Classification: Biological Sciences: Ecology

Title: Fungal infection counters insecticide resistance in African malaria mosquitoes

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Abstract

The evolution of insecticide resistance in mosquitoes is threatening the effectiveness and sustainability of malaria control programmes in various parts of the world. Through their unique mode of action, entomopathogenic fungi provide promising alternatives to chemical control. However, potential interactions between fungal infection and insecticide resistance, such as cross-resistance, remain to be investigated. We show that insecticide-resistant *Anopheles* mosquitoes remain susceptible to the fungus *Beauveria bassiana*. Four different mosquito strains with high resistance levels against either pyrethroids, organochlorines or carbamates were equally susceptible to *B. bassiana* infection as their baseline counterparts. Moreover, fungal infection reduced the expression of resistance to the key public health insecticides permethrin and DDT. Mosquitoes pre-infected with *B. bassiana* or *Metarhizium anisopliae* showed a significant increase in mortality after insecticide exposure compared with uninfected control mosquitoes. Our results show a high potential utility of fungal biopesticides for complementing existing vector control measures and provide novel products for use in resistance management strategies.

Text

Introduction

Increasing incidences of insecticide resistance are reported in the major African malaria vector species *Anopheles gambiae s.s.* (1-3), *A. funestus* (4, 5) and *A. arabiensis* (6, 7), thereby threatening the efficiency of insecticides approved for malaria vector control. As the use of indoor residual spraying (IRS) and insecticide treated nets (LLIN's) is scaling up (8), so will the selection pressure for insecticide resistance. Resistance-management strategies, such as the use of chemical mixtures or rotations, are suggested to improve the sustainability of these approaches (9, 10), but with significant cross-resistance between the currently approved classes of insecticide (9-11) and no new public health insecticides introduced in the last twenty years (12), practical options are few. It is becoming increasingly evident that for sustainable malaria vector control, new products with unique modes of action are required.

Previous work has suggested that entomopathogenic fungi could play this role (13-15). A range of isolates belonging to the fungal species *Metarhizium anisopliae* and *Beauveria bassiana* have been shown to infect and significantly reduce the longevity of adult *Anopheles* mosquitoes, killing them within 14 days (13, 14, 16). This slow-speed of kill is considered to dramatically reduce the selection pressure for fungal resistance development (15) yet, since *Plasmodium* maturation within the mosquito also takes approximately 14 days (17), to be sufficient to significantly reduce the mosquito's vectorial capacity. Pre-lethal effects of fungal infection, which reduce sporozoite formation (13), feeding propensity (13, 18) and fecundity (18), increase the potential impact on malaria transmission further.

For fungus-based biopesticides to play a prominent role in malaria control, an important criterion is that fungal susceptibility will remain unaffected by resistance to insecticides. The mode of action of entomopathogenic fungi, i.e. infecting insects through external contact, penetrating the cuticle and subsequently proliferating through the haemocoel (15, 19), makes cross-reaction with insecticide resistance unlikely. However, enzymatic detoxification of insecticides is an important resistance mechanism in *Anopheles* mosquitoes (11), and enhanced detoxification may interact with fungal metabolites, such as cyclic peptide toxins, which are important virulence factors (15, 19). Similarly, with regards to pathogens, enhanced concentrations of esterases in organophosphate (OP)-resistant *Culex* mosquitoes have been implicated in limiting growth of filarial worms (20, 21), and enhanced monooxygenase levels associated with pyrethroid resistance in certain *Anopheles* species have been suggested to increase oxidative stress to the detriment of *Plasmodium* survival (21). Reciprocally, studies on the wax moth, *Galleria mellonella*, have indicated that the elevation of detoxifying enzymes in response to infection with *M. anisopliae* increases host resistance to organophosphate insecticides (22). In contrast, however, numerous studies in other insect hosts indicate that fungal infection can act synergistically with insecticides, increasing impact of otherwise sub-lethal insecticide doses (23-25). In this study, we investigated the effects of insecticide resistance on fungal susceptibility, and effects of fungal infection on insecticide resistance in a diverse suite of insecticide-resistant *Anopheles* colonies.

