes. In
494 ATQ exposed mosquitoes, prevalence (indicated by pie charts) was zero despite robust infection in
495 mock-exposed controls (Chi^2, n=158, df=1, X^2=156, p<0.0001). Median lines and values are indicated,
496 "n" indicates the number of independent samples. To isolate Oocyst Prevalence and Oocyst Intensity,
497 midgut samples with zero oocysts have been excluded from intensity analysis. Statistical significance
498 is indicated where relevant as follows: ns=not significant, * = p<0.05, **=p<0.01, ***=p<0.001,
499 ****=p<0.0001.

Figure 3


(a)
Post-infection exposure
Infection
6 d post-infection
Oocyst counts
Sporozoite counts
(b)
ns
Oocyst Intensity
8.5
11
Control
ATQ
n=67
n=69
Prev: 86.6%
81.2%
ns
(c)
****
Oocyst Size/um2
1686.9
941.0
Control
ATQ
n=58
n=56
(d)
ns
Sporozoite intensity
1800
1000
Control
ATQ
n=62
n=63
Prev: 90.3%
60.3%
***

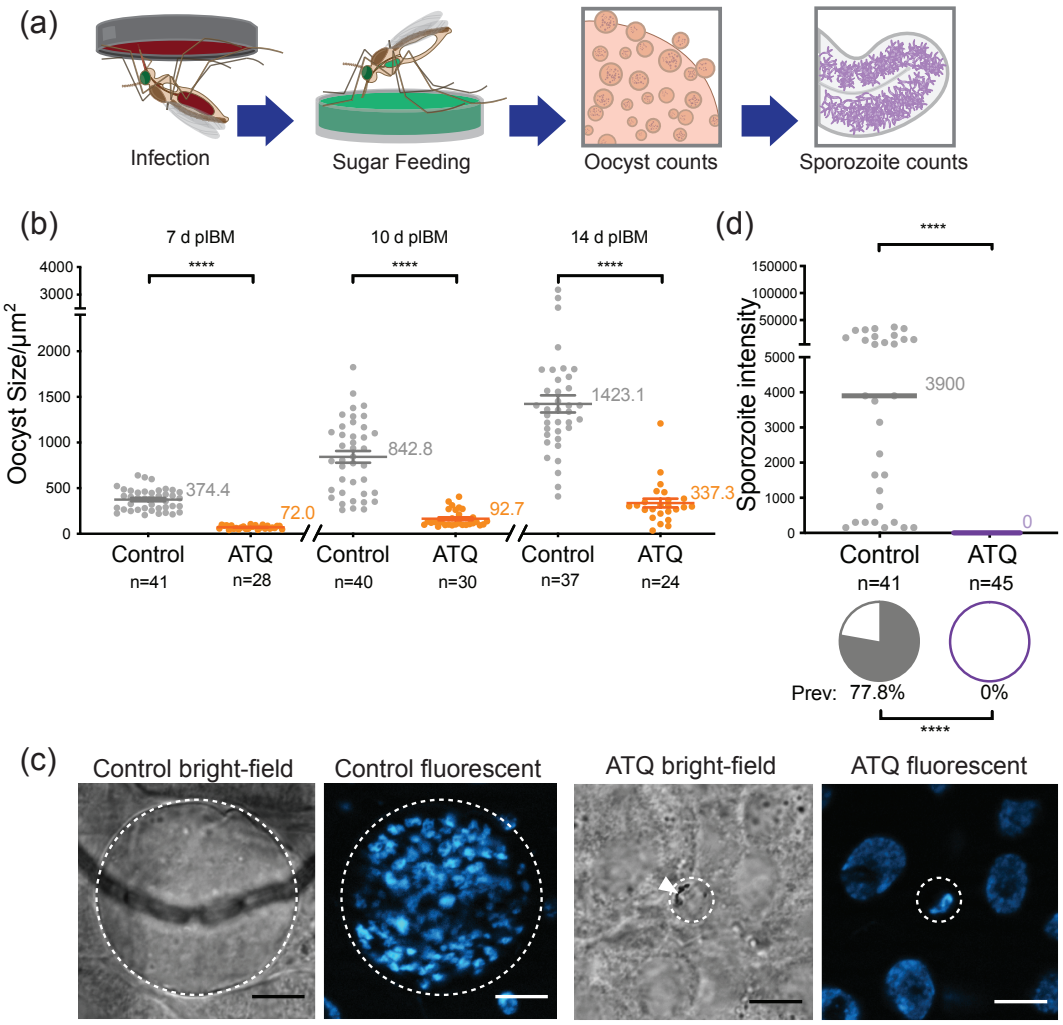
500 **Figure 3: Sporozoite prevalence is significantly reduced after tarsal ATQ exposure during oocyst**
501 **development when *An. gambiae* (G3) are infected with *P. falciparum* (NF54). (a)** Experimental
502 scheme. **(b)** There was no effect of 6 d pIBM ATQ exposure on either prevalence (indicated by pie
503 charts) or intensity (indicated by points) of infection determined at 10 d pIBM. Prevalence: Chi^2,
504 n=136, df=1, X^2=0.733, p=0.3919, intensity: Mann-Whitney, n=114, df=1, U=1482, p=0.4335. **(c)**
505 ATQ exposure at 6 d pIBM significantly reduced the median cross-sectional area of oocysts relative
506 to control (n=114, df=1, U=531, p<0.0001). **(d)** The prevalence, but not the median intensity of
507 sporozoites in salivary glands was significantly reduced in mosquitoes exposed to ATQ at 6 d pIBM
508 (Chi^2, n=125, df=1, X^2=7.190, p=0.0073). Median lines and values are indicated, "n" indicates the
509 number of independent samples. To isolate Oocyst Prevalence and Oocyst Intensity, midgut samples
510 with zero oocysts have been excluded from intensity analysis. Statistical significance is indicated
511 where relevant as follows: ns=not significant, * = p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.

