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 malaria parasites is contributing to a global resurgence of malaria, and the generation of control tools that can overcome these issues is an urgent public health priority. We recently demonstrated that the transmission of *Plasmodium falciparum* parasites by *Anopheles gambiae* can be efficiently blocked when female mosquitoes contact the antimalarial atovaquone deposited on a treated surface, with no negative consequences on mosquito fitness. Here, we demonstrate that the transmission-blocking efficacy of mosquito-targeted atovaquone is maintained when highly insecticide resistant, field-derived *Anopheles* mosquitoes are exposed to this antimalarial, indicating that insecticide resistance mechanisms do not interfere with drug function. Moreover, this approach prevents transmission of field *P. falciparum* isolates, including an artemisinin resistant *Kelch13* C580Y mutant, demonstrating that this strategy could prevent the spread of parasite mutations that induce resistance to front-line antimalarials. Finally, we show that ingestion of atovaquone in sugar solution is highly effective at limiting parasite development, including in ongoing infections. These data support the use of mosquito-targeted antimalarial interventions to potentiate and extend the efficacy of our best malaria control tools.

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 control is the most effective tool at reducing the transmission of *Plasmodium* parasites, with long-lasting insecticide-impregnated nets (LLINs) and indoor residuals spraying (IRS) as primary tools for malaria prevention. LLINs alone are predicted to have contributed to 68% of malaria cases averted between 2000 and 2015 (2). Alongside mosquito-targeted preventative interventions, artemisinin combination therapies (ACT) have been the cornerstone of human malaria treatment since their widespread introduction at the beginning of this century (3, 4) and have similarly contributed substantially to the reduction in malaria mortality and morbidity observed since then (2).

Despite sizeable investment, malaria control and elimination efforts are, however, faltering due to reduced operational effectiveness of these key control tools, largely caused by mosquito resistance to insecticides and parasite resistance to drugs (5-7). As pyrethroids are the sole class of insecticides currently approved for use as the primary insecticidal ingredient of LLINs, resistance to insecticides from this group, such as permethrin or deltamethrin, has spread rapidly across sub-Saharan Africa. In the malaria hyperendemic regions of southern Mali and southwest Burkina Faso, for example, resistance to pyrethroids is extreme (8), driven by multifactorial and synergistic resistance mechanisms (9) including reduced insecticide sensitivity though mutation in the target enzyme binding pocket, enhanced metabolic detoxification through upregulated cytochrome P450s, and reduced tarsal uptake through cuticular thickening (10-12). Such levels of insecticide resistance are likely one of the key factors contributing to the recent plateau in malaria incidence in Africa following its steady reduction over the preceding years (1).

53 Similarly, resistance to ACTs has emerged in *P. falciparum* parasites in Cambodia and other areas of
54 the Greater Mekong subregion (GMS)(13). Mediated through mutations in *kelch13* (*PfK13*) (14),
55 artemisinin resistance has the primary effect of increasing the treatment time required to clear a patient
56 of *Plasmodium* parasites, with a secondary effect of increasing the likelihood of resistance evolving to
57 artemisinin partner drugs such as piperaquine and lumefantrine (15). The emergence and spread of
58 artemisinin resistance to sub-Saharan Africa — a region where as many as 93% of annual malaria
59 deaths occur (1) — is a major concern. Until recently, resistance to these first-line antimalarials was
60 limited geographically to the GMS, however *de novo PfK13* mutations associated with *in vitro*
61 resistance have now been detected in both Tanzania and Rwanda (16, 17). Of further concern is the
62 recent invasion and spread of the Asian vector *Anopheles stephensi* to the horn of Africa (18), as this
63 mosquito species is highly competent for the transmission of *P. falciparum* parasites endemic to the
64 GMS and invasive populations may facilitate the spread of parasites harboring resistance mutations
65 from Asia to Africa.

66 A control strategy that blocks the transmission of malaria parasites, including those carrying drug-
67 resistant mutations, without imposing strong selective pressure on mosquitoes or parasites could
68 overcome the limitations of current interventions, providing a critical addition to the malaria
69 elimination toolkit. In an effort to generate such a strategy, we recently demonstrated that transmission
70 of *P. falciparum* parasites can be prevented when *Anopheles gambiae* mosquitoes, a major malaria
71 vector in sub-Saharan Africa, are exposed to the antimalarial drug atovaquone (ATQ), a potent
72 cytochrome bc1 inhibitor (19). Exposure through direct contact between the compound and the
73 mosquito tarsi (lower legs) — analogous to the mode of insecticide exposure on LLINs or IRS —
74 completely abrogated parasite development when it occurred around the time of infection (between 24
75 h before a *P. falciparum*-infected blood meal (IBM) and 12 h post IBM). Tarsal exposure to ATQ
76 killed parasites within the midgut lumen prior to ookinete formation and was not associated with any
77 harmful effect on the mosquito. These results demonstrate that, unlike with the use of insecticides,

there would be no selective pressure leading to mosquito resistance to direct antimalarial exposure. Importantly, incorporating these data in a mathematical model of malaria transmission showed that parasite prevalence in the human population in sub-Saharan Africa would be remarkably decreased by the incorporation of antimalarials on LLINs (19). In field settings, however, antimalarials on LLINs or IRS must maintain efficacy when absorbed by insecticide-resistant mosquitoes, as resistance mechanisms may interfere with drug efficacy. Furthermore, they must be active against *P. falciparum* strains circulating in endemic regions, including parasites carrying resistance-conferring mutation. In the latter case, the use of antimalarials on LLINs or IRS could also be exploited to slow or prevent the spread of drug-resistance haplotypes by specifically targeting parasites at a severe bottleneck in the malaria transmission cycle, where typically less than 100 ookinetes escape the mosquito midgut lumen to form oocysts (20-22). It is important to stress that although ATQ could not be responsibly incorporated into a mosquito-targeted intervention due to its use as a human prophylactic and therapeutic drug (23), this compound remains an ideal tool for testing the limits of this strategy, and a crucial gold standard for screening for novel active compounds.

Here we show that *P. falciparum* development is completely blocked when ATQ is delivered, before infection, to a field-derived *An. coluzzii* population that is highly resistant to pyrethroids, suggesting that the drug is not detoxified by mosquito pathways inducing insecticide resistance. ATQ is also fully active against field-derived *P. falciparum* parasites from Burkina Faso. Importantly, mosquito exposure to this drug completely abrogates development—and therefore transmission—of an artemisinin-resistant parasite strain from Cambodia, demonstrating the potential of this method to stop the spread of deleterious parasite mutations. Additionally, ATQ exposure during an ongoing mosquito infection considerably slows down parasite oocyst growth, reducing the probability of onward transmission. Finally, we show that delivering ATQ via sugar solutions causes a striking reduction in both parasite numbers and growth, proving that antimalarials could be incorporated into additional interventions that target outdoor malaria transmission, such as attractive targeted sugar baits (ATSB).