Results

Mosquito susceptibility to fungus infection

We studied the infectivity and virulence of *B. bassiana* in a permethrin-resistant *A. funestus* colony (Af ^{Perm}), two DDT-resistant *A. arabiensis* colonies (Aa_1 ^{DDT} and Aa_2 ^{DDT}), one bendiocarb-resistant *A. gambiae s.s.* colony (Ag ^{Bend}), and their corresponding baseline colonies from which each was selected. Replicate samples of mosquitoes were infected with the fungus using a standard exposure bioassay previously shown to provide reliable infection in the laboratory (16). *Beauveria bassiana* caused 100% mortality in all eight colonies within 8-20 days (Fig. 1), with levels of infection exceeding 95% (confirmed by fungal sporulation on mosquito cadavers (16)). Cox regression analyses showed the effect of *Beauveria* infection on mosquito survival to be significant in all tested colonies ($P<0.001$). Additionally, a highly significant interaction between fungus treatment and insecticide resistance was found in *A. funestus* ($P=0.002$) and *A. gambiae s.s.* ($P<0.001$), indicating quantitative differences in the effect of fungus infection on mosquito survival between the baseline and insecticide-resistant colonies. *Beauveria* infection reduced survival more strongly in permethrin-resistant *A. funestus* mosquitoes (Hazard ratio (HR)=47241.42; $P<0.001$) than in its baseline colony (HR=28.22; $P<0.001$), yet reduced survival less strongly in bendiocarb-resistant *A. gambiae s.s.* (HR=5.70; $P<0.001$) than in the baseline mosquitoes (HR=71.70; $P<0.001$). However, similar differences in Hazard Ratio's were observed in the corresponding uninfected control mosquitoes; a significantly lower daily mortality rate in permethrin-resistant *A. funestus* mosquitoes (HR=0.003; $P=0.005$) and higher mortality rate in bendiocarb-selected *A. gambiae s.s.* mosquitoes (HR=28.20; $P<0.001$) compared with their baseline

colonies, suggesting that these quantitative differences in the effect of fungus infection on survival are caused by inherent differences in laboratory colony longevity rather than any effect of resistance *per se*.

Effect of fungal infection on insecticide resistance

To investigate whether fungal infection affects the expression of insecticide resistance, we conducted a series of experiments to examine pre-lethal effects of fungal infection on insecticide sensitivity in resistant mosquitoes. First, we assessed the effect of *B. bassiana* or *M. anisopliae* infection on the expression of permethrin resistance in three mosquito strains with known levels of resistance to this insecticide; permethrin-resistant *A. funestus* (Af^{Perm}), DDT resistant *A. arabiensis* (Aa_2^{DDT}) and a recently established, unselected, multiple resistant strain of *A. gambiae s.s.* (Ag^{MR}). Replicate sets of mosquitoes were infected with either *Beauveria* or *Metarhizium* and exposed to permethrin three days later, using standard WHO insecticide assay protocols. Mortality was recorded 24 hours post insecticide exposure and compared with permethrin-induced mortality in uninfected controls. In all three mosquito strains, permethrin induced significantly higher mortality in mosquitoes pre-infected with *Beauveria* ($P<0.001$) or *Metarhizium* ($P<0.001$) than in the uninfected groups (Fig. 2*A*). Both fungal strains induced similar increases in susceptibility to permethrin. In *A. arabiensis* we found significantly higher mortality levels induced by *Beauveria* infection compared with *Metarhizium* infection ($\chi^2=36.04$, $P<0.001$), which may be caused by differences in pathogenicity of the two fungal species or by a different insecticide resistance mechanism in this mosquito species.

Using the Af ^{Perm} line we also examined the effects of *B. bassiana*- infection five days after exposure. This revealed a further, significant ($\chi^2 = 18.38$, $P < 0.001$) increase in permethrin mortality compared with the three day group (Fig. 2*B*). However, mortality of the uninfected control group was also significantly higher at five days ($\chi^2 = 57.22$, $P < 0.001$), which is consistent with age-dependent responses to insecticide exposure found in several mosquito species (26, 27) and suggests equivalent relative effects of fungus, at least over the initial time course of infection.

