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Exposing *Anopheles* mosquitoes to antimalarials blocks *Plasmodium* parasite transmission

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Abstract

Every year the bites of *Anopheles* mosquitoes kill hundreds of thousands of people, mostly young African children, by transmitting deadly *Plasmodium falciparum* malaria parasites. Since the turn of the century, efforts to prevent transmission of these parasites via the mass distribution of insecticide-treated bed nets have been extremely successful, causing an unprecedented reduction in malaria deaths[1]. However, resistance to insecticides has become widespread in *Anopheles* populations[2–4], threatening a global resurgence of the disease and making the generation of effective new malaria control tools an urgent public health priority. Here, we show that development of *P. falciparum* can be rapidly and completely blocked when *Anopheles gambiae* females uptake low concentrations of specific antimalarials from treated surfaces, simulating contact with a bed net. Mosquito exposure to atovaquone prior to or shortly after *P. falciparum* infection causes full parasite arrest in the female midgut, preventing transmission of infection. Similar transmission-blocking effects are achieved with other cytochrome B inhibitors, demonstrating that parasite mitochondrial function is a good target for parasite killing. Incorporating these effects into a model of malaria transmission dynamics predicts that the inclusion of *Plasmodium* inhibitors on mosquito nets would significantly mitigate the global health impact of insecticide resistance. This study identifies a powerful new strategy for blocking *Plasmodium* transmission by *Anopheles* females, with promising implications for malaria eradication efforts.

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Author Contributions

DGP carried out and analyzed infection and fitness experiments. MAI carried out immunofluorescent assays. LMC and IEH generated code and carried out mathematical modeling. COB and FC supervised the study.

Significant strides have been made in malaria control since the introduction of insecticide-based strategies targeting the *Anopheles* mosquito species that transmit *Plasmodium* parasites. Long-lasting insecticide treated bed nets (LLINs) alone are predicted to be responsible for 68% of all malaria cases averted since the beginning of the 21st century, and together with indoor residual insecticide spraying (IRS) of house walls represent a cornerstone of malaria control efforts[1]. The pervasive use of these strategies has, however, caused an alarming spread of resistance to insecticides in all major *Anopheles* populations in malaria-endemic countries[2–5]. Containment and management of this issue has been undermined by the lack of approved active ingredients for LLINs, which, until recently[6–8], were limited to pyrethroids. Undoubtedly, the rapid decline in insecticide efficacy constitutes a pressing public health emergency threatening to roll back much of the progress made towards eliminating malaria since the introduction of LLINs. Indeed, after a period of steady decline in annual clinical cases, sub-Saharan Africa and other geographies have experienced a plateau or even an increase in malaria incidence[9]. As many countries move towards a focus on not just malaria control but elimination, it is imperative that more and improved tools to stop parasite transmission by the *Anopheles* mosquito are generated.

Besides LLINs and IRS, malaria control strategies heavily rely on drugs to cure *Plasmodium* infections in humans, the current gold standard treatment being the use of artemisinin-based combination therapy (ACT)[9]. We reasoned that it may be possible to use antimalarial compounds to also clear *Plasmodium* infections directly in the *Anopheles* mosquito, employing delivery methods equivalent to mosquito contact with insecticides on a bed net or wall. This rationale exploits the fact that generally fewer than 100 *P. falciparum* ookinetes successfully cross the midgut epithelium to form oocysts, representing a significant bottleneck to transmission. To test this approach, we coated a glass substrate with the potent parasite cytochrome B inhibitor atovaquone (ATQ) and allowed *An. gambiae* females to rest on this surface immediately prior to *P. falciparum* infection. This tarsal exposure (i.e. via the mosquito legs) is based on a modified WHO insecticide assay[10] which simulates how mosquitoes uptake insecticides on LLINs and IRS. Due to its highly lipophilic nature, we hypothesized that ATQ would be capable of traversing the insect cuticle, killing the parasite during sporogony. Strikingly, no *P. falciparum* oocysts were detected in ATQ-treated females (1 mmol/m^2 for 60 minutes) at 7 days post an infectious blood meal (pIBM), while control, mock-exposed individuals showed high infection prevalence and intensity (Fig. 1a). To characterize the protective effect of ATQ, we performed a dilution series of exposures, and observed complete blockade of *P. falciparum* development using a tenfold lower ATQ concentration (100 μmol/m^2), while at as low as 10 μmol/m^2 we still found significant inhibition of infection prevalence (87.6% inhibition) and intensity (87.5% inhibition) (Fig. 1b). Further ATQ dilutions had a progressively reduced, dose-dependent inhibitory effect (Fig. 1b). By interpolating these data onto a dose-response curve we calculated the IC_{50} of ATQ exposure as a surface concentration of 1.77 μmol/m^2 (Fig. 1c). This is comparable to the LC_{50} of the potent neurotoxic LLIN insecticide permethrin in susceptible *An. gambiae* (63 μmol/m^2 for a 60-minute tarsal exposure[11]).

Importantly, killing of *P. falciparum* parasites was similarly effective when exposure time was reduced to 6 minutes (Fig. 2a), indicating that transmission-blocking doses of ATQ are taken up across the insect cuticle within a short timeframe that is compatible with reported

contact times for host-seeking mosquitoes on LLINs[12]. Parasites were killed at the early zygote-ookinete transition, as determined in immunofluorescent assays (IFAs) of infected midguts (Fig. 2b). These data are consistent with previous studies showing that ookinetes are arrested when mosquitoes feed on *P. berghei*-infected mice injected with ATQ[13] or when parasites are cultured *in vitro* in the presence of this antimalarial drug[14]. Parasite development was also completely aborted when mosquitoes were exposed to ATQ either 24 hours prior to or 12 hours after infection (Fig. 2c, d). These findings indicate that ATQ-like antimalarials could be incorporated into other control interventions beside treated nets, including attractive toxic sugar baits where females become exposed to chemicals while sugar feeding[15], or IRS where contact occurs while females are resting before or after blood feeding. Notably, ATQ exposure had no fitness costs to the mosquito in terms of survival and reproductive output (Extended Data Fig. 1).

We next incorporated these results into a mathematical model of malaria transmission that includes mosquito population dynamics and human malaria infection[16] (Extended Data Fig. 2). The effects of the introduction of either conventional insecticide-treated nets, or nets combining insecticides with a compound that has ATQ-like properties, were modeled in populations with varying malaria transmission intensity (20–80% human infection prevalence), at varying intervention coverage (0–100%) and insecticide resistance levels in the vector population (0–100%). We assumed 100% blocking of new *Plasmodium* infections on the same day of exposure to the antimalarial compound, and no effects on ongoing infections. In the presence of insecticide resistance, the application of ATQ-like compounds to nets is always predicted to reduce malaria prevalence, with the extent of this reduction being dependent on the level of malaria transmission, the coverage, and the degree of insecticide resistance (Extended Data Fig. 3). ATQ-like compounds significantly increased the effectiveness of the control intervention under a broad range of scenarios, facilitating malaria suppression across transmission settings relevant in Africa and in other malaria-endemic regions (Fig. 3a, Extended Data Fig. 4). Moreover, we modeled 567 specific locations in West and East Africa for which recent (2013–2018) insecticide resistance data is available[17], incorporating estimates of *P. falciparum* prevalence and LLIN coverage from 2015[1]. Consistent with our other model outputs, these data predict that adding an ATQ-like compound to LLINs would appreciably reduce *P. falciparum* prevalence in these areas (Fig. 3b). Incorporation of ATQ-like compounds would therefore markedly expand the lifespan of insecticide-based strategies, a factor particularly important in transmission hot spots where resistance to pyrethroids is nearly total[2].

ATQ acts by displacing ubiquinone (CoQ) from the Q^O site of complex III (Cytochrome bc_1) of the mitochondrial electron transport chain (mtETC), disrupting the mitochondrial membrane potential ($\Delta\Psi m$) and thus inhibiting both mitochondrial ATP production[18] and *de novo* pyrimidine synthesis[19]. We next tested additional compounds with anti-cytochrome B activity not in clinical use, but with potential to be rapidly adapted for use in mosquito-targeting interventions: the registered insecticides acequinocyl (ACE) and hydramethylnon (HYD), and the veterinary drug decoquinate (DEC). We also included the dihydrofolate reductase inhibitor pyrimethamine (PYR)[20], a compound with extremely potent transmission-blocking activity in humans that acts by disrupting parasite DNA replication (Extended Data Table 1). All these compounds have nanomolar activity against *P. falciparum*

asexual stages *in vitro*[20–22], and they lack acute insecticidal activity against *An. gambiae*, as determined in our experiments (Extended Data Fig. 5a). Remarkably, HYD and ACE showed strong *P. falciparum* killing activity, reducing oocyst prevalence by 63.9% and 64.3%, respectively, relative to controls (Fig. 4). DEC and PYR on the other hand had no detectable effect on infection (Extended Data Fig. 5b), possibly due to higher polar surface area relative to ATQ, ACE and HYD - and insecticides used in LLINs - which may negatively affect uptake (Extended Data Table 1).