103 Targeting *Plasmodium* parasites in the mosquito vector is therefore a promising strategy that
104 circumvents key limitations of current malaria control and preventative interventions.

105 **Results**

106 **Exposure to ATQ blocks *P. falciparum* development in insecticide resistant *An. coluzzii***

107 To determine whether ATQ can abrogate *P. falciparum* development in insecticide resistant
108 mosquitoes, we tested the Bama-R population of *An. coluzzii* (a sibling species to *An. gambiae*)
109 recently colonized from Burkina Faso (Fig. 1). Bama-R mosquitoes are 100% resistant to permethrin
110 at the WHO discriminating concentration (DC, 696 μmol/m^2) and have appreciable acute survival at
111 five times this dose (SFig. 1), likely due to multiple resistance mechanisms as reported in this region
112 (8, 10). Bama-R females were exposed to either glass plates treated with ATQ at the EC_{99} concentration
113 (100 μmol/m^2, 6 min (19)) or to vehicle-treated plates (control group) immediately preceding infection
114 with *P. falciparum* (NF54), and parasite infection prevalence and intensity were determined at 7 days
115 (d) pIBM. While control females were highly infected, with a median of 12 oocysts per infected midgut
116 and 81.25% overall prevalence of infection, no oocysts were observed in females exposed to ATQ,
117 demonstrating that extant insecticide-resistant mechanisms do not interfere with the antimalarial
118 activity of ATQ (Fig. 1B). These results support the possibility of using mosquito-targeted
119 antimalarials to extend the lifespan of LLINs and IRS, our best malaria control interventions, in regions
120 with widespread insecticide resistance.

121 **Exposure to ATQ abrogates transmission of field-derived *P. falciparum* isolates**

122 We next set out to test the transmission-blocking efficacy of ATQ exposure on a field *P. falciparum*
123 isolate collected from a blood donor (here named P5) from the same region of Burkina Faso where we
124 isolated Bama-R. This isolate (P5) is polyclonal (n=3, determined by PCR genotyping of merozoite
125 surface protein (MSP) 1 (24)), allowing us to test efficacy of treatment against different parasite strains.
126 After a short period in culture, we infected laboratory-reared *An. gambiae* with these parasites and

127 determined that P5 development was completely blocked in female mosquitoes treated with ATQ (Fig.
128 1C) such that zero oocysts were observed in mosquito midguts, compared to heavy infections—both
129 in terms of infection intensity (median 59.5 oocysts per midgut) and infection prevalence (95.7%)—
130 in control mosquitoes (G3 colony). Delivery of ATQ to mosquitoes is therefore fully effective against
131 field-derived *P. falciparum* isolates currently circulating in West Africa.

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

133 As a next step, we determined whether ATQ exposure could prevent transmission of parasite mutations
134 conferring resistance to frontline human therapeutics. To this end, we used a Cambodian *P. falciparum*
135 patient clone (KH001_029 (7), hereafter ART29) carrying the C580Y mutation in *PfK13* conferring
136 resistance to artemisinin (Fig. 2A). We used the major Asian malaria vector *An. stephensi* for these
137 experiments as initial tests with *An. gambiae* did not produce appreciable infections (SFig. 2). ART29
138 generated robust infections in control, mock-exposed *An. stephensi*, (median 16 oocysts per midgut,
139 100% prevalence of infection). However, no oocysts were detected in females exposed to ATQ prior
140 to infection (100 μmol/m^2, 6 min) (Fig. 2B). These data show that mosquito exposure to antimalarials
141 could be an effective strategy for reducing the spread of artemisinin resistance both within and between
142 malaria endemic areas, including sub-Saharan Africa.

143 **ATQ exposure during an ongoing infection delays oocyst growth and decreases sporozoite**
144 **prevalence**

145 In the field, mosquitoes that contact an antimalarial compound through mosquito-targeted
146 interventions may harbor parasites from a previous blood meal that have already traversed the midgut
147 lumen and formed oocysts. We therefore investigated the effects of ATQ on parasites in which oocyst
148 development is already underway, exposing mosquitoes 6d pIBM (Fig. 3A). In contrast to females
149 exposed before infection, ATQ had no effect on the prevalence or intensity of infection, as measured
150 at 10d pIBM (Fig. 3B). However, we did observe a significant, 45% decrease in the mean oocyst cross-

151 sectional area (Fig. 3C). Oocyst size is a good proxy for rate of growth (25) and as such, when we
152 sampled mosquitoes a second time at 14 d pIBM, we observed a 33% reduction in the prevalence of
153 sporozoites in the salivary glands of ATQ-treated females (Fig. 3D). Similar results were obtained
154 when ATQ exposure instead occurred at 3 d pIBM (SFig. 3). Taken together, these results suggest that
155 ATQ exposure after oocyst formation has a transient cytostatic effect on *P. falciparum*.

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

157 Malaria transmission in the presence of universal effective LLIN coverage, referred to as residual
158 transmission, is driven by mosquitoes that exhibit outdoor or day-time biting preferences, and is a
159 considerable hurdle to malaria eradication efforts (26). Increased focus on residual malaria has
160 stimulated interest in the use of attractive targeted sugar baits (ATSBs) to attract and kill adult
161 mosquitoes irrespective of blood-feeding behavior. In field trials, ATSBs have shown some promise
162 as a tool for suppressing vector populations (27, 28). We therefore assessed whether anti-parasitic
163 compounds in sugar could suppress *Plasmodium* transmission in a similar manner to tarsal exposure
164 to antimalarials (19, 29). We gave *An. gambiae* females access to an ATQ-treated sugar solution (100
165 µM ATQ/0.5% v/v DMSO/10% w/v glucose) *ad libitum* for the 24 h preceding *P. falciparum* infection
166 (Fig. 4A). Ingestion of this solution caused a striking 92.4% reduction in parasite prevalence (Fig. 4B).
167 No difference in infection intensity was observed (Control: median = 10.5, ATQ 100 µM median =
168 5.5), although this may be underpowered due to the very low number of infected mosquitoes in the
169 ATQ-treated group. Further 10- and 100-fold ATQ dilutions displayed a progressively reduced, dose
170 dependent effect on the prevalence of infection (Fig. 4C).