The effect of fungus infection on DDT resistance was assessed using DDT-resistant colonies of *A. arabiensis* (Aa_2 ^{DDT}) and *A. gambiae s.s.* (Ag ^{MR}). We pre-infected replicate samples of mosquitoes to *B. bassiana* and compared susceptibility to DDT between the infected and uninfected group three days later. For both tested species, infection with *B. bassiana* led to significantly higher mortality following DDT exposure, compared with the uninfected controls ($P<0.001$) (Fig. 2*C*). This increase in the mosquito's susceptibility to DDT shows that fungal infection negatively affects the expression of DDT resistance.

Discussion

Our results show that resistance against three of the four classes of public health insecticides does not confer enhanced resistance to infection by *B. bassiana.* The fungus was highly infective and virulent to a diverse suite of resistant mosquito strains. Furthermore, fungus infection pre-lethally interferes with the expression of permethrin and DDT resistance in genetically resistant mosquitoes, increasing their susceptibility to these insecticides. The exact mechanisms involved in the interactions between insecticide

resistance and fungal infection remain unclear. In the tested *A. funestus* (Af ^{Perm}) colony, resistance is mediated by elevated levels of mono-oxygenases (5, 28, 29) and in *A. arabiensis* (Aa_2 ^{DDT}) through elevated glutathione-*S*-transferase and esterases (27) (the West African *kdr* target site mutation is also present in this laboratory colony and in wild *A. arabiensis* sampled from the same locality in Sudan, but there is no correlation with the resistance phenotype (6, 27)). Resistance in the *A. gambiae s.s.* (Ag ^{MR}) colony may be mediated by the West African *kdr* target site mutation as well as metabolic detoxification. One possibility, therefore, is that a re-allocation of insecticide-detoxifying enzymes towards fungal toxins reduced the quantity of enzymes available to target insecticides and resulted in the observed post-fungus infection decrease in resistance. However, as is the case for wild-type resistant mosquitoes (30), there are diverse, potentially interacting mechanisms conferring resistance in our tested mosquito species, and the lack of a clear correlation between resistance genotype and phenotype complicates assessing the exact interactions between insecticide resistance mechanisms and fungal infection.

Overall, our findings support the potential use of fungal biopesticides against mosquito vectors in areas where insecticide resistance levels are increasing, potentially adding new product options to the very limited selection of chemicals currently available. The susceptibility of insecticide-resistant mosquitoes to fungal pathogens adds weight to the possibility of using biopesticides within insecticide resistance management strategies, such as rotations or mosaics, to slow the spread of resistance (15). The use of oil-formulated spores in point sources such as black cotton cloths (14) or African water storage pots (31) have shown potential for field implementation and would allow for the

integration of fungi in existing control measures. With fungal infection reducing the expression of permethrin and DDT resistance, developing such novel ‘combination treatments’ may enhance the efficacy and effective lifespan of key insecticides where resistance has reached high levels. Together, these findings provide a compelling case for viewing biopesticides and chemical insecticides not as mutually exclusive, but as complementary technologies that may improve the efficiency and sustainability of integrated malaria vector control programmes.

Materials and Methods

Mosquitoes

An overview of mosquito colony names, abbreviations, resistance selection and origins is given in Supporting Information Table S1. The *A. funestus* colonies (Af ^{Perm}) originated from collections in southern Mozambique. Mosquitoes from the baseline colony (FUMOZ) were selected for high levels of resistance to the pyrethroid permethrin in the insectary of the National Institute for Communicable Diseases (NICD, Johannesburg) for a period of two years, resulting in the colony FUMOZ-R of which adults show 0-1% mortality when exposed to 1% lambda-cyhalothrin for 1h(28). The two *A. arabiensis* colonies used (Aa_1 ^{DDT} and Aa_2 ^{DDT}), originated from Mamfene, KwaZulu/Natal, South-Africa (MBN) and from Sennar, south-central Sudan (SENN) respectively. The SENN baseline colony was selected for DDT resistance for 16 generations after which SENN-DDT adults showed 12.1% mortality when exposed to 4% DDT and 0% when exposed to 0.75% permethrin for 1 hour(27). For this study adults of the F50-F54 generation were used, which were shown to have lower baseline resistance levels to DDT and permethrin (Fig. 2A and C). The *Anopheles gambiae s.s.* colony used in survival assays (Ag ^{Bend}) originated from Obuasi, Ghana (SOG), of which a bendiocarb-resistant colony (BENROG) was selected in the laboratory. The *A. gambiae s.s.* colony used in insecticide resistance assays (Ag ^{MR}) originated from Ahafo, Ghana (GAH). This colony has not been selected for resistance to insecticides in the laboratory, but carries quantified levels of resistance to all four classes of insecticides. Both the SOG and FUMOZ baseline colonies exhibit low levels of resistance, which is increased by orders of magnitude in the