ATQ is used extensively (in combination with proguanil) for prophylaxis in travelers to malaria-endemic areas, and as a stopgap therapy in the case of treatment failure with ACT and other therapies. A handful of mutations conferring resistance to ATQ in the *P. falciparum* erythrocytic cycle have been shown to cause parasite arrest early in mosquito infection[23], although partial transmissibility has been observed in some ATQ-resistant *P. berghei* parasites[23,24]. Similar developmental arrest during mosquito stages is associated with functional knockout of other components of the mtETC - including Type II NADH dehydrogenase[25], succinate dehydrogenase (complex II)[26] and ATP-synthase (complex V)[27] - which are non-essential for asexual growth. These observations demonstrate the critical role for the mtETC during *P. falciparum* development within the *Anopheles* female, making mitochondrial function an attractive target for parasite killing. The use of ATQ in mosquito-targeting interventions may, however, not be advisable as mutations conferring resistance to ATQ could nevertheless arise in the event of its widespread incorporation on LLINs, compromising its efficacy as a human therapeutic. The identification of additional effective compounds that can kill mosquito stages of *P. falciparum* using different modes of action is therefore a priority area of future research. In contrast, mosquito resistance to parasite inhibitors would be unlikely given the lack of observed fitness costs associated with compound exposure in the *An. gambiae* female, either in terms of fecundity or survival.

Our study demonstrates the vast potential of anti-parasitic compounds for malaria control methods aimed at the *Anopheles* vector, and greatly expands the library of compounds that can be considered for use on bed nets and other mosquito control interventions such as IRS and attractive toxic sugar baits. It is important to note that there is a significant gap between the proof-of-concept demonstrated here and implementation of a field-ready product. Indeed, while we were able to demonstrate that exposure to ATQ deposited on a net substrate was also able to completely block infection (Extended Data Fig. 6), many additional parameters including compound toxicity, formulation, cost and stability will need to be determined before this strategy can be deployed. Critically, once these hurdles are overcome, the use of *Plasmodium* inhibitors on LLINs or IRS could be rapidly integrated into the extensive manufacturing and distribution pipelines already in operation in all malaria-endemic regions, thereby providing an effective and safe tool to accelerate the drive toward malaria elimination.

Materials and Methods

Insect lines and rearing

Mosquitoes used for this study were *Anopheles gambiae sensu stricto*, G3 strain. Adult and larval mosquitoes were maintained in a purpose-built insectary at 27°C ± 2 and 80% RH.

Larvae were reared from hatching in 1L ddH_2O using an optimized density and feeding regimen. Pupae were collected and placed in cages (Bugdorm™, Megaview Science. Co. Taiwan), and after eclosure adult mosquitoes were provided water and 10% w/v glucose solution *ad libitum*. For colony maintenance, 5–7 d old adults were provided a blood meal of donated human blood using an artificial membrane feeding system (Hemotek Limited, Great Harwood, UK).

Compound Exposures

Compounds (ATQ, ACE, HYD, PYR, PER, Millipore-Sigma, St. Louis, MO, USA) were dissolved in a suitable, volatile vehicle (Extended Data Table 1) at stock concentrations of 3–10 mg/ml (0.3–1% w/v). Working concentrations of each compound were created through serial dilutions. To generate a compound-coated surface, a volume of working solution containing a known quantity of compound was added to 1 ml excess vehicle and transferred to a 6 cm diameter glass petri dish (0.0283m^2). Treated dishes were placed on a lateral shaker and left for 4 h or overnight until evaporation of the volatile vehicle, coating the compound to the glass substrate. Control plates were treated identically using only the vehicle. A translucent plastic cup was placed over the coated surface to contain mosquitoes during exposure. A flap was cut into the base of the cup to allow the introduction of mosquitoes. Plates were used for 1 d and discarded. For exposures, 15–25 mosquitoes were introduced through the cup flap using a mouth aspirator (J. W. Hock & Co., Florida, USA) and incubated on the treated surface for 6–60 min depending on the experimental parameters. Exposure plates were agitated once during exposure to discourage resting on the untreated walls and base of the cup. After exposure, mosquitoes were transferred to a clean 17.5 cm^3 cage (Bugdorm™, Megaview Science. Co. Taiwan). For net exposures, 10 × 10 cm squares of 100 denier, polyester netting were dipped in a 0.5 mg/ml solution of ATQ in acetone - or acetone alone - and allowed to air-dry for 10 min. Mosquito exposure was carried out as described above.

Plasmodium falciparum Infection Assays

Infections were carried out using the NF54 *P. falciparum* cell line provided by Carolina Barillas-Mury at the NIH via a BSL-II MTA from BEI Resources. The original source of this cell line is BEI Resources (MRA-1000). The parasite line was authenticated using a nested PCR protocol that uses primers specific for *P. falciparum*. Our NF54 cultures were confirmed to be free of any mycoplasma contamination. *An. gambiae* females (5-d old) were exposed to compounds as described above. Immediately after exposure, females were transferred to a sealed, secure infection glovebox and provided an *in vitro* culture of *P. falciparum* (NF54) gametocytes[28,29] through a custom made, glass, water-heated membrane feeder. After 60 min, females that failed to engorge fully were vacuum aspirated out of their containers directly into 80% ethanol, and discarded. At 7–9 d pIBM, females that had blood fed were vacuum aspirated into 80% ethanol, incubated for 10 minutes at −20°C, and transferred out of the secure feeding box into PBS on ice. Midguts were dissected out in PBS and stained with 0.2% w/v mercurochrome (in ddH_2O) for 17 minutes. After staining, midguts were mounted on glass microscope slides in 0.02% w/v mercurochrome, and oocyst prevalence and intensity were determined by examination at 40x air objective on an inverted compound light microscope (Olympus Corporation, Waltham, MA).

Ookinete Immunofluorescent Staining

21 hr pIBM females (either ATQ- or mock-exposed) were aspirated into PBS at 4°C, beheaded, and transferred to a dissecting microscope. Female midguts including the blood bolus were isolated and transferred to 20 μl PBS on ice. Guts were disrupted by repeated pipetting and the crude isolate homogenized by vortexing briefly (~5 s). 10μl of the homogenate was spotted onto a poly-L-lysine-coated slide and air-dried. Once dry, the tissues were fixed by incubation with 4% paraformaldehyde (PFA) for 15 minutes. Slides were then rinsed with 0.05% w/v BSA in PBS and stained with a mouse antibody raised against the *P. falciparum* surface protein PfS25 (BEI Resources, Manassas VA, USA). Secondary staining was carried out with a FITC-donkey-anti-mouse antibody (ThermoFisher Scientific, Waltham MA, USA). After staining and rinsing, tissues were mounted in Vectashield™ with DAPI (Vector Laboratories, Burlingame CA, USA) and examined under oil at 63x magnification using a Zeiss Observer.Z1 inverted fluorescent microscope (Carl Zeiss Microscopy GmbH, Jena, Germany).

Survival Assays

To assess acute survival following exposure, 40 females were exposed (as described above) to ATQ, PYR, ACE, HYD, and PER for 1 h at a dosage of 1 mmol/m^2 for each compound. Each exposure had an independent negative control of 40 mock exposed females. Immediately after exposure, each treatment group was transferred to a 500 ml paper cup and provided with glucose. At 48 h post exposure the proportion of surviving mosquitoes in each group was determined. Differences in survival between control and compound-exposed mosquitoes were detected using Chi^2 analysis. For long-term survival, ~100 ATQ- or control-exposed females were placed in clean 17.5 cm^3 cages (Bugdorm™, Megaview Science. Co., Taiwan). Water and a 10% w/v glucose solution were provided *ad libitum.* Cages were checked daily for mortality, and dead mosquitos were removed and counted. Each experiment continued until all mosquitoes had died. Differences in median time-to-death between treatment groups were analyzed using a Log-Rank Mantell-Cox test.