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

172 As mosquitoes may often visit an ATSB after acquiring an infectious blood meal, we also investigated
173 the impact of ATQ ingestion on ongoing *P. falciparum* infections, providing ATQ-treated sugar to
174 mosquitoes from 2 d pIBM, when ookinetes have escaped the midgut lumen and formed oocysts on

175 the midgut basal lamina (Fig. 5A). This time, as we expected a possible cytostatic effect on oocyst
176 growth, we performed a sampling time course to capture oocyst growth through mid- to late sporogony
177 (7d, 10d and 14 d pIBM). We also counted salivary gland sporozoites, the end point of parasite
178 development in the mosquito, at 14 d pIBM. In agreement with our results for post-infection exposure
179 through tarsal contact (Fig. 3), we observed no change in oocyst prevalence and intensity as a result of
180 ATQ ingestion (SFig. 4). However, we observed a striking 80.8% decrease in oocyst cross-sectional
181 area relative to controls at 7 d pIBM, which persisted at later time points (89% and 76.3% decreases
182 at 10- and 14 d pIBM, respectively) (Fig 5B). By 14 d pIBM, ATQ-exposed oocysts had a similar size
183 to 7 d pIBM control oocysts, suggesting a remarkable suppression of growth. Inspection of DAPI-
184 stained infected midguts revealed a stark decrease in the number of nuclear foci, with a single, diffuse
185 DNA signal compared to many condensed foci in oocysts on control-exposed midguts (Fig. 5C).
186 Moreover, despite a robust infection in controls, with a median 3,900 salivary gland sporozoites per
187 mosquito and 77.8% prevalence of infection, we detected no sporozoites in the salivary glands of
188 mosquitoes given access to ATQ-glucose (Fig. 5D). Combined, these data point to a strong suppression
189 of oocyst growth, DNA replication and sporozoite differentiation after ATQ ingestion, suggesting this
190 delivery method would be extremely effective at curbing ongoing infections and transmission.

191 **Discussion**

192 In this study we demonstrate the feasibility of incorporating antimalarials into LLINs, IRS and ATSBs
193 to stop parasite transmission by the *Anopheles* female. Our proof of principle compound ATQ was
194 effective at killing a polyclonal, field-derived parasite isolate from sub-Saharan Africa, showing that
195 an antimalarial-based LLIN or IRS could suppress transmission even in areas of malaria
196 hyperendemicity such as Burkina Faso, where parasite haplotype diversity and complexity of infection
197 are high (30, 31). Crucially, complete transmission-blocking activity was maintained in Bama-R
198 mosquitoes that are highly resistant to insecticides. This recently lab-adapted *An. coluzzii* population
199 was established from an area of Western Burkina Faso where pyrethroid resistance is exceptionally

200 high (8) due to a combination of mechanisms that include target site mutations (11), cuticular
201 thickening that reduces insecticide penetration (10) and systemic upregulation of metabolic enzymes
202 (cytochrome P450 mono-oxygenases and glutathione-S-transferases) that detoxify the insecticide (12).
203 Both cuticular and metabolic resistance could potentially interfere with drug function by limiting
204 compound uptake and stability, respectively. Our finding that ATQ abolished *P. falciparum* infection
205 despite high insecticide resistance levels (100% of Bama-R mosquitoes survived insecticide exposure
206 in standard WHO bioassays) demonstrates, in principle, that mosquito antimalarial exposure can
207 bypass currently circulating insecticide resistance mechanisms and remain active even in areas where
208 conventional insecticides have ceased to be effective. As our previous work had shown that ATQ
209 exposure had no detrimental effects on female longevity and reproduction, adding anti-parasitic
210 compounds to LLINs or IRS would not only circumvent the emergence of mosquito resistance, but
211 would also potentiate and extend the lifespan of our current best malaria control tools.

212 Tarsal ATQ exposure also killed artemisinin-resistant *P. falciparum* parasite ART29 in *An. stephensi*.
213 Although these results were somewhat expected given ART29 asexual parasites are ATQ-sensitive *in*
214 *vitro* (32), they are an important proof of concept that drug-based mosquito-targeted interventions
215 could be developed to specifically contain and eliminate parasite haplotypes conferring resistance to
216 human therapeutics. In this strategy, which we define here as human-mosquito combination therapies,
217 transmission of drug resistant parasites surviving treatment in humans would be prevented through
218 mosquito exposure to antimalarials with distinct and separate modes of action, limiting the
219 consequences of resistance mutations on the broader human population. Importantly, mosquito-
220 targeted antimalarials attack the parasite during an extreme population bottleneck and in a non-cyclical
221 stage in its life cycle (20-22), greatly reducing the probability of selection of both extant and *de novo*
222 resistance mutations compared to treatment during the human asexual cycle. Combined with our
223 previous findings that exposure to ATQ does not impair mosquito fitness (19), the deployment of
224 human-mosquito combination therapies could provide an innovative, integrated approach to apply

225 drug pressure to parasites across the entirety of their dual host life cycle and stop malaria transmission
226 in endemic regions where current malaria prevention and treatment interventions are faltering.

227 Ingestion of 100 μM ATQ/glucose before *P. falciparum* infection blocked transmission in similar
228 manner to tarsal contact, albeit incompletely at this concentration (96% total inhibition compared to
229 100% killing achieved with tarsal exposure). That parasite inhibition was incomplete could be due to
230 variability in the volume of sugar imbibed or may be a consequence of slow release of ATQ from the
231 crop, a food storage organ in the mosquito foregut from where sugar is gradually transferred to the
232 midgut for digestion (33). The use of mass spectrometry to determine tissue concentrations of ATQ
233 over time after ingestion or tarsal contact may clarify this issue. These results support the use of
234 antimalarials in ATSB-like strategies to reduce residual malaria transmission carried out by
235 mosquitoes that predominantly rest and feed outside and thereby avoid both LLINs and IRS. This
236 concept was originally introduced in the mid 20th century as a way to screen and identify new human
237 therapeutic or prophylactic antimalarial drugs (34-37) and has been sporadically implemented for this
238 purpose in the decades since (reviewed in (38)). However, leveraging this system to suppress malaria
239 transmission in endemic settings has only been recently proposed (19, 29). While current proposed
240 ATSB designs rely on insecticidal ingredients, the use of antimalarials in sugar baits could act as a
241 more specific and environmentally benign paradigm for this promising intervention.

242 ATQ uptake via tarsal exposure or sugar feeding during oocyst development had an apparent cytostatic
243 effect. A 6-minute tarsal ATQ treatment at 3- or 6-days post infection significantly reduced oocyst
244 growth, resulting in an appreciable reduction in the proportion of salivary gland sporozoite-positive
245 mosquitoes. Taking this effect to its most extreme endpoint, when we provided infected mosquitoes
246 continual access to 100 μM ATQ-glucose throughout sporogony, no sporozoites were observed by 14
247 d pIBM. ATQ targets the Qo site of cytochrome b (39), a key element of the mitochondrial electron
248 transport chain with dual roles in both ATP generation through oxidative phosphorylation and DNA
249 replication through ubiquinone-mediated oxidation of dihydroorotate dehydrogenase (40). Thus,

250 inhibition of either DNA replication or ATP production could explain the cytostatic effect observed
251 here. Consistent with our findings, previous studies have shown that both chemical inhibition of *P.*
252 *vivax* DNA replication during sporogony (37), and disruption of ATP production through knock-out
253 of components of the tricarboxylic acid cycle in *P. falciparum* (41) and mitochondrial electron
254 transport chain in *P. berghei* (42-44), can cause oocyst arrest. Regardless of the inhibitory mechanism,
255 these results demonstrate that *P. falciparum* can be targeted via LLINs, IRS or ATSB throughout their
256 development in the mosquito. This considerably increases the effective window of opportunity for a
257 mosquito-targeted antimalarial intervention, given that even relatively modest increases in oocyst
258 development time can significantly reduce the probability of parasite transmission due to the short
259 mosquito lifespan (25, 45). Furthermore, slowing or preventing oocyst development could interact
260 synergistically with both the transmission blocking effect of pre-infection antimalarial exposure, and
261 with the life-shortening effects of insecticides.