Figure 4


(a)
Sugar Feeding
Infection
Oocysts counts
(b)
ns
Oocyst Intensity
7
4
Control
ATQ 100 µM
n=88
n=93
Prev: 64.8%
9.7%
****
Insecticide resistant An. coluzzi Field pupae collections (AcVK5,Burkina Faso)
Donor-isolated P. falciparum (Burkina Faso)
(c)
ns
Oocyst Intensity
10.5
5.5
Control
ATQ 100 µM
n=151
n=167
Prev: 77.6%
5.9%
****
Lab-reared, insecticide-susceptible An. gambiae (G3)
Lab-cultured P. falciparum (NF54)
(d)
ns
Oocyst Intensity
7
5
5
Control
ATQ 10 µM
ATQ 1 µM
n=55
n=79
n=86
Prev: 74.0%
34.2%
59.3%
****
ns
Lab-reared, insecticide-susceptible An. gambiae (G3)
Lab-cultured P. falciparum (NF54)

512 **Figure 4: Ingestion of an ATQ-glucose solution prior to *P. falciparum* infection significantly**
513 **reduces transmission. (a)** Experimental scheme. **(b)** AcVK5, collected as pupae from field sites in
514 Bama, Burkina Faso, mosquitoes were significantly less likely to become infected with *P. falciparum*
515 (human gametocyte donor sample) after ingestion of 100 µM ATQ/10% w/v glucose (access to treated
516 sugar 24 h prior to infection). The proportion of AcVK5 mosquitoes infected with *P. falciparum*
517 oocysts at 7 d pIBM was reduced from 64.8% in the control group, to 9.7% in mosquitoes given access
518 to ATQ/sugar solution (85.0% reduction, GLM (Binomial;Logit), n=181, df=1, X^2= 59.242,
519 p<0.0001). While median intensity of infection in mosquitoes harboring ≥1 oocyst was reduced in
520 ATQ-treated mosquitoes, this reduction was not significant. **(c)** Infection with culture adapted NF54
521 *P. falciparum* in insecticide susceptible G3 *An. gambiae* produced remarkably similar results.
522 Ingestion of 100 µM ATQ/0.5% DMSO/10% w/v glucose (access to treated sugar 24 h prior to
523 infection) significantly reduced the prevalence of infection at 7 d pIBM (92.4% reduction Chi^2, n=318,
524 df=1, X^2=162.467, p<0.0001) but had no detectable effect on the median intensity of infection (Mann-
525 Whitney, n=128, df=1, *U*=480.5, p=0.3364). **(d)** Significant, dose-dependent inhibition of oocyst
526 prevalence was observed with a 10-fold reduction in ATQ concentration (10 µM: Chi^2, n=134, df=1,
527 X^2=19.275, p<0.0001). A non-significant reduction in prevalence was also observed at 1 µM (Chi^2,
528 n=141, df=1, X^2=2.642, p=0.1041). Median lines and values are indicated, "n" indicates the number
529 of independent samples. To isolate Oocyst Prevalence and Oocyst Intensity, midgut samples with zero
530 oocysts have been excluded from intensity analysis. Statistical significance is indicated where relevant
531 as follows: ns=not significant, * = p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.