Egg development assay

Females (5-day old) were exposed to ATQ at 1 mmol/m^2 for 60 minutes and provided with an infectious blood meal as described above. Gravid females were collected at 3 d pIBM and the ovaries dissected out in 1x PBS. Developed eggs were liberated from the ovarian tissue by gentle agitation with a fine dissection needle and counted.

Modeling

We built upon a discrete time model of the mosquito life cycle and malaria transmission[16]. We used a simple model in order to distinguish the qualitative impact on transmission of adding ATQ to bed nets in the presence of insecticide resistance, in comparison with standard approaches. For clarity we use a single, well-mixed mosquito population without spatial structure, waning of efficacy of interventions, seasonality, or outdoor biting behavior. Briefly, mosquitoes progressed through egg, larval, and adult stages, which included four-day gonotrophic cycles (feeding, two days of resting, laying), with a time step of one day (Extended Data Fig. 2a). Malaria transmission was incorporated through a simple

Susceptible-Infectious-Susceptible (SIS) framework for human malaria infection. Modifications from the model[17] included (i) the possibility for exposure in every feeding compartment, (ii) a revised formulation of age-dependent adult daily mortality, and (iii) updated computations of the mosquito-human transmission risk functions (β_M and β_H, Extended Data Fig. 2). All simulations were carried out using Matlab 2016a.

During every feeding, mosquitoes could be exposed to insecticide, alone or with ATQ. Insecticide caused the death of a fraction of the feeding population, determined by insecticide resistance (the fraction of mosquitoes that are impervious to insecticide) and coverage level. In the case of no insecticide resistance, all mosquitoes that encountered insecticide along with ATQ were killed by the insecticide. We assumed no lasting effects of insecticide and that mosquito survival after an initial insecticide exposure was not correlated with survival after additional exposures. ATQ induced full refractoriness to *Plasmodium falciparum* infection, i.e. 100% protection against infection on the same day of exposure.

Age-dependent mortality for adult mosquitoes was determined by a Gompertz distribution[30] (Extended Data Fig. 1a) with scale parameter $b = 0.1868$ and shape parameter $\eta = 0.0293$ (Extended Data Fig. 2b), such that the survival function, i.e. one minus the cumulative distribution function, was $\boldsymbol{S(x) = \exp\left(\eta(1 - e^{bx})\right)}$

The daily risk of a human becoming infected was computed using $\boldsymbol{\beta_H(t) = 1 - (1 - b)^{af(t)}}$ where $b = 0.55$ is the probability of infection given a bite from an infectious mosquito[31], a is the bites per human per mosquito, and $f(t)$ is the number of infectious feeders on day t (Extended Data Fig. 2c(i)). We fitted the biting rate a to give the desired transmission setting in the absence of intervention assuming a larval carrying capacity of $K = 5\times10^5$ mosquitoes and a human recovery rate of *25* days. We found $a = 1.1\times10^{-4}$ in moderate transmission (45% human infection prevalence (HIP)). For other transmission settings, we find a = 4.2×10^{-5} at 20% HIP, a = 6.4×10^{-5} at 30% HIP, a = 9.2×10^{-4} at 40% HIP, a=1.34×10^{-4} at 50% HIP, a=1.96×10^{-4} at 60% HIP, a = 3.04×10^{-4} at 70% HIP, and a = 5.32×10^{-4} at 80% HIP.

The daily risk of a mosquito becoming infected was computed with $\boldsymbol{\beta_M(t) = k_2\left(1 - k_1 / k_2 I_H(t) + k_1\right)}$. We restricted β_M to be between 0 and 0.2 by choosing $k_1 = 0.02$, which controls the initial steepness of the curve, and $k_2 = 0.2$, which restricts the maximum risk to be ~18%[32,33] (Extended Data Fig. 2c(ii)).

To calculate a rough estimate of the possible impact of adding antimalarials on insecticide-treated nets in Africa, we examined locations where there was available data on insecticide resistance, malaria prevalence, and bed net coverage. To this end, insecticide resistance measurements in sub-Saharan Africa from 2013 to 2018 from IR Mapper[17] were combined with estimates from 2015 for bed net coverage and *P. falciparum* parasite rate (*Pf*PR) in 2–10 year old children from the Malaria Atlas Project[1]. The data were aggregated with resampling to a grid square size of 5 km by 5 km at the equator. We considered each grid square to be a location, and only included these grid squares in the analysis, since insecticide resistance is likely to be highly spatially heterogeneous. In total we found 2641 insecticide resistance measurements from 597 locations. For 10 locations there was insufficient data on

*Pf*PR and bed net coverage to fit the model. We additionally excluded a further 20 sites outside of our selected geographies for a final total of 567 locations. Insecticide resistance, bed net coverage and *Pf*PR ranged between 0 – 100%, 0 – 100% and 0.13 – 74.4 respectively in these locations. When there were multiple insecticide resistance measurements in a location, we used the average level of insecticide resistance. For simplicity, and because we calculate a relative impact, we assume that *Pf*PR in 2–10 year old children reflects overall prevalence in the population. For each location, given the level of bed net coverage and insecticide resistance, we fit the biting rate to return the reported *Pf*PR value as prevalence for the entire population of that grid square. We compared the *Pf*PR with the prevalence predicted by the model when including an ATQ-like compound (as above, considering 100% blocking of new *Plasmodium* infections on the same day of exposure to the antimalarial compound, and no effects on ongoing *Plasmodium* infections) on all insecticide-treated bed nets predicted from the Malaria Atlas Project[1]. We considered the relative reduction in prevalence in each location and grouped them by West Africa (186 locations) and East Africa (381 locations), to account for broad differences in ecology and epidemiology. This approach is necessarily simplistic, and does not account for the fact that the relationship between current prevalence, bed net use, and insecticide resistance may not be at equilibrium, or that the measurements for the underlying data may not have been taken during the same time period. Further, the estimated impact of our approach may not be generalizable to areas outside of our sample area for which insecticide resistance data is not available. Overall, however, this calculation gives a rough estimate for the relative impact that adding antimalarials may have in the regions for which data exist.

Statistics and Reproducibility

Statistical analyses were carried out using GraphPad Prism v7.0 for MacOSX (GraphPad Software Inc., La Jolla CA, USA) unless otherwise stated. For infections, differences in prevalence were analyzed by Chi^2. In experiments where both treatment groups had individuals that produced >0 oocysts, differences in median oocyst burden between groups (intensity of infection) was analyzed using a Mann-Whitney Mean Ranks test. For multiple comparisons (e.g. Fig. 4) differences in prevalence between multiple groups were determined using pair-wise Chi^2 corrected for multiple comparisons (Bonferroni). Similarly multiple comparisons of intensity were carried out using Wilcoxon with Dunn's *post hoc.* To determine IC_{50} from dose-response data, the mean relative inhibition (ATQ exposed prevalence/Control prevalence) was calculated for each tested dose and fit with a sigmoidal curve function using non-linear regression. To compare the relative proportions of each parasite form detected in the mosquito midgut at 21 h pIBM we constructed a logistic regression model using JMP Pro 14 (SAS Institute, Cary NC, USA) with "Parasite Form" (ookinete, retort, zygote) as the independent variable and "Treatment" (ATQ/Control) as the dependent variable. We also included the term "Mosquito Sample" (n = 7 per treatment) to account for random between-sample variation. This cofactor was nested within treatment. All infection experiments were replicated a total of three times as independent biological replicates. Survival and fitness experiments were replicated independently twice. All collected data is included in the presented figures.

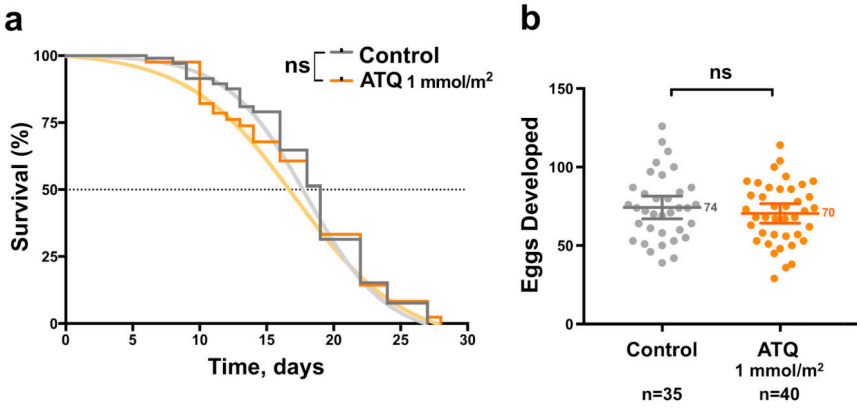
Data Availability

Raw data for infection experiments are available as a Supplemental Data spreadsheet. All further data is available upon request.