selected lines. A summary of mosquito insecticide resistance/susceptibility to insecticides approved by the WHO is given in Table S2.

Larvae were reared in plastic bowls filled with distilled water. For *A. funestus* the water was supplemented with green algae. Larval food was supplied daily and contained a mixture of finely crushed dog biscuit and brewer's yeast (28). Adults were collected daily from the bowls and transferred to holding cages in which cotton wool soaked in 10% glucose solution was provided. All species were maintained at 25°C and 80% RH with a 12-h day/night photoperiod and artificial 45-min dusk/dawn cycles. For experiments, 2-5 day old mosquitoes were used.

Fungus

Metarhizium anisopliae var. anisopliae (Metsch.) Sorokin, isolate ICIPE-30 (14) (courtesy Dr. N. Maniania, ICIPE, Kenya) and *Beauveria bassiana* isolate IMI 391510 (13) were used. Both fungi were produced through solid state fermentation with glucose-impregnated hemp (courtesy F. van Breukelen and M. Jumbe, Wageningen, The Netherlands) of which conidia were dried and stored in the dark at 4°C.

The viability of the conidia was assessed by mixing some dry spores of each stock in oil (Shell Ondina® Oil 917, Shell, The Netherlands) and plating a drop on Sabouraud Dextrose Agar. After 20-26 hrs of incubation at 27°C, the proportion of germinated conidia was determined using a light-microscope at magnification 400X. Stocks showing 85% or higher sporulation were used for experiments.

Fungal exposures

Adult mosquitoes were exposed to 100 mg of dry conidia using the suspensor set-up previously shown to give reliable infections, as described by Scholte *et al*. (16). Female mosquitoes were exposed to one suspensor in a holding cage for 24 hours, after which the suspensor was replaced with clean cotton wool soaked in 10% glucose solution. Control mosquitoes were exposed in the same way, but to a suspensor without fungus.

Survival bioassays

The effect of fungal infection on mosquito survival was tested in baseline and insecticide resistant colonies of *A. funestus* (Af ^{Perm}), *A. arabiensis* from South Africa (Aa_1 ^{DDT}), *A. arabiensis* from Sudan (Aa_2 ^{DDT}) and *A. gambiae s.s.* from Obuasi, Ghana (Ag ^{Bend}). For each colony nine test and nine control replicates were performed on three consecutive days, exposing approximately 30 mosquitoes per replicate to dry spores of *B. bassiana* or control suspensors for 24 hours. For the baseline *A. funestus* colony, six replicates were performed. Mosquito mortality was recorded daily and cadavers removed from each holding cage, dipped in 70% ethanol and placed on moist filter paper in sealed petri dishes. To verify infection, these were incubated at 25°C for three or more days and assessed for fungus sporulation, i.e. emerging hyphae, using a dissection microscope (16).

Permethrin resistance assays

The resistant colonies of *A. funestus* (Af ^{Perm}), *A. arabiensis* from Sudan (Aa_2 ^{DDT}) and *A. gambiae s.s.* from Ahafo, Ghana (Ag ^{MR}) were used to test the effect of fungal infection on permethrin resistance. A three day waiting period was chosen between fungal

exposures and assessments for insecticide resistance, to allow for some progression of the fungal infection whilst not losing large numbers through death. Mosquitoes from the same cohort received either a control, *Beauveria* or *Metarhizium* treatment, of which 25 females per treatment were exposed three days later to a control paper and 25 females to a filter paper treated with 0.75% permethrin for 1 hour, according to the WHO protocol (32). Mosquitoes were then transferred to clean holding tubes, provided with 10% glucose solution using cotton balls, and the proportion of dead mosquitoes was scored 24 hours post insecticide exposure. Five replicates were performed per mosquito species and for each group, mortality per replicate exposed to insecticide was corrected using mortality data of counterparts exposed to control papers, according to Abbott's formula (32). After mortality measurements, mosquitoes were removed from the exposure tubes with an aspirator, killed through drowning in 70% alcohol and checked for fungal infection as described above.