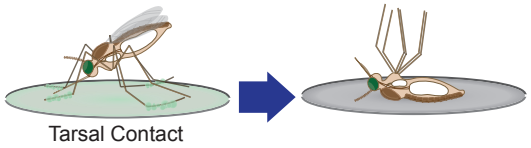
Figure 5


(a)
Infection
Sugar Feeding
Oocyst counts
Sporozoite counts
(b)
7 d pIBM
10 d pIBM
14 d pIBM
Oocyst Size/μm²
374.4
72.0
842.8
92.7
1423.1
337.3
Control
ATQ
n=41
n=28
n=40
n=30
n=37
n=24
(d)
Sporozoite intensity
3900
0
Control
ATQ
n=41
n=45
Prev: 77.8%
0%
(c)
Control bright-field
Control fluorescent
ATQ bright-field
ATQ fluorescent

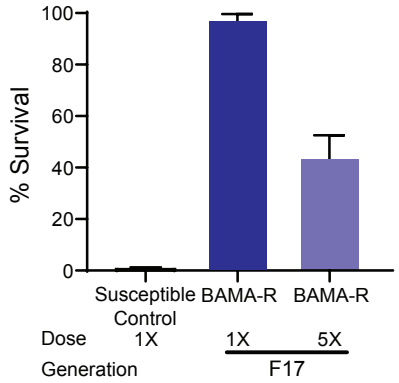
532 **Figure 5: Ingestion of ATQ-glucose after *P. falciparum* (NF54) infection blocks parasite**
533 **development in *An. gambiae* (G3). (a)** Experimental scheme. **(b)** Oocyst size over time. Access to
534 ATQ-glucose from 2 d pIBM caused a significant reduction in mean oocyst cross-sectional area
535 ("size"/m^2) relative to control at 7 d pIBM (2-way T-test, n=69, df=67, t=13.63, p<0.0001), 10 d pIBM
536 (2-way T-test, n=70, df=68, t=8.998, p<0.001), and 14 d pIBM (2-way T-test, n=61, df=59, t=8.793,
537 p<0.0001). Means are indicated, error bars represent the standard error of the mean (SEM). **(c)**
538 Example brightfield, and fluorescent micrographs of control and ATQ-exposed bright-field and DAPI-
539 stained oocysts at 10 d pIBM (outline indicated by dashed line). At this timepoint, the control oocyst
540 is large, and contains many discrete nuclear foci, indicating nuclear division and sporozoite
541 differentiation. In contrast, the ATQ-exposed oocyst is small relative to control and contains a single
542 nuclear focus. Dense hemozoin crystals (white triangle), typically associated with younger oocysts,
543 are visible. Scale bars represent 10 µm, **(d)** Salivary gland sporozoite prevalence and intensity at 14 d
544 pIBM. No sporozoites (0% prevalence, median intensity = 0) were observed in salivary glands samples
545 collected from mosquitoes given access to 100 µM ATQ/0.5% DMSO/10% w/v glucose *ad libitum*
546 (Chi^2, n=86, df=1, X^2=53.202, p<0.0001). Where relevant, median lines and values are indicated, "n"
547 indicates the number of independent samples. To isolate Oocyst/Sporozoite Prevalence and
548 Oocyst/Sporozoite Intensity, midgut samples with zero oocysts have been excluded from intensity
549 analysis. Statistical significance is indicated where relevant as follows: ns=not significant, * = p<0.05,
550 **=p<0.01, ***=p<0.001, ****=p<0.0001.