Code Availability

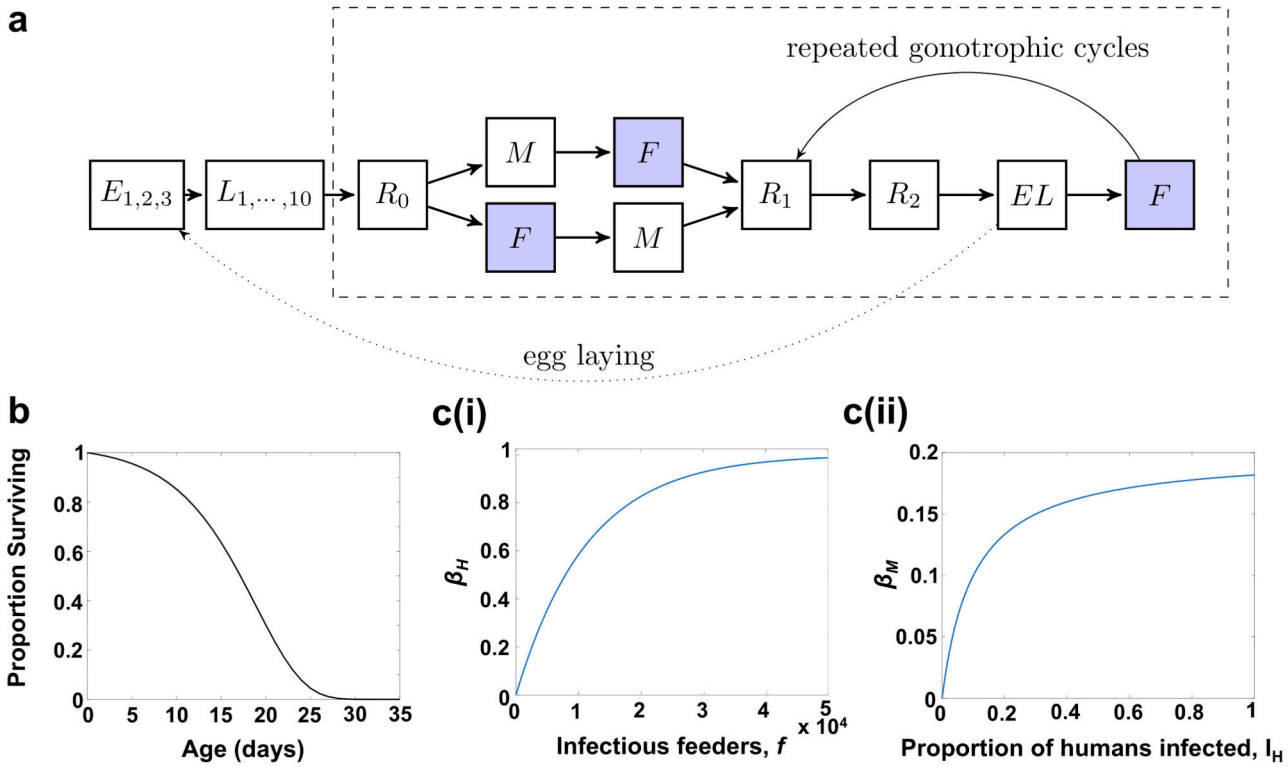
All custom computer code used in this study has been uploaded to GitHub and can be accessed from the following URL: https://github.com/laurenchilds/ATQAnopheles

Extended Data



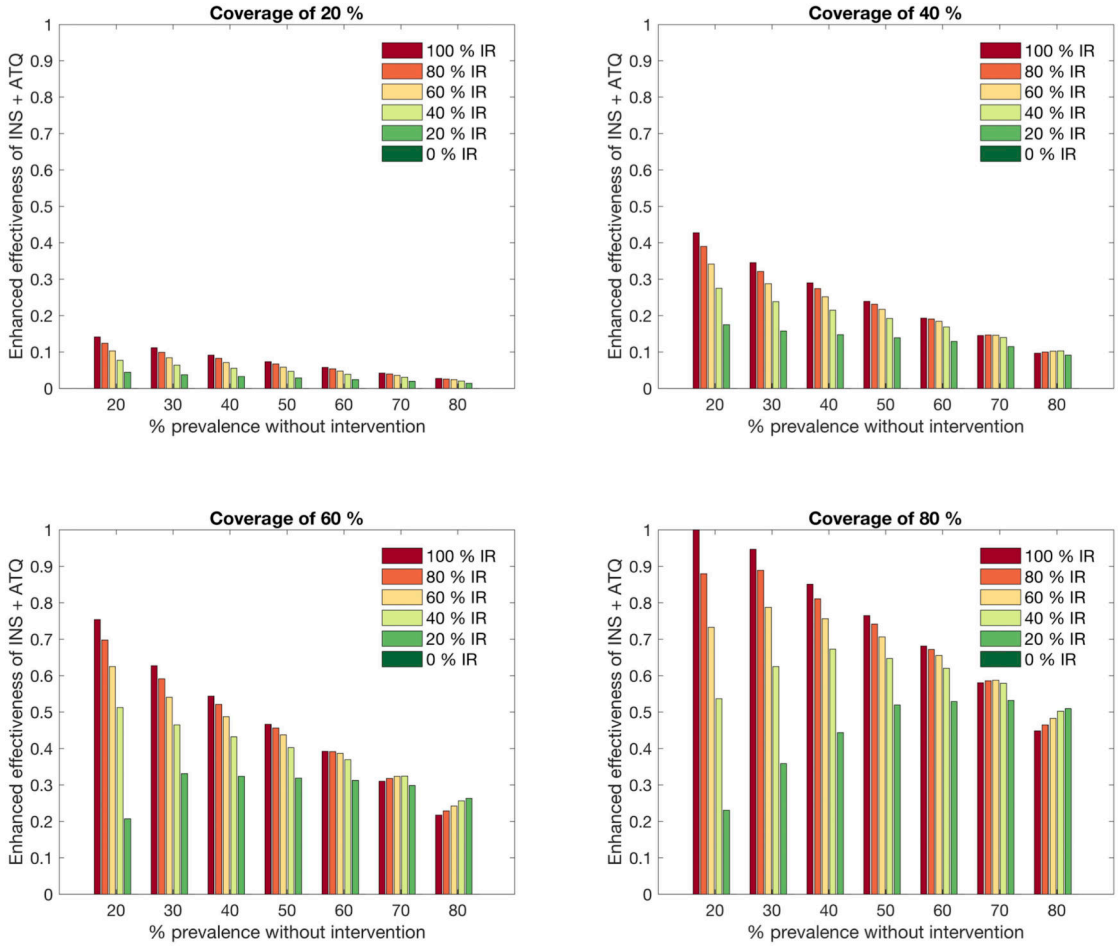
Extended Data Figure 1: Effects of ATQ exposure on survival and post blood-feeding egg production in *An. gambiae* females.

a) ATQ exposure has no effect on the acute or long-term survival of *An. gambiae* females (2-sided Log-Rank Mantel-Cox, n = 189, df = 1, $\chi^2 = 0.00$, p = 0.9951). The sigmoidal fit used for subsequent modeling is shown. **b)** The production of eggs after an infections blood meal is unaffected by ATQ exposure (2-sided, unpaired Student's t, n = 75, df = 1, t = 0.826, p = 0.4115). Means and 95% CI of the mean are indicated. Where relevant, statistical significance is indicated as so: ns = not significant, * = p < 0.05, ** = p < 0.01, *** = < 0.001, **** = p < 0.0001; n indicates the number of biologically independent mosquito samples.