262 Although at an early stage, the concept of human-mosquito combination therapies described here has
263 the potential to be an effective element to drive malaria incidence down. To this end, identifying
264 additional compounds with strong antiparasitic activity during the mosquito stages of *P. falciparum*
265 development will be a crucial next step, and one that should leverage the extensive libraries of known
266 antimalarials. Indeed, one of the key strengths of this approach is the potential to exploit and repurpose
267 compounds that are otherwise unsuitable for human therapeutic use, whether due to toxicity, poor
268 bioavailability, poor kinetics or other limiting factors. By integrating human and mosquito-based
269 interventions, this strategy will extend and protect the efficacy of human therapeutics and mosquito
270 interventions, 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) *An. gambiae* G3 ("G3"), a highly lab-adapted, insecticide-susceptible strain competent for *P. falciparum* of African origin. 2) *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. 3) *An. gambiae s.l.* 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).

P. falciparum strains culture

294 *P. falciparum* strains used in this study were: 1) *P. falciparum* NF54. NF54 is the drug-susceptible
295 standard strain for mosquito transmission studies, obtained from BEI Resources in 2014. This
296 parasite culture was received through a material transfer agreement (MTA) with the laboratory of Dr.
297 Carolina Barillas-Mury. 2) *P. falciparum* P5. P5 is polyclonal (n=3, KMMM, KMKM, RMMM), as
298 determined by MSP1 PCR genotyping following standard procedures (24), and has been culture-
299 adapted from a blood sample donated by a gametocytemic malaria patient in Burkina Faso in 2017 as
300 part of a previous study. This strain was received through an MTA with Thierry Lefèvre, Institut de
301 Recherche pour le Développement (IRD), Burkina Faso. 3) *P. falciparum* KH001_029 "ART29".
302 ART29 is a *P. falciparum* monogenomic parasite isolate obtained from an infected human donor in
303 Cambodia between 2011 and 2013 as part of the TRAC I initiative (7). This parasite carries the
304 *PfK13* mutation C580Y associated with resistance to artemisinin. ART29 has clear phenotypic
305 artemisinin resistance, both as determined by *in vivo* clearance time (11.8 h) and *in vitro* ring-stage
306 survival (22.6%) (46), alongside resistance to other antimalarial drugs (but not ATQ) (32). These
307 parasites generate robust infections in *An. stephensi* mosquitoes, but not *An. gambiae* (SFig. 2).

308 For mosquito infection, females were transferred to a secure malaria infection facility and provided a
309 14-21 d post-induction stage V *P. falciparum* gametocyte culture using a custom blown, water heated
310 glass feeder. Within 24 h of infection, partially engorged or unfed mosquitoes were collected by
311 vacuum aspiration and discarded. To determine oocyst burden, between 7 and 14 d pIBM, infected
312 mosquitoes were collected by vacuum aspiration, and dissected to isolate the midgut. The oocyst
313 burden was determined after staining midguts with 0.2% w/v mercurochrome and examination under
314 a 40x air objective inverted compound light microscope (Olympus, US). For sporozoites, at 14 d
315 pIBM infected mosquitoes were collected by vacuum aspiration and beheaded. The mosquito
316 salivary glands were extracted into RPMI media by applying pressure to the lateral thorax. *P.*
317 *falciparum* sporozoites were isolated by homogenization and centrifugation of salivary gland
318 material, followed by resuspension in a known volume of RPMI. Sporozoites were counted using a

319 disposable haemocytometer under a 20x air objective inverted compound light microscope
320 (Olympus, US). All *P. falciparum* strains were cultured and induced to form gametocytes using
321 standard protocols (47, 48). All strains have been confirmed to be *P. falciparum* by PCR followed by
322 DNA sequencing of the amplified products (49) and have been confirmed free of mycoplasma
323 infection.

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

325 Tarsal exposure plates were prepared as described previously (19). Briefly, for 100 μmol/m^2 plate
326 concentrations, 0.1 ml of this solution of a 0.1% w/v solution of ATQ in acetone was diluted with 1
327 ml additional acetone and spread onto a 6 cm diameter (0.002628 m^2) glass petri dish. For 1
328 mmol/m^2, 1 ml of 0.1% w/v ATQ/acetone solution was added directly to the plate. Plates dried for a
329 minimum of 4 h, with agitation on a lateral shaker at room temperature. For pre-infection exposure,
330 30 min prior to infection, 3-5 d old virgin female mosquitoes were incubated on either ATQ-coated
331 plates, or an acetone treated control, for 6 min. To prevent crowding and agitation, a maximum of 25
332 mosquitoes were exposed per plate, with all exposures occurring in parallel. For post-infection
333 exposure, due to biosafety considerations, compound exposures were carried out in serial, with a
334 maximum of 10 infected mosquitoes per plate.

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

336 For experiments involving compound-treated sugar solutions, 20 mM stock solutions of each active
337 ingredient (AI) were prepared in 100% DMSO. Each 20 mM stock solution was diluted 200-fold in
338 10% w/v glucose to achieve the final working concentration of 100 μM AI/0.5% DMSO/10% w/v
339 glucose. For pre-infection sugar feeding experiments, 2-4 d post-emergence females were denied
340 access to glucose or water for 24 h and then provided access to either the test solution, or a control
341 solution of 0.5% v/v DMSO/10% glucose, for 24 h. After this time had elapsed, all mosquitoes were
342 provided with an infectious *P. falciparum* blood meal as described above. Infected mosquitoes were

343 provided with untreated 10% w/v glucose *ad libitum* for the remainder of the experiment. For post-
344 infection sugar-feeding experiments, 3-5 d post-emergence female mosquitoes were infected with *P.*
345 *falciparum* and denied access to glucose or water for 48 h pIBM. After this time, infected mosquitoes
346 were continuously provided either 100 µM ATQ/0.5% DMSO/10% w/v glucose or 0.5%
347 DMSO/10% w/v glucose as control, *ad libitum.* Sugar feeders were replaced every 48 h for the
348 remainder of the experiment, up to 14 d pIBM.