Supplementary Figure 1

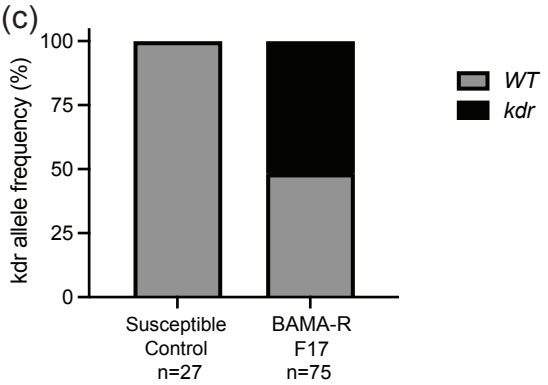
(a)



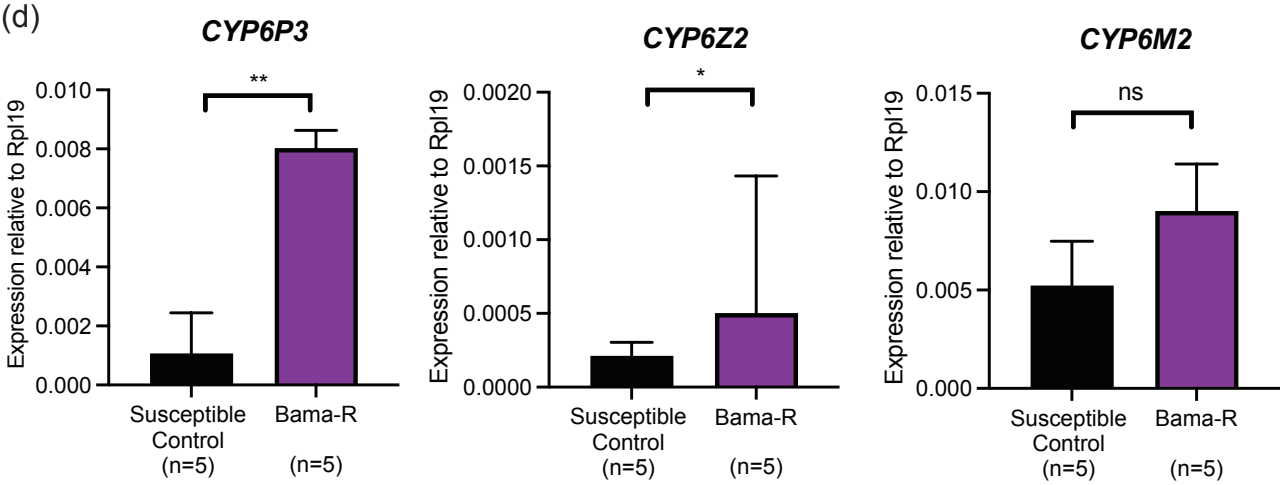
(b)



(c)

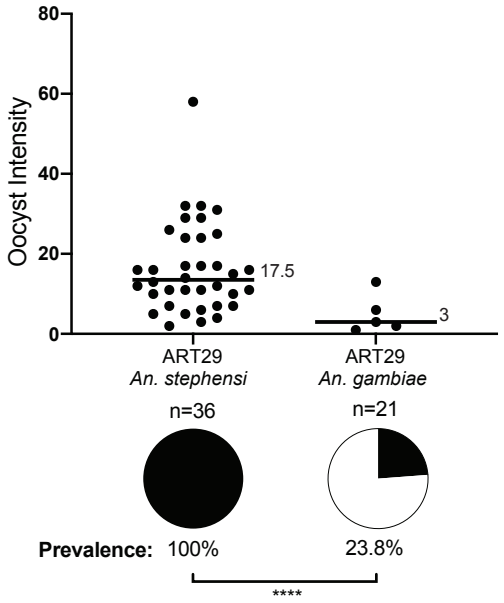


(d)