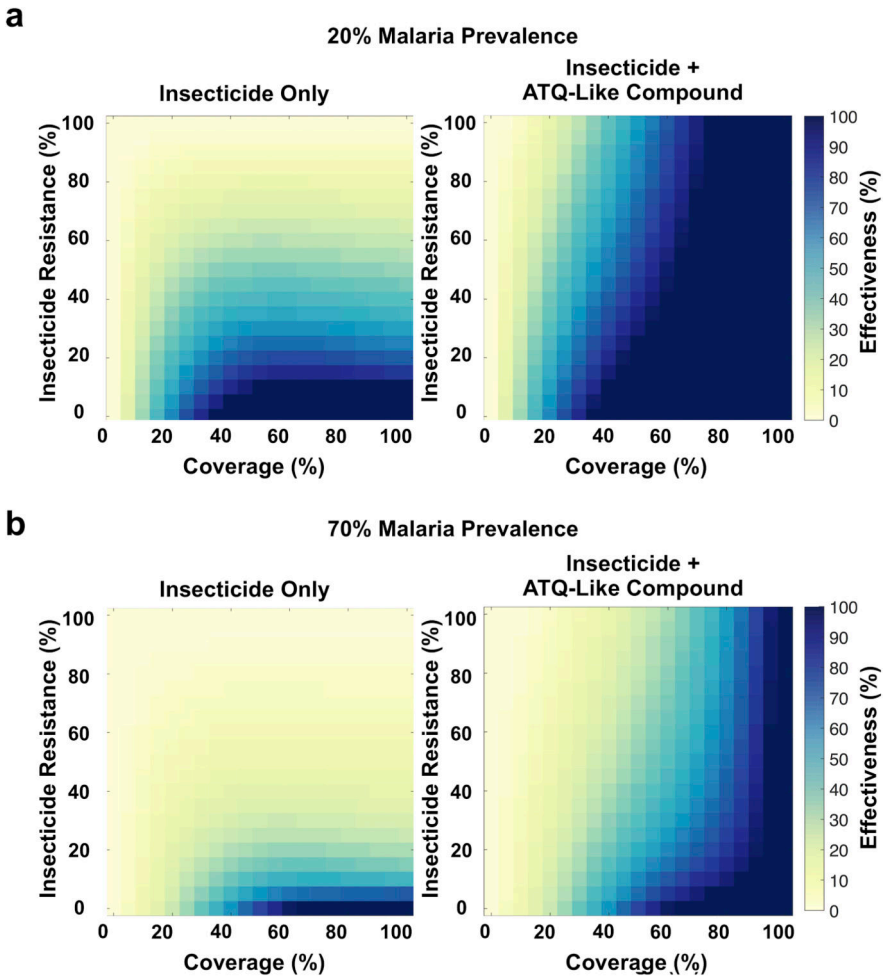
Extended Data Figure 2: Model Structure and Population Parameters.

(a) Schematic representation of the mosquito life cycle model with the time step of one day. Mosquitoes spend three days as eggs (E_i), ten days as larvae (L_i), which includes the pupal stage. Adult female mosquito compartments fall within the dashed box and begin with a rest day (R_0) followed by mating (M) or feeding (F). After feeding, females undergo two days of rest (R_i) followed by a day for egg laying (EL). Then the cycle repeats. Shaded boxes denote when exposure to insecticide or ATQ could occur. These are the same compartments were mosquitoes can become infected or transmit infections, assuming they have been infected for a period longer than the incubation time. **(b)** Survival of the mosquito population as a function of age. The curve is a Gompertz distribution with scale parameter $b = 0.1868$ and shape parameter $\eta = 0.0293$. **(c)** Functions relating human and mosquito infection levels with risk of infection. **(i)** The risk of a human becoming infected, β_H, as a function of the number of infectious feeders, f. **(ii)** The risk of a mosquito becoming infected, β_M, as a function of the fraction of the human population that is infected, I_H.



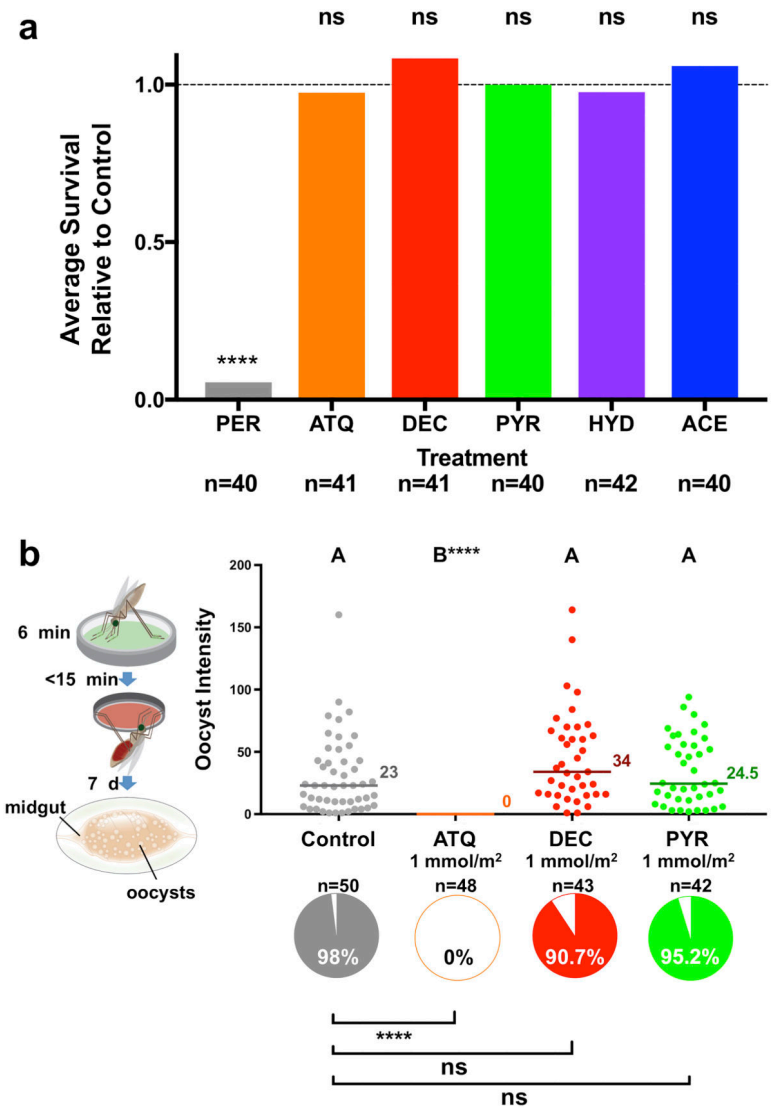
Extended Data Figure 3: Sensitivity of model results to variation in prevalence, coverage and insecticide resistance.

The graphs show the enhanced effectiveness of insecticide combined with ATQ (relative to insecticide alone) in reducing human prevalence under varying levels of coverage (across panels), prevalence (along x-axis), coverage and insecticide resistance (bar color). The enhanced effectiveness of the interventions is defined as (the quantity of human prevalence with only insecticide - human prevalence with insecticide and ATQ over human prevalence with only insecticide) and is represented by positive values when the addition of ATQ is beneficial. Prevalence is quantified after ten years of simulation. The coverage is varied from 20%−80% (upper left panel 20%; upper right panel 20%; lower left panel 60%; and lower right panel 80%). In each panel, the position of the bars determines the malaria prevalence under no intervention, from 20–80%. The bar color represents insecticide resistance levels (dark green 0%; green 20%; light green 40%; yellow 60%; orange 80%; and red 100%). In the complete absence of insecticide resistance all mosquitoes that contact insecticide are killed, and thus, all dark green bars equal zero.



Extended Data Figure 4: Malaria transmission model predicting the effects of adding ATQ to insecticide-treated nets in additional malaria prevalence settings.