349 *Statistical analyses*

350 Statistical analyses were carried out using GraphPad Prism v8.4.2 for MacOSX (GraphPad Software
351 Inc., USA) and JMP Pro 14 (SAS Corp. US). For infections, differences in prevalence were analyzed
352 by Chi^2. In experiments where both treatment groups had individuals that produced >0 oocysts,
353 differences in median oocyst burden between groups (intensity of infection) was analysed using a
354 Mann-Whitney Mean Ranks test. For multiple comparisons, differences in prevalence between
355 multiple groups were determined using pairwise Chi^2 corrected for multiple comparisons
356 (Bonferroni). Similarly, multiple comparisons of intensity were carried out using Wilcoxon with
357 Dunn's *post hoc.*

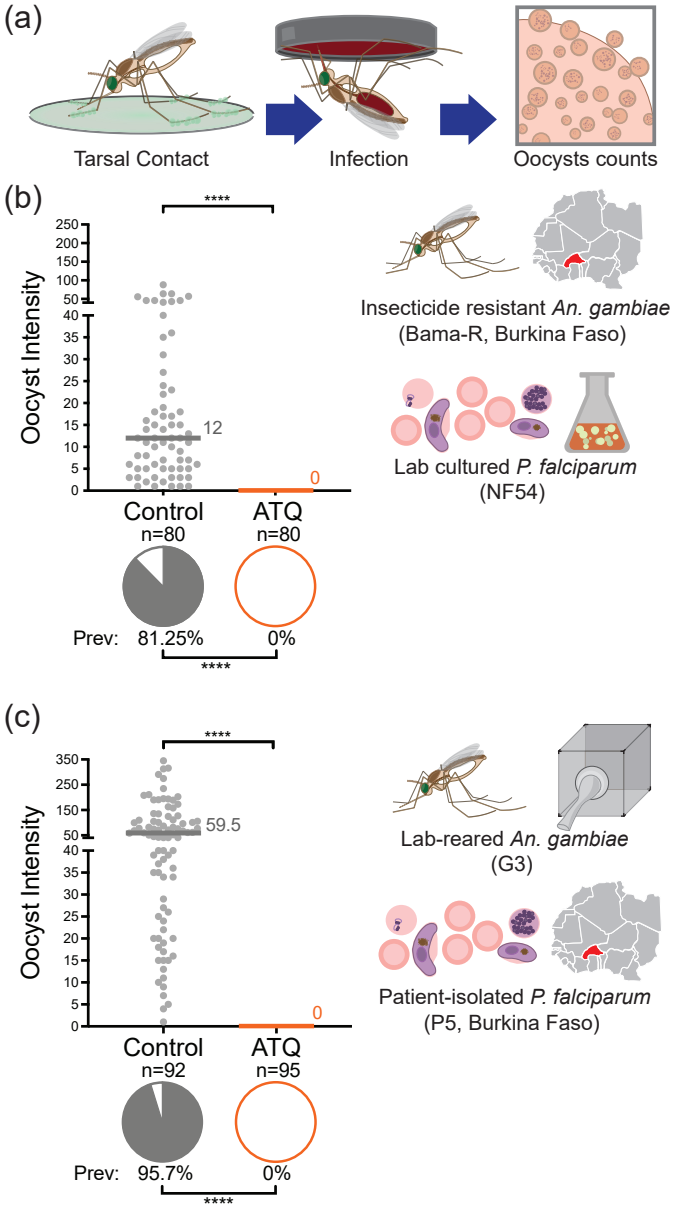
358 **Author Contributions:**

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

364

365 The authors state that they have no conflicting interests.

Figure 1

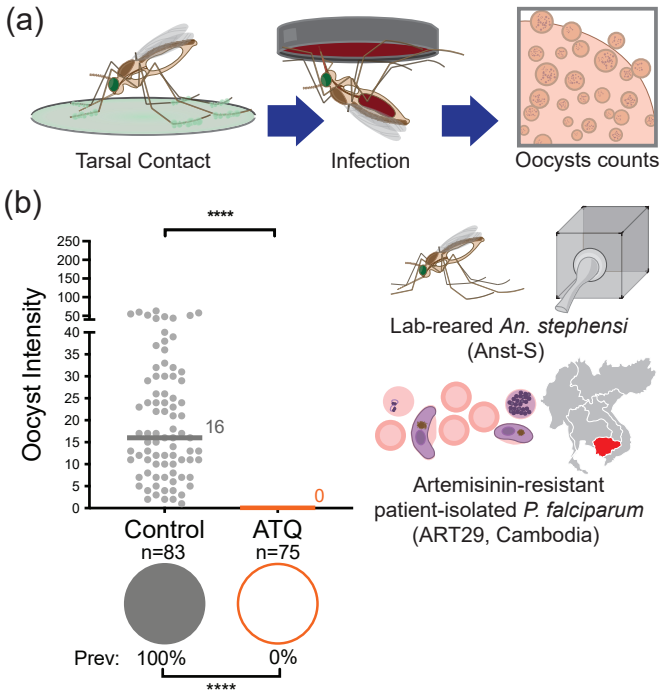

(a)
Tarsal Contact
Infection
Oocysts counts
(b)
Oocyst Intensity
****
12
0
Control
n=80
ATQ
n=80
Prev: 81.25%
0%
****
Insecticide resistant An. gambiae
(Bama-R, Burkina Faso)
Lab cultured P. falciparum
(NF54)
(c)
Oocyst Intensity
****
59.5
0
Control
n=92
ATQ
n=95
Prev: 95.7%
0%
****
Lab-reared An. gambiae
(G3)
Patient-isolated P. falciparum
(P5, Burkina Faso)

366 **Figure 1: Exposing *An. gambiae* mosquitoes to ATQ blocks *P. falciparum* development in field-**
367 **derived parasites and insecticide-resistant mosquitoes. (A)** Experimental scheme. **(B)** Transmission
368 of *P. falciparum* (NF54) is completely blocked when field-derived, highly insecticide-resistant *An.*
369 *gambiae* (Bama-R) are exposed to ATQ for 6 min to the maximal effective concentration EC_{99} for
370 insecticide-susceptible mosquitoes. In ATQ-exposed mosquitoes, prevalence (indicated by pie charts)
371 was zero, in contrast to the robust infection in mock-exposed controls (n=160, df=1, X^2=93.545,
372 p<0.0001). **(C)** Similarly, transmission of a polyclonal *P. falciparum* West African patient isolate (P5)
373 was blocked (zero evidence of infection at 7 d pIBM) after *An. gambiae* G3 mosquitoes took an
374 infectious blood meal, despite a strong (prevalence 95.7%, median intensity 59.5 oocysts per midgut)
375 infection in controls (n=187, df=1, X^2=167.995, p<0.0001). Median lines and values are indicated, "n"
376 indicates the number of independent samples. Statistical significance is indicated where relevant as
377 follows: ns=not significant, * = p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.