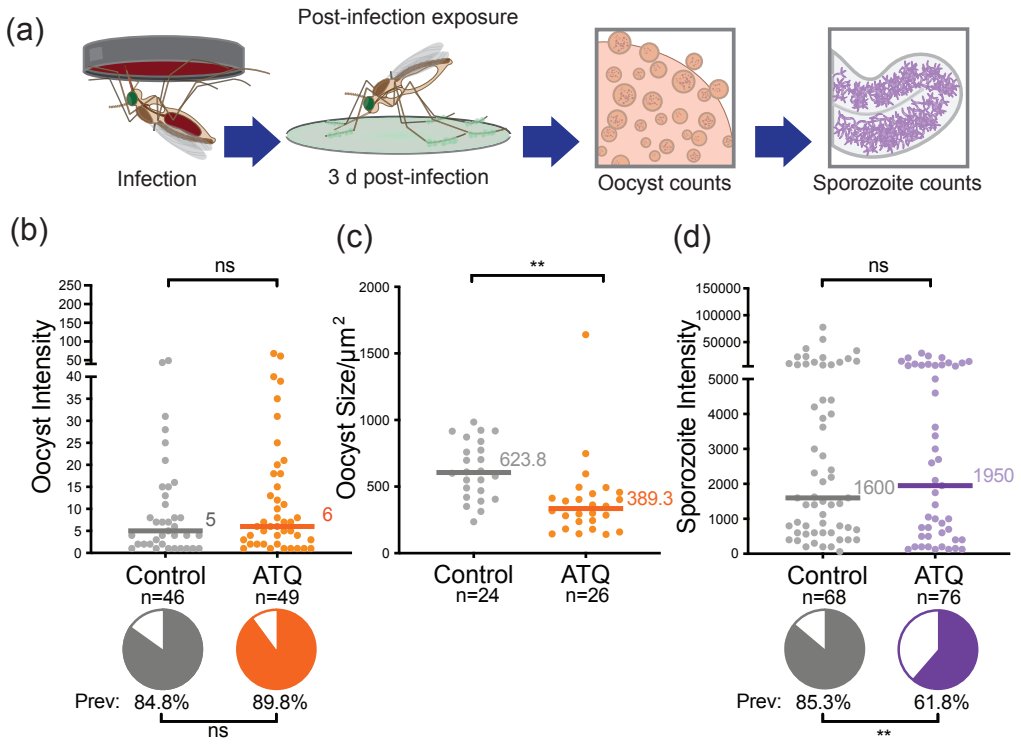
551 **Supplementary Figure 1: *An. gambiae* Bama-R mosquitoes are highly resistant to permethrin.**
552 **(a)** Experimental scheme. **(b)** G3 females were 0% resistant at the DC, while 97% of exposed Bama-
553 R females survived at the same dose, and 43% survived exposure to 5xDC, indicating extreme
554 permethrin resistance. Mean survival ± SEM from 3 replicates is indicated. **(c)** Bama-R mosquitoes
555 are segregating for the *kdr* allele conferring target site resistance to pyrethroids. Allele frequency at
556 generation F17 was 51.7% (n=75), indicating that the observed resistance phenotype is the result of
557 additional modalities. **(d)** qPCR analysis shows that key cytochrome P450 genes associated with
558 metabolic insecticide resistance are constitutively upregulated in Bama-R females compared to a
559 susceptible control (G3). Median expression level normalized to the *Anopheles* housekeeping gene
560 *rpl19* are shown, error bars represent the interquartile range (IQR). Expression levels for *Cyp6P3* and
561 *Cyp6Z2* were significantly elevated compared to a phenotypically susceptible control (*Cyp6P3*, Mann-
562 Whitney, n=10, df=1, U=0, p=0.0079; *Cyp6Z2*, Mann-Whitney, n=10, df=1, U=2, p=0.0317) while
563 *Cyp6M2* was not significantly upregulated. Statistical significance is indicated where relevant as
564 follows: ns=not significant, * = p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.

Supplementary Figure 2



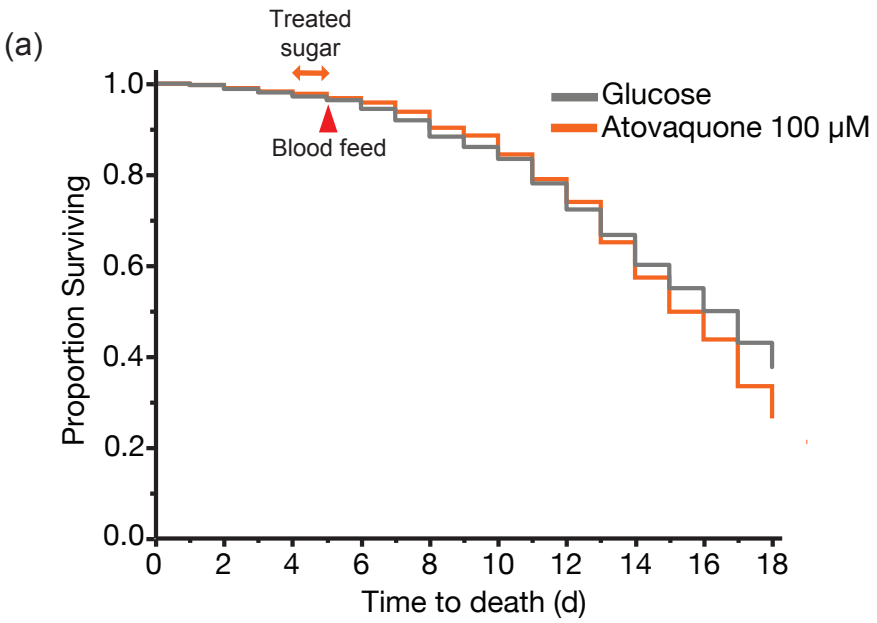
565 **Supplementary Figure 2: Development of the artemisinin-resistant *P. falciparum* parasite**
566 **ART29 in *An. stephensi* and *An. gambiae*.** Female G3 (*An. gambiae*) and Anst (*An. stephensi*) were
567 provided with a blood meal containing mature ART29 gametocytes. Outcome of infection was
568 determined at 7 d pIBM by oocyst count. While ART29 exhibited poor infectivity in *An. gambiae*
569 (23.8% infection prevalence), they established robust infections in *An. stephensi* (100% infection
570 prevalence).