Using the same methods, the effect of a more advanced *Beauveria* infection was tested on *A. funestus* (Af ^{Perm}) by measuring the permethrin resistance levels of control and *Beauveria*-infected mosquitoes 5 days after fungus exposure. Five replicates of 25 mosquitoes each were performed and mortality of permethrin-exposed mosquitoes corrected for control mosquito mortality.

DDT resistance assays

The effect of *Beauveria*-infection on DDT resistance was tested in five separate experiments in DDT-resistant *A. arabiensis* from Sudan (Aa_2 ^{DDT}) and *A. gambiae s.s.* from Ghana (Ag ^{MR}). Three days after *Beauveria* exposure, control and infected mosquitoes (25 females per group) were exposed to 4% DDT papers or untreated papers

and mortality measured 24 hours later, and corrected for control mortality as described for the permethrin assays.

Data analysis

Differences in the computed survival curves of treated and control mosquitoes were analysed using Cox' regression analyses (33) with SPSS 15.0 software. Mortality data were analysed separately for each tested mosquito species, including all replicates of the control and fungus-infected groups for both the baseline and the insecticide-resistant colony. A full model, with all main effects and possible interactions included, was calculated to estimate the effects of *Beauveria* on the hazard ratio. *Beauveria*-infected mosquito survival was analysed separately to quantify and compare the effect of fungus infection between the resistant and baseline colonies.

Mortality of the permethrin and DDT-exposed groups was corrected for the mortality of their corresponding control groups using the Abbott's formula (32). Corrected mosquito mortality was compared using a Chi-square Goodness of Fit test with GenStat 9.0 software.

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Figure legends

Fig. 1. Effect of fungus infection on baseline and insecticide-resistant mosquito survival**.** Mean (±SEM) cumulative proportional survival of *B. bassiana*-infected (red) and uninfected control mosquitoes (black) of four different mosquito species; *A. funestus* (Af), two strains of *A. arabiensis* (Aa_1 and Aa_2), and *A. gambiae s.s.* (Ag). Survival of baseline colonies is shown on the left and colonies selected for resistance to either permethrin (^{Perm}), DDT (^{DDT}) or bendiocarb (^{Bend}) on the right. Data represent nine replicates, each containing approximately 30 females, except for the baseline Af colony where n=6 for both curves.

Fig. 2. Effect of fungus infection on mosquito insecticide resistance levels. *(a)* Corrected mean (±SEM) percentage mortality of uninfected (black), *Beauveria*-infected (red) and *Metarhizium*-infected (blue) mosquitoes following exposure to permethrin three days after fungal infection. The tested mosquito strains *A. funestus* (Af ^{Perm}), *A. arabiensis* from Sudan (Aa_2 ^{DDT}) and *A. gambiae s.s.* (Ag ^{MR}) exhibit high baseline levels of permethrin resistance. Data represent mosquito mortality of 5 replicates, each containing 25 females. *(b)* Effect of permethrin exposure on *Beauveria*-infected (red) and uninfected (black) permethrin-resistant *A. funestus* (Af ^{Perm}) mosquitoes at 3 days (left) and 5 days (right) post fungal infection. Data represent corrected mean (± SEM) percentage mortality from five replicates, each containing 25 females. *(c)* Corrected mean (±SEM) percentage mortality of *Beauveria*-infected (red) and uninfected (black) DDT-resistant mosquitoes (*A. arabiensis* (Aa_2 ^{DDT}) and *A. gambiae s.s.* (Ag ^{MR})). Data represent five

replicates, each containing 25 females. For each treatment, mortality of mosquitoes exposed to insecticide was corrected for mortality of counterparts exposed to control papers, using the Abbott's formula (32). Asterisks indicate significant differences **: χ^2-test $P<0.01$, ***: χ^2-test $P<0.001$.