378

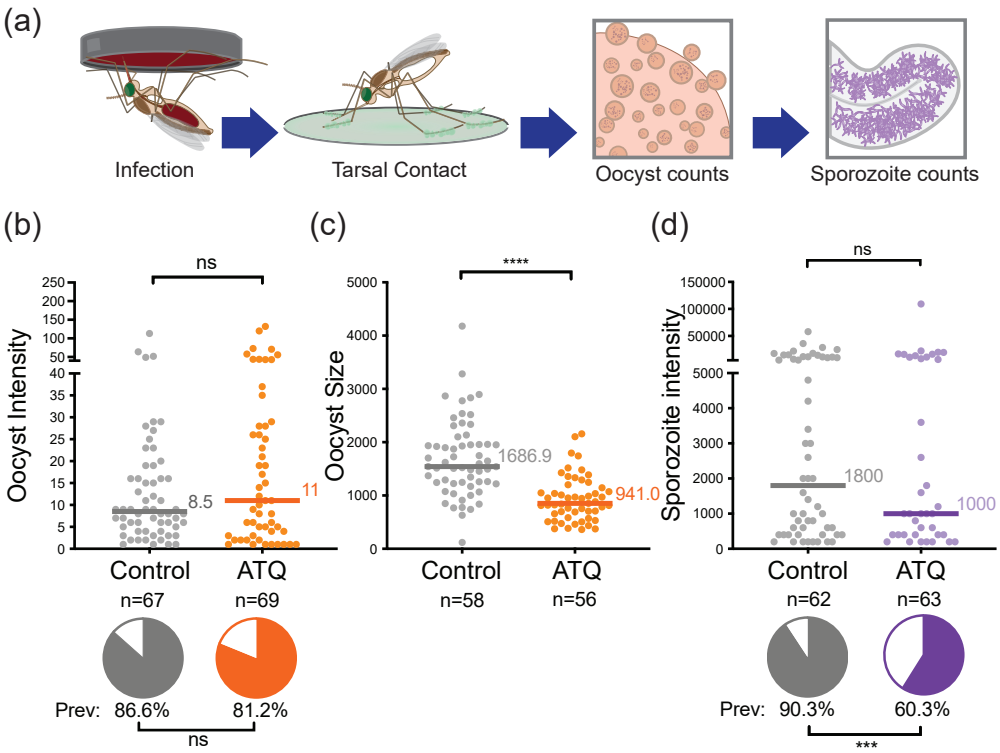
379

Figure 2


(a)
Tarsal Contact
Infection
Oocysts 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
patient-isolated *P. falciparum*
(ART29, Cambodia)

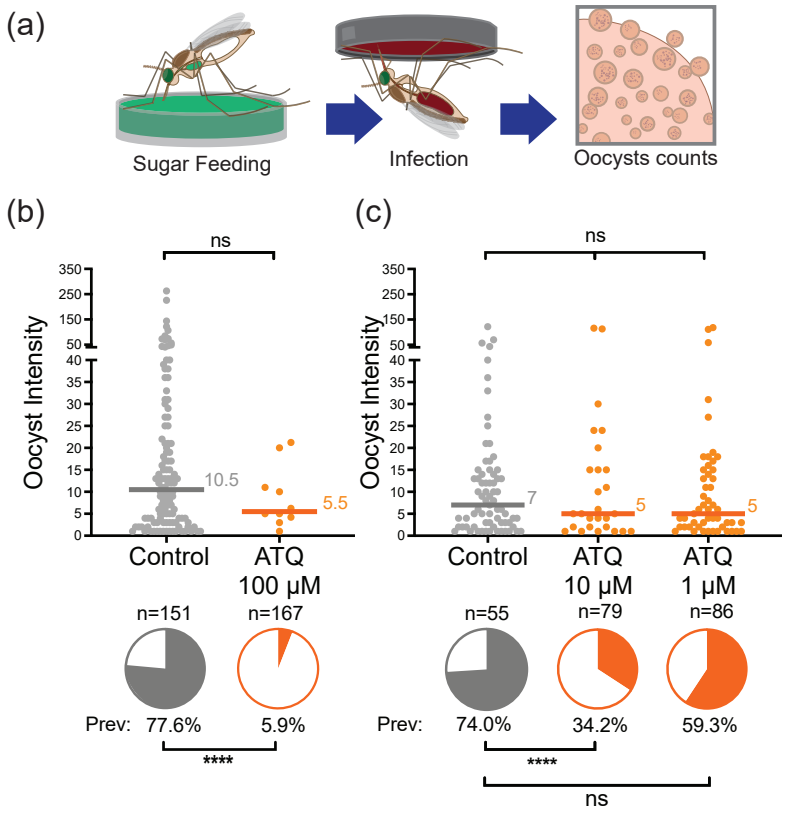
380 **Figure 2: Exposing *An. stephensi* to ATQ blocks an artemisinin-resistant *P. falciparum* patient**
381 **isolate from Cambodia. (A)** Experimental scheme. **(B)** Transmission of artemisinin resistant *P.*
382 *falciparum* (ART29) is completely blocked when *An. stephensi* females (Anst-S) are exposed to ATQ
383 for 6 min to the maximal effective concentration (EC_{99}) for insecticide-susceptible mosquitoes. In
384 ATQ exposed mosquitoes, prevalence (indicated by pie charts) was zero despite robust infection in
385 mock-exposed controls (n=158, df=1, X^2=156, p<0.0001). Median lines and values are indicated, "n"
386 indicates the number of independent samples. Statistical significance is indicated where relevant as
387 follows: ns=not significant, * = p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.

Figure 3


(a)
Infection
Tarsal Contact
Oocyst counts
Sporozoite counts
(b)
Oocyst Intensity
ns
8.5
11
Control
ATQ
n=67
n=69
Prev: 86.6%
81.2%
ns
(c)
Oocyst Size
****
1686.9
941.0
Control
ATQ
n=58
n=56
(d)
Sporozoite intensity
ns
1800
1000
Control
ATQ
n=62
n=63
Prev: 90.3%
60.3%
***