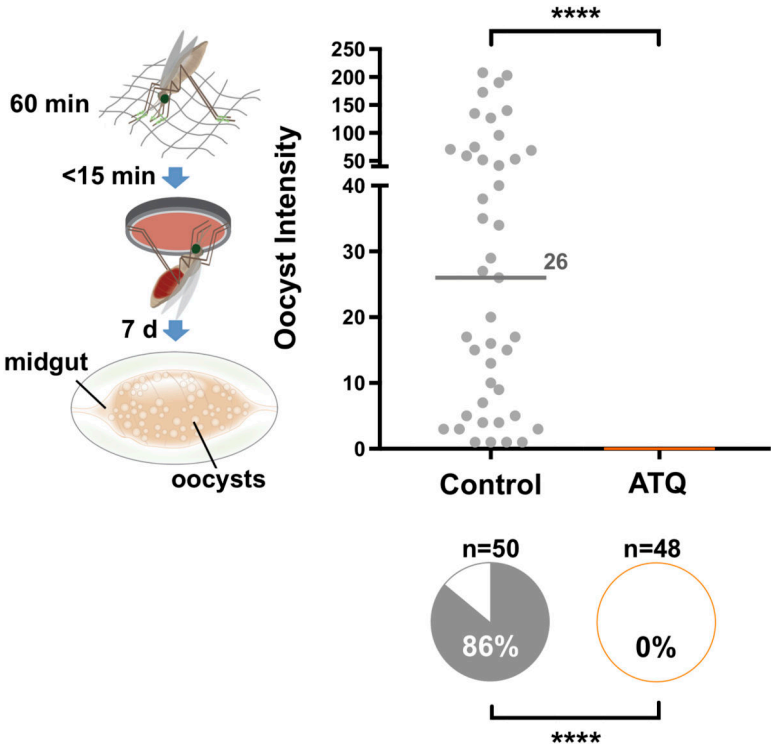
The heat maps show changes in malaria transmission for bed net-like interventions using insecticide alone or insecticide plus an ATQ-like compound, relative to no intervention at varying coverage and varying insecticide resistance levels. The model considers both (**a**) 20% and (**b**) 70% prevalence of malaria. The effectiveness of the interventions is defined as (1 - proportion reduction in malaria transmission relative to no intervention) and is represented as colors ranging from yellow (no change in malaria transmission) to dark blue (elimination of malaria transmission) at varying levels of coverage (x-axis) and insecticide resistance (y-axis). Insecticide resistance is the percentage of mosquitoes that are impervious to insecticide. Coverage is the probability of a mosquito encountering an intervention during a single feeding episode. The model output demonstrates that addition of ATQ significantly increases the ability of an LLIN-like intervention to reduce and even eliminate malaria transmission.



Extended Data Figure 5: Testing additional compounds for fitness costs and transmission blocking activity through tarsal contact.

(a) Mosquito survival relative to an untreated control after 48 h following exposure to ATQ, DEC, PYR, HYD, ACE and permethrin (PER). The proportion of female *An. gambiae* surviving exposure to each compound (1 mmol/m^2, 60 minutes) relative to the proportion of individuals surviving exposure to an untreated control is shown. PER exposure causes almost complete mortality (proportionate survival relative to controls = 0.055, Pairwise, 2-sided Chi^2 w/ Bonferroni correction, n = 80, df = 1, $\chi^2 = 76.10$, $p < 0.0001$), while all other compounds behave comparably to controls. **(b)** Neither PYR nor DEC (1 mmol/m^2, 6 min) are capable of reducing the prevalence *P. falciparum* through tarsal contact, relative to controls (Pairwise Chi^2 w/ Bonferroni correction, DEC: n= 93, df = 1, $\chi^2 = 2.42$, $p = 0.12$. PYR: n = 92, df = 1, $\chi^2 = 0.55$, $p = 0.46$). Similarly, DEC and PYR had no impact on the intensity of infection, compared to a mock-treated control (Wilcoxon with Dunn's *post hoc*, n = 183, df = 3, $p = 0.31$ (DEC) and $p = 0.99$ (PYR)). Letters indicate groups that are statistically different from one another. Statistical significance is indicated as ******** = p <

0.0001. Medians are indicated; n denotes the number of biologically independent mosquito samples.

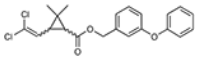
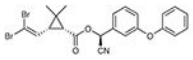
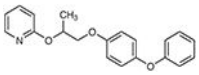
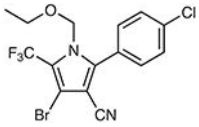
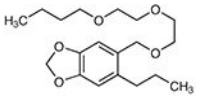
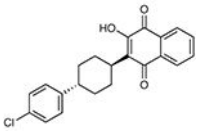
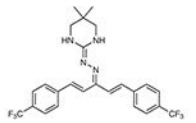
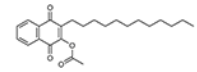
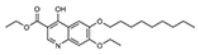
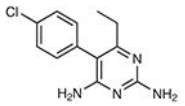


Extended Data Figure 6: ATQ exposure via a netting substrate completely inhibits *P. falciparum* development.

An. gambiae females were allowed to rest for 60 min on 100 denier polyester netting that had been treated with either a 0.5 mg/ml (0.05% w/v) solution of ATQ in acetone or acetone alone. Females exposed to ATQ in this way failed to become infected after an infectious *P. falciparum* blood meal, demonstrating that a netting substrate is also capable of delivering sufficiently high doses of ATQ to inhibit infection (2-sided Chi^2, n = 98, df = 1, $\chi^2 = 75.55$, $p < 0.0001$). Medians are indicated; n denotes the number of biologically independent mosquito samples.

Extended Data Table 1:
Chemical properties and structures of study compounds and bed-net approved chemicals.

Chemical properties of currently approved insecticides and synergists for bed net use - permethrin, deltamethrin, pyriproxifen, piperonyl butoxide and chlorfenapyr. - and all compounds tested in this study - atovaquone, hydramethylnon, acequinocyl, decoquinate and pyriproxifen.

Compound	Vehicle	H Donors	H Acceptors	Rotational Bonds	Polar Surface Area (Å^2)	Active Tarsally?	Target	Chemical Structure
Permethrin[a]	Acetone	0	3	7	35.5	Yes	*Para* sodium-gated ion channel (cell membrane)	
Deltamethrin[a]	Acetone	0	4	7	59.3	Yes	*Para* sodium-gated ion channel (cell membrane)	
Pyriproxifen[ab]	Acetone	0	4	7	40.6	Yes	*Methoprene Tolerant* (nucleus)	
Chlorfenapyr[ab]	Acetone	0	5	4	38	Yes	Mitochondrial inter-membrane space	
Piperonyl butoxide[ab]	Acetone	0	5	13	46.2	Yes	Cytochrome P450s	
Atovaquone	Acetone	1	3	2	54.4	Yes[c]	*Cytochrome B* (mitochondrial inner membrane)	
Hydramethylnon	Acetone	2	8	6	48.8	Yes[c]	*Cytochrome B* (mitochondrial inner membrane)	
Acequinocyl	Acetone	0	4	13	60.4	Yes[c]	*Cytochrome B* (mitochondrial inner membrane)	
Decoquinate	Chloroform	1	6	15	**73.89**	**No**[c]	*Cytochrome B* (mitochondrial inner membrane)	
Pyrimethamine	Acetone (sparingly)	2	4	2	**77.8**	**No**[c]	*Dihydrofolate Reductase-Thymidylate Synthase* (cytosol)	

[a] approved for use in long-lasting inseciticide treated nets

[b] only in combination with permethrin/deltamethrin

[c] against Plasmodium falciparum infection

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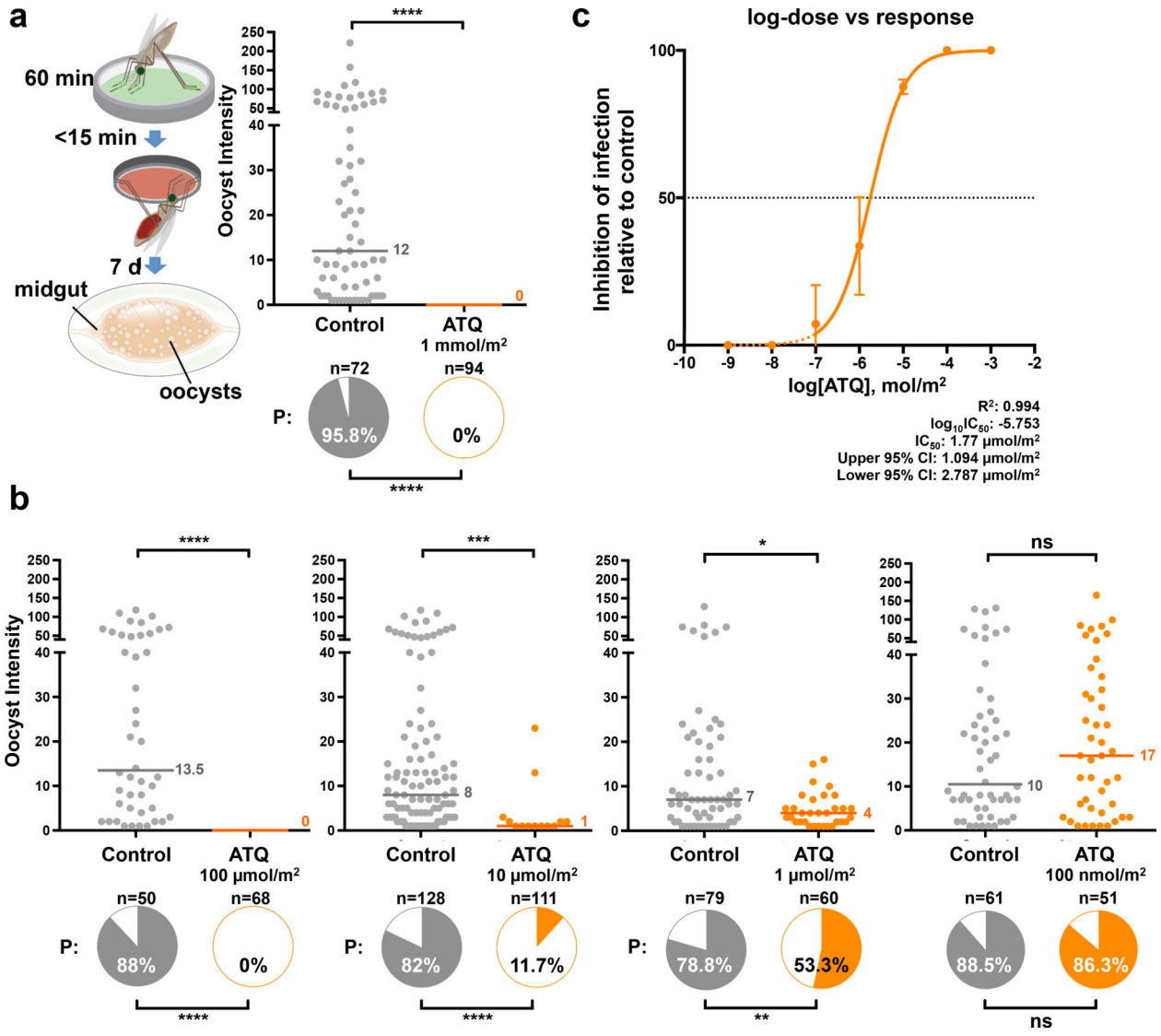