Supplementary Figure 3



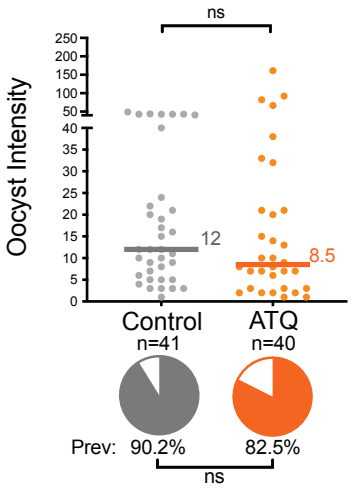
571 **Supplementary Figure 3: The proportion of *An. gambiae* mosquitoes with salivary gland**
572 **sporozoites is significantly reduced after tarsal ATQ exposure at 3 d pIBM. (a)** Experimental
573 scheme. **(b)** There was no effect of 3 d pIBM ATQ exposure on either prevalence (indicated by pie
574 charts) or intensity (indicated by points) of infection determined at 10 d pIBM. Prevalence: Chi^2, n=95,
575 df=1, X^2=0.540, p=0.4623, intensity: Mann-Whitney, n=80, df=1, U=717.5, p=0.4530. **(c)** ATQ
576 exposure at 3 d pIBM significantly reduced the median cross-sectional area of oocysts at 7 d pIBM
577 relative to control (Chi^2, n=50, df=1, *U*=110, p<0.0001). **(d)** The prevalence, but not the median
578 intensity of *P. falciparum* sporozoites in mosquito salivary glands was significantly reduced in
579 mosquitoes exposed to ATQ at 3 d pIBM (Chi^2, n=144, df=1, X^2=9.995, p=0.0016). Median lines and
580 values are indicated, "n" indicates the number of independent samples. To isolate Oocyst/Sporozoite
581 Prevalence and Oocyst/Sporozoite Intensity, midgut samples with zero oocysts have been excluded
582 from intensity analysis. Statistical significance is indicated where relevant as follows: ns=not
583 significant, * = p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.

Supplementary Figure 4



584 **Supplementary Figure 4: AcVK5 survival and infectivity after ingestion of ATQ/glucose. (a)**
585 Survival prior to- and following 24 h access to 100 µM/10% w/v Glucose/0.5 v/v/ DMSO (4 d post
586 emergence, orange arrow), followed by *P. falciparum* (donor blood) infection (5 d post emergence,
587 red arrow). Ingestion of ATQ/glucose had no impact on the survival of AcVK5 mosquitoes relative to
588 a control group provided with 10% w/v glucose/0.5% v/v DMSO (Log-Rank Survival, n=1374, df=1,
589 X^2=1.3795, p=0.2402).

Supplementary Figure 5



590 **Supplementary Figure 5: Ingestion of an ATQ-glucose solution after *P. falciparum* infection has**
591 **no effect on oocyst prevalence or intensity.** There was no difference relative to controls in either
592 intensity (n=68, df=1, U=495.5, p=0.3257) or prevalence (n=81, df=1, X^2=2.441, p=0.1182) of oocysts
593 at 10 d pIBM in females with continued access to 100 µM ATQ/0.5% DMSO/10% w/v glucose from
594 2 d pIBM - 14 d pIBM. Median lines and values are indicated, "n" indicates the number of independent
595 samples. To isolate Oocyst Prevalence and Oocyst Intensity, midgut samples with zero oocysts have
596 been excluded from intensity analysis. Statistical significance is indicated where relevant as follows:
597 ns=not significant, * = p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.

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