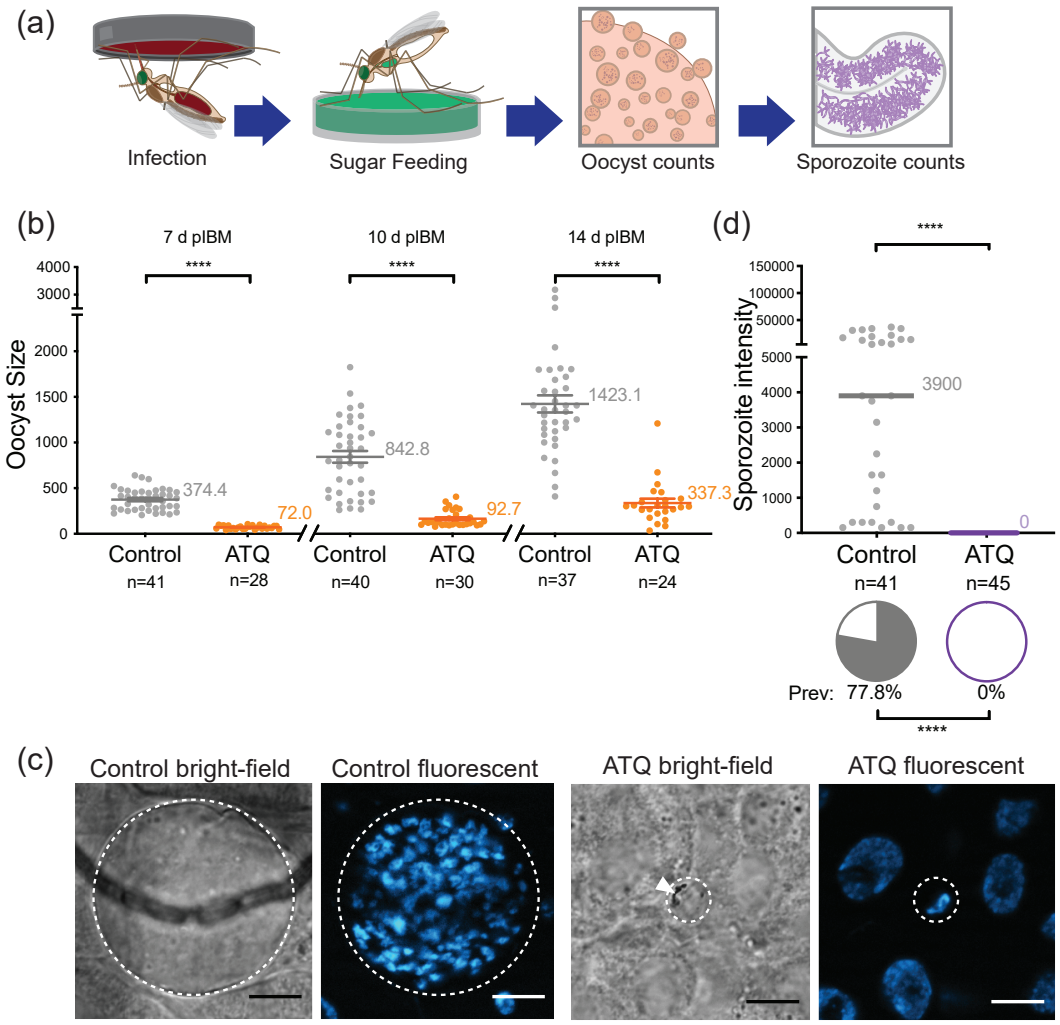
388 **Figure 3: Sporozoite prevalence is significantly reduced after ATQ exposure during oocyst**
389 **development. (A)** Experimental scheme. **(B)** There was no effect of 6 d pIBM ATQ exposure on either
390 prevalence (indicated by pie charts) or intensity (indicated by points) of infection determined at 10 d
391 pIBM. Prevalence: n=136, df=1, X^2=0.733, p=0.3919, intensity: n=114, df=1, U=1482, p=0.4335. **(C)**
392 ATQ exposure at 6 d pIBM significantly reduced the median cross-sectional area of oocysts relative
393 to control (n=114, df=1, U=531, p<0.0001). **(D)** The prevalence, but not the median intensity of
394 sporozoites in salivary glands was significantly reduced in mosquitoes exposed to ATQ at 6 d pIBM
395 (n=125, df=1, X^2=7.190, p=0.0073). Median lines and values are indicated, "n" indicates the number
396 of independent samples. Statistical significance is indicated where relevant as follows: ns=not
397 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
350
250
150
50
40
35
30
25
20
15
10
5
0
10.5
5.5
Control
ATQ
100 µM
n=151
n=167
Prev: 77.6%
5.9%
****
(c)
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

398 **Figure 4: Ingestion of an ATQ-glucose solution prior to *P. falciparum* infection significantly**
399 **reduces transmission. (A)** Experimental scheme. **(B)** Ingestion of 100 µM ATQ/0.5% DMSO/10%
400 w/v glucose significantly reduced the prevalence of infection at 7 d pIBM (n=318, df=1, X^2=162.467,
401 p<0.0001) but had no detectable effect on the median intensity of infection (n=128, df=1, *U*=480.5,
402 p=0.3364). **(C)** Significant, dose-dependent inhibition of oocyst prevalence was observed with a 10-
403 fold reduction in ATQ concentration (10 µM: n=134, df=1, X^2=19.275, p<0.0001). A non-significant
404 reduction in prevalence was also observed at 1 µM (n=141, df=1, X^2=2.642, p=0.1041). Median lines
405 and values are indicated, "n" indicates the number of independent samples. Statistical significance is
406 indicated where relevant as follows: ns=not significant, * = p<0.05, **=p<0.01, ***=p<0.001,
407 ****=p<0.0001.

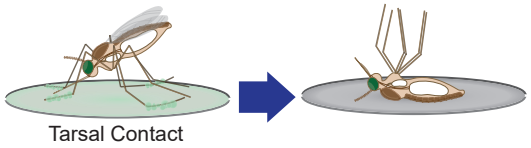
Figure 5


(a)
Infection
Sugar Feeding
Oocyst counts
Sporozoite counts
(b)
7 d pIBM
10 d pIBM
14 d pIBM
Oocyst Size
****
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

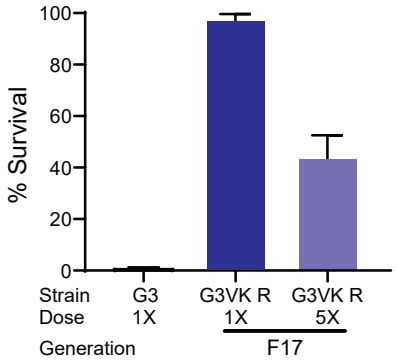
408 **Figure 5: Ingestion of ATQ-glucose after *P. falciparum* infection significantly blocks parasite**
409 **development. (A)** Experimental scheme. **(B)** Oocyst size over time. Access to ATQ-glucose from 2 d
410 pIBM caused a significant reduction in oocyst cross-sectional area ("size") relative to control at 7 d
411 pIBM (n=69, df=1, t=13.63, p<0.0001), 10 d pIBM (n=70, df=1, t=8.998, p<0.001), and 14 d pIBM
412 (n=61, df=1, t=8.793, p<0.0001). Means are indicated, error bars represent the standard error of the
413 mean (SEM). **(C)** Example brightfield, and fluorescent micrographs of control and ATQ-exposed
414 bright-field and DAPI-stained oocysts at 10 d pIBM (outline indicated by dashed line). At this
415 timepoint, the control oocyst is large, and contains many discrete nuclear foci, indicating nuclear
416 division and sporozoite differentiation. In contrast, the ATQ-exposed oocyst is small relative to control
417 and contains a single nuclear focus. Dense hemozoin crystals (white triangle), typically associated with
418 younger oocysts, are visible. Scale bars represent 10 µm, **(D)** Salivary gland sporozoite prevalence and
419 intensity at 14 d pIBM. No sporozoites (0% prevalence, median intensity = 0) were observed in
420 salivary glands samples collected from mosquitoes given access to 100 µM ATQ/0.5% DMSO/10%
421 w/v glucose *ad libitum* (n=86, df=1, X^2=53.202, p<0.0001). Where relevant, median lines and values
422 are indicated, "n" indicates the number of independent samples. Statistical significance is indicated
423 where relevant as follows: ns=not significant, * = p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.