Figure 1: *An. gambiae* exposure to atovaquone (ATQ) aborts *P. falciparum* development.

(a) *P. falciparum* parasites are completely eliminated (0% oocyst intensity, and 0% prevalence of infection, shown in the pie charts) in females exposed to 1 mmol/m^2 ATQ for 60 minutes immediately prior to infection (Prevalence: Two-sided Chi^2, n = 166, df = 1, χ^2 = 155.14, p < 0.0001). The exposure method is shown in the graphic: green represents ATQ coated onto a glass surface. **(b)** Dose-dependent inhibition (range: 100 µmol/m^2 - 100 nmol/m^2) of *P. falciparum* infection by exposure to ATQ. Significant reductions in prevalence and intensity were observed at doses as low as 1 µmol/m^2 (Prevalence: Two-sided Chi^2. 100 µmol/m^2: n = 118, df = 1, χ^2 = 95.42, p < 0.0001. 10 µmol/m^2: n = 239, df = 1, χ^2 = 117.6, p < 0.0001. 1 µmol/m^2: n = 139, df = 1, χ^2 = 9.85, p = 0.0017. Intensity: Two-sided Mann-Whitney: 10 µmol/m^2: n = 239, df = 1, U = 287.5, p = 0.0004. 1 µmol/m^2: n = 139, df = 1, U = 686, p = 0.0104). **(c)** Dose-response curve fit for ATQ exposure (Non-linear regression, n = 13, df = 12, Sum of Squares = 1003, R^2 = 0.9441). The IC$_{50}$ for ATQ pre-infection exposure, calculated by interpolation, is indicated. Mean inhibition relative to control prevalence is indicated. Error bars are 95% CI. Dashed portions of the sigmoidal fit

are estimated. In all panels where relevant, statistical significance is indicated as so: ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = < 0.001, **** = $p < 0.0001$. Medians are indicated. For (a) and (b), n indicates the number of biologically independent mosquito samples. For (c), n indicates the relative inhibition observed in ATQ-treated mosquitoes in independent experiments.

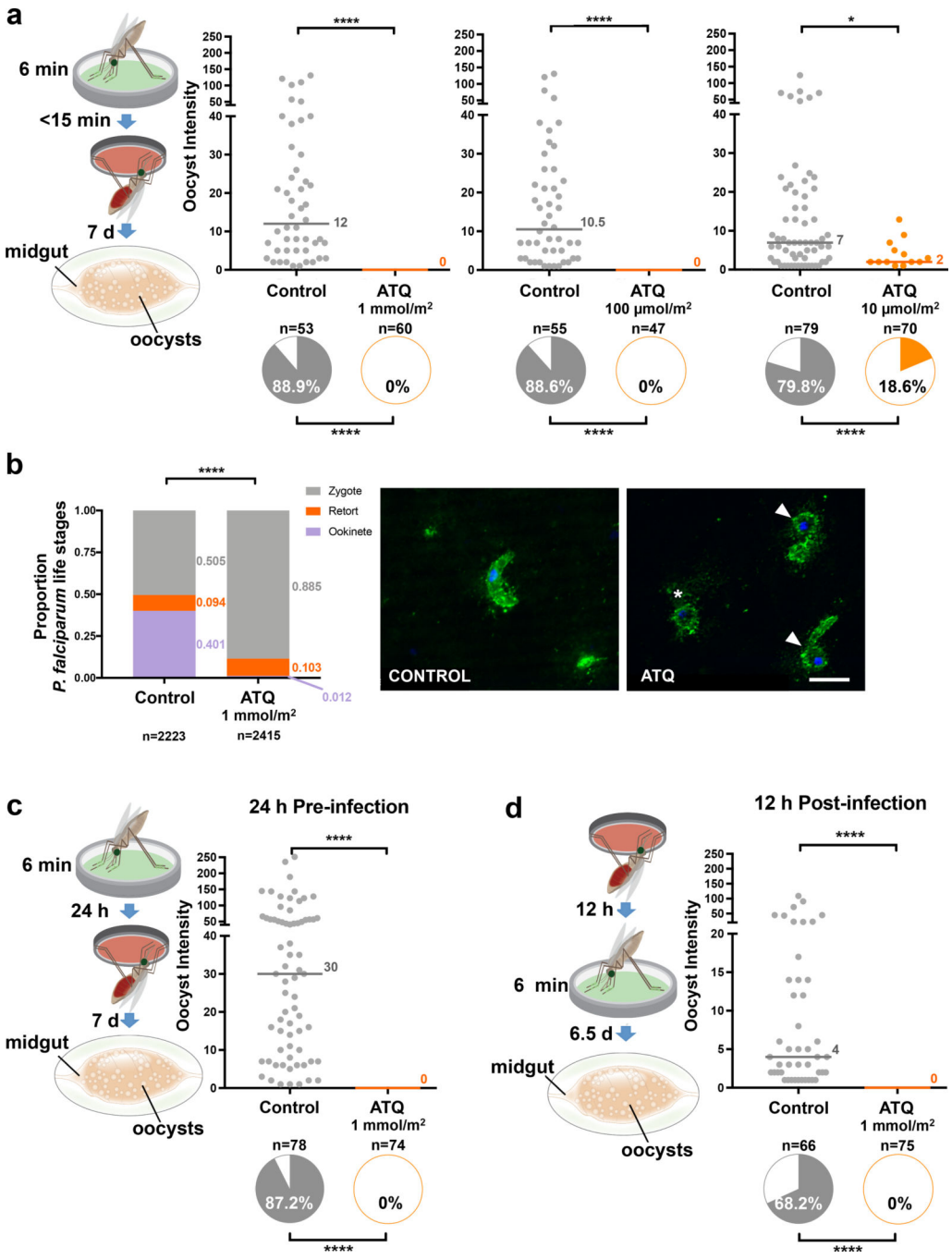

Figure 2: The transmission blocking activity of ATQ is maintained at shorter exposure times and at time points of exposure before and after infection.

(a) *P. falciparum* parasites are completely eliminated (0% oocyst intensity, and 0% prevalence of infection, shown in the pie charts) in females exposed to either 1 mmol/m^2 or 100 µmol/m^2 ATQ for 6 min (Prevalence: Two-sided Chi^2. 1 mmol/m^2: n = 113, df = 1, χ^2 = 91.00, p < 0.0001. 100 µmol/m^2: n = 102, df = 1, χ^2 = 80.59, p < 0.0001). At 10 µmol/m^2, prevalence of infection (10 µmol/m^2: n = 149, df = 1, χ^2 = 55.58, p < 0.0001) and median oocyst intensity (2-sided Mann-Whitney, n = 149, df = 1, U = 258, p = 0.0349) are

significantly reduced in the ATQ-treated group. Medians are indicated. **(b)** IFAs of mosquito midgut lumens 21 h post *P. falciparum* infection using parasite-specific antibodies (anti-PfS25, green) and DNA (DAPI, blue) staining. Example images from 14 independent mosquito midgut samples (7 control, 7 ATQ-treated); *P. falciparum* forms are shown. Left panel: mature ookinete in controls. Right panel: zygote (asterisk) and retort forms (white arrows) in ATQ-treated females. ATQ-treated females have few ookinetes (1.2% total parasites) and a large proportion of zygotes (88.5% total parasites), indicating parasite arrest, while controls contain a significantly larger proportion of normal ookinetes (40.1%, Nominal Logistic Regression, n = 5091, df = 14, $\chi^2 = 1620.88$, $p < 0.0001$). Scale bar: 10 μm. **(c, d)** *P. falciparum* parasites are completely eliminated also when females are exposed to ATQ (1 mmol/m^2, 6 min) either **(c)** 24 h prior (2-sided Chi^2 w/Bonferroni correction, n= 152, df = 1, $\chi^2 = 116.74$, $p < 0.0001$) or **(d)** 12 h after (2-sided Chi^2 w/ Bonferroni correction, n = 141, df = 1, $\chi^2 = 75.11$, $p < 0.0001$) an infectious blood meal. Medians are indicated. Where relevant, statistical significance is indicated as so: * = $p < 0.05$, ** = $p < 0.01$, *** = < 0.001, **** = $p < 0.0001$. For (a), (c) and (d), n indicates the number of biologically independent mosquito samples. For (b), n indicates the number of independent parasite forms.

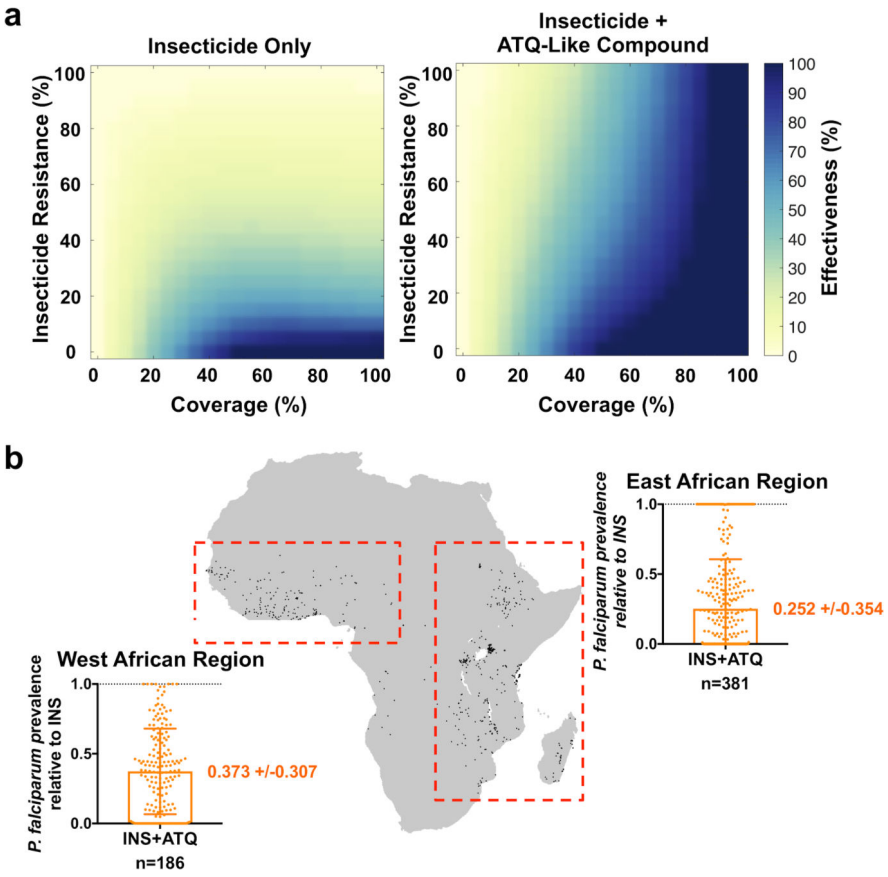

Figure 3: Malaria transmission model predicts that adding ATQ to insecticide-treated nets would increase bed net effectiveness.

(a) Heat maps of changes in malaria transmission for bed net-like interventions using insecticide alone or insecticide plus an ATQ-like compound, relative to no intervention at varying coverage and varying insecticide resistance levels. The model considers an intermediate 45% prevalence of human infection (effects at lower and higher malaria prevalence are described in Extended Fig. 4). The "effectiveness" of the interventions is defined as (1 - proportion reduction in malaria transmission relative to no intervention) and is represented as colors ranging from yellow (no change in malaria transmission) to dark blue (elimination of malaria transmission) at varying levels of coverage (x-axis) and insecticide resistance (y-axis). Insecticide resistance is the percentage of mosquitoes that are impervious to insecticide; coverage is the probability of a mosquito encountering an intervention during a single feeding episode. **(b)** Predicted effects of adding ATQ to existing insecticide-treated nets in 567 African locations with available insecticide resistance data (indicated by black dots on the map of Africa). For each location, the model considers the estimated bed net coverage and *P. falciparum* prevalence in 2–10 year old children reported in 2015[1], and insecticide resistance levels reported between 2013 and 2018[17]. The graphs show mean malaria prevalence for insecticide/ATQ combination bed nets (INS+ATQ), relative to insecticide only bed nets (dotted line at y = 1), for sampled sites in West and East Africa (red boxes, n = 186 and n = 381, respectively). Error bars represent one standard deviation from the mean prevalence. In both (a) and (b), the model outputs demonstrate that

addition of ATQ substantially increases the ability of treated nets to reduce malaria transmission across a broad range of transmission settings.

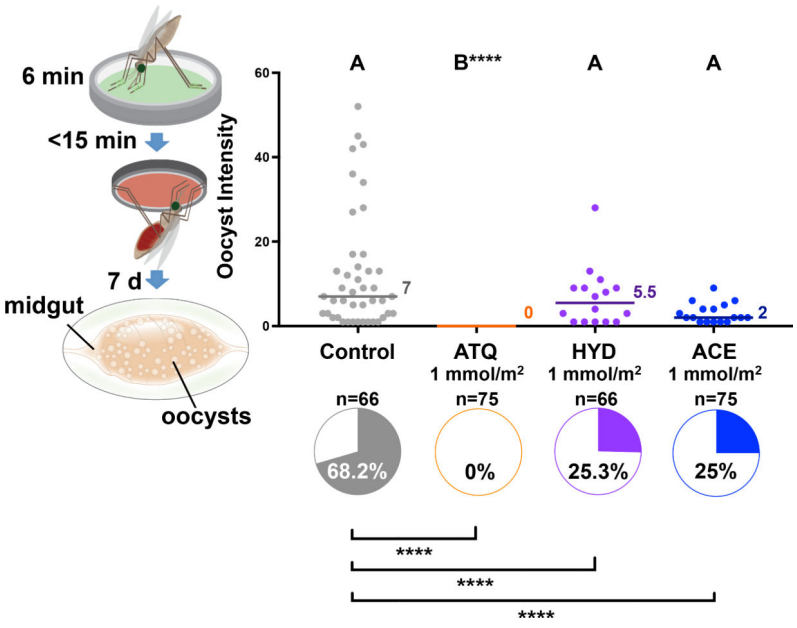

Figure 4: Other cytochrome B inhibitors have *P. falciparum* transmission-blocking activity.

An. gambiae females exposed to 1 mmol/m^2 of the arthropod cytochrome B inhibitors acequinocyl (ACE) and hydramethylnon (HYD), as well as ATQ, for 6 minutes show strongly reduced prevalence (pie charts) of *P. falciparum* relative to controls (Pairwise, 2-sided Chi^2 w/ Bonferroni correction: ATQ: n = 141, df = 1, $\chi^2 = 75.11$, $p < 0.0001$. HYD: n = 132, df = 1, $\chi^2 = 23.85$, $p < 0.0001$. ACE: n = 141, df = 1, $\chi^2 = 26.00$, $p < 0.0001$). HYD and ACE had no impact on the intensity of infection (Wilcoxon with Dunn's *post hoc*, n = 282, df = 3, HYD: p = 0.99, ACE: p = 0.19). Letters indicate groups that are statistically different from one another ******** = $p < 0.0001$; n indicates the number of biologically independent mosquito samples.