Figure 1

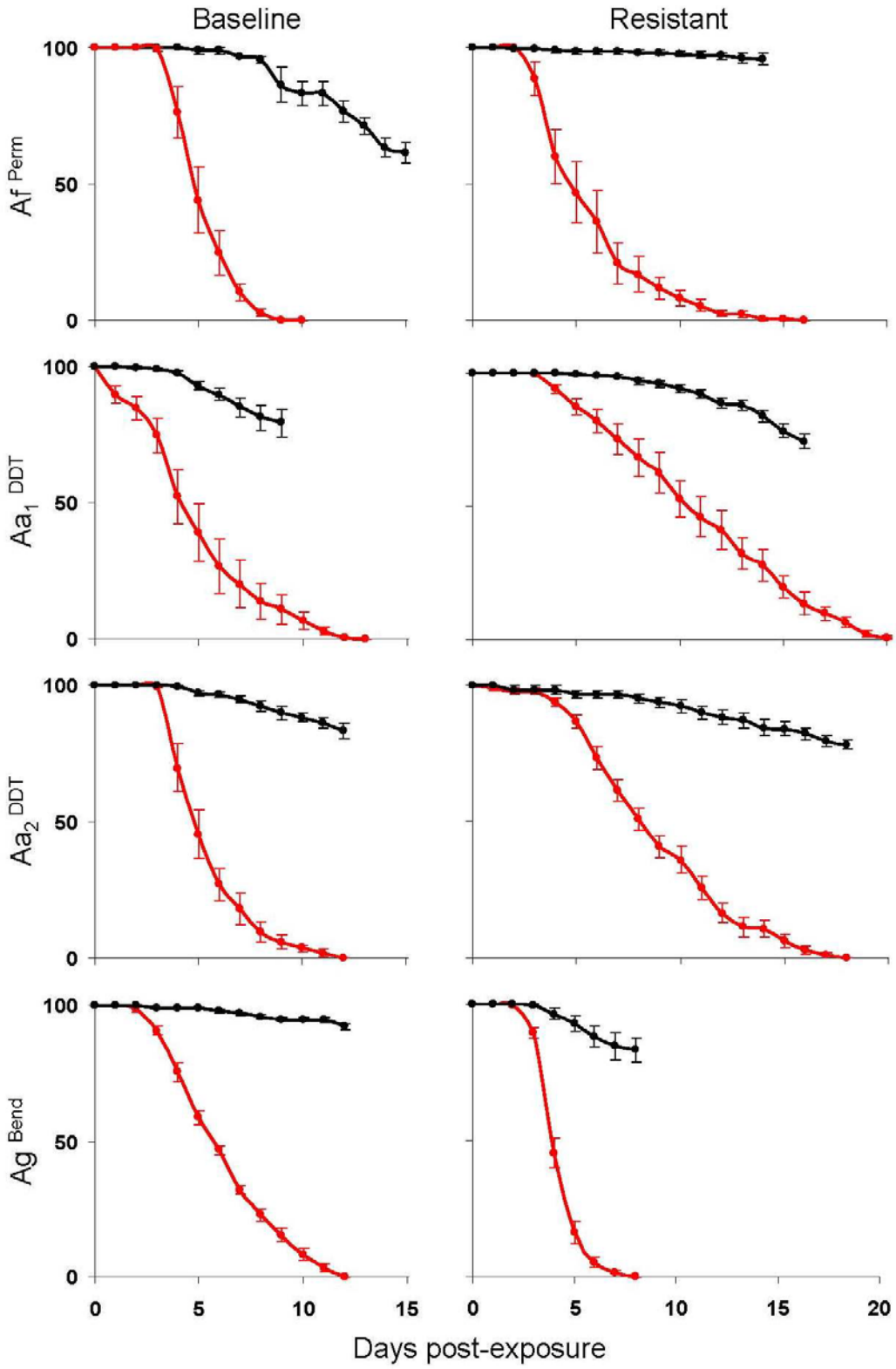