Supplementary Figure 1

(a)

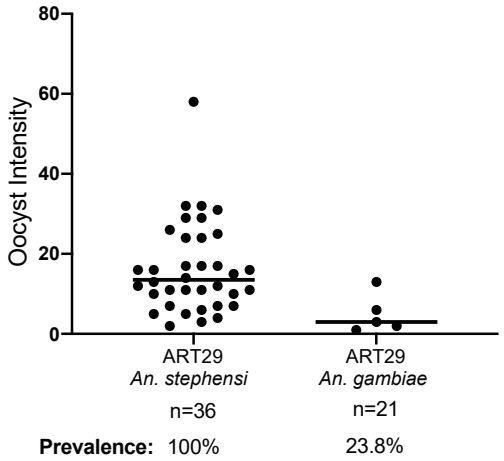


(b)



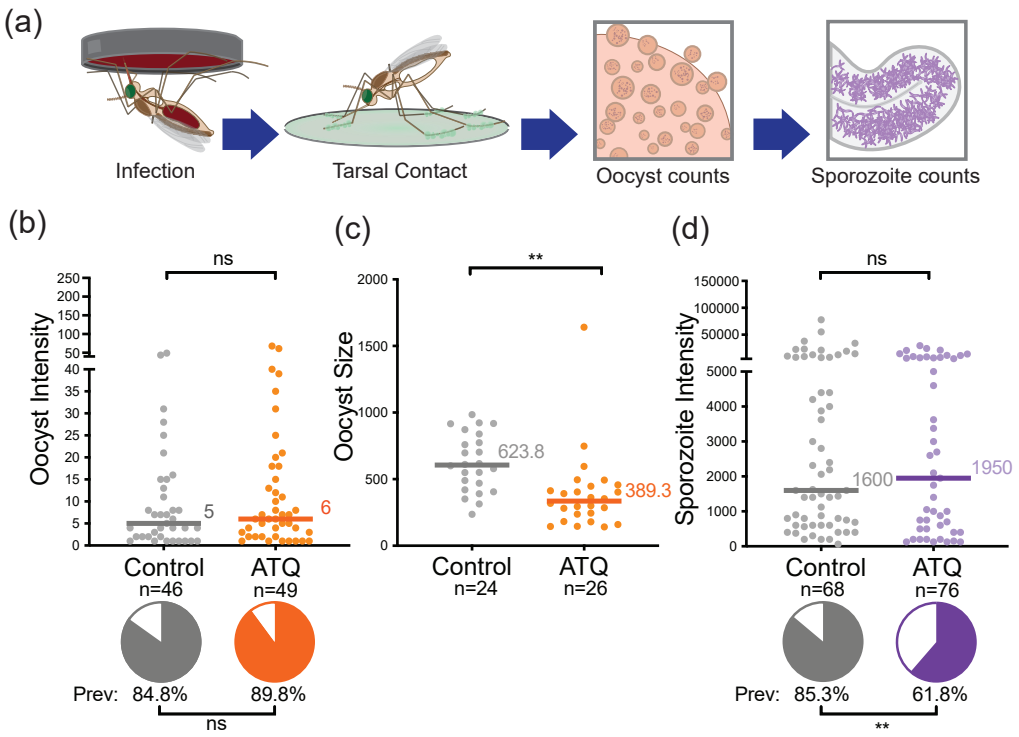
424 **Supplementary Figure 1: *An. gambiae* Bama-R mosquitoes are highly resistant to permethrin.**
425 **(A)** Experimental scheme. **(B)** G3 females were 0% resistant at the DC, while 97% of exposed Bama-
426 R females survived at the same dose, and 43% survived exposure to 5xDC, indicating extreme
427 permethrin resistance. Mean survival ± SEM from 3 replicates is indicated.

Supplementary Figure 2



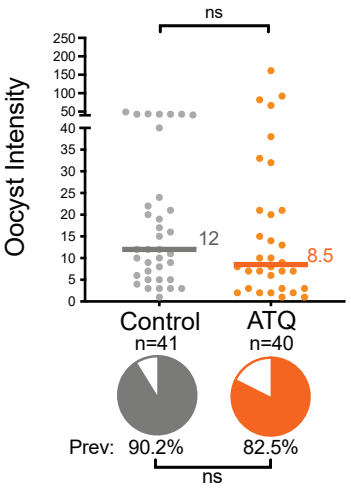
428 **Supplementary Figure 2: Development of the artemisinin-resistant *P. falciparum* parasite**
429 **ART29 in *An. stephensi* and *An. gambiae*.** Female G3 (*An. gambiae*) and Anst (*An. stephensi*) were
430 provided with a blood meal containing mature ART29 gametocytes. Outcome of infection was
431 determined at 7 d pIBM by oocyst count. While ART29 exhibited poor infectivity in *An. gambiae*
432 (23.8% infection prevalence), they established robust infections in *An. stephensi* (100% infection
433 prevalence).

Supplementary Figure 3



434 **Supplementary Figure 3: The proportion of *An. gambiae* mosquitoes with salivary gland**
435 **sporozoites is significantly reduced after mosquito ATQ exposure at 3 d pIBM. (A)** Experimental
436 scheme. **(B)** There was no effect of 3 d pIBM ATQ exposure on either prevalence (indicated by pie
437 charts) or intensity (indicated by points) of infection determined at 10 d pIBM. Prevalence: n=95, df=1,
438 X^2=0.540, p=0.4623, intensity: n=80, df=1, U=717.5, p=0.4530. **(C)** ATQ exposure at 3 d pIBM
439 significantly reduced the median cross-sectional area of oocysts at 7 d pIBM relative to control (n=50,
440 df=1, *U*=110, p<0.0001). **(D)** The prevalence, but not the median intensity of *P. falciparum* sporozoites
441 in mosquito salivary glands was significantly reduced in mosquitoes exposed to ATQ at 3 d pIBM
442 (n=144, df=1, X^2=9.995, p=0.0016). Median lines and values are indicated, "n" indicates the number
443 of independent samples. Statistical significance is indicated where relevant as follows: ns=not
444 significant, * = p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.

Supplementary Figure 4



445 **Supplementary Figure 4: Ingestion of an ATQ-glucose solution after *P. falciparum* infection has**
446 **no effect on oocyst prevalence or intensity.** There was no difference relative to controls in either
447 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
448 at 10 d pIBM in females with continued access to 100 µM ATQ/0.5% DMSO/10% w/v glucose from
449 2 d pIBM - 14 d pIBM. Median lines and values are indicated, "n" indicates the number of independent
450 samples. Statistical significance is indicated where relevant as follows: ns=not significant, * = p<0.05,
451 **=p<0.01, ***=p<0.001, ****=p<0.0001.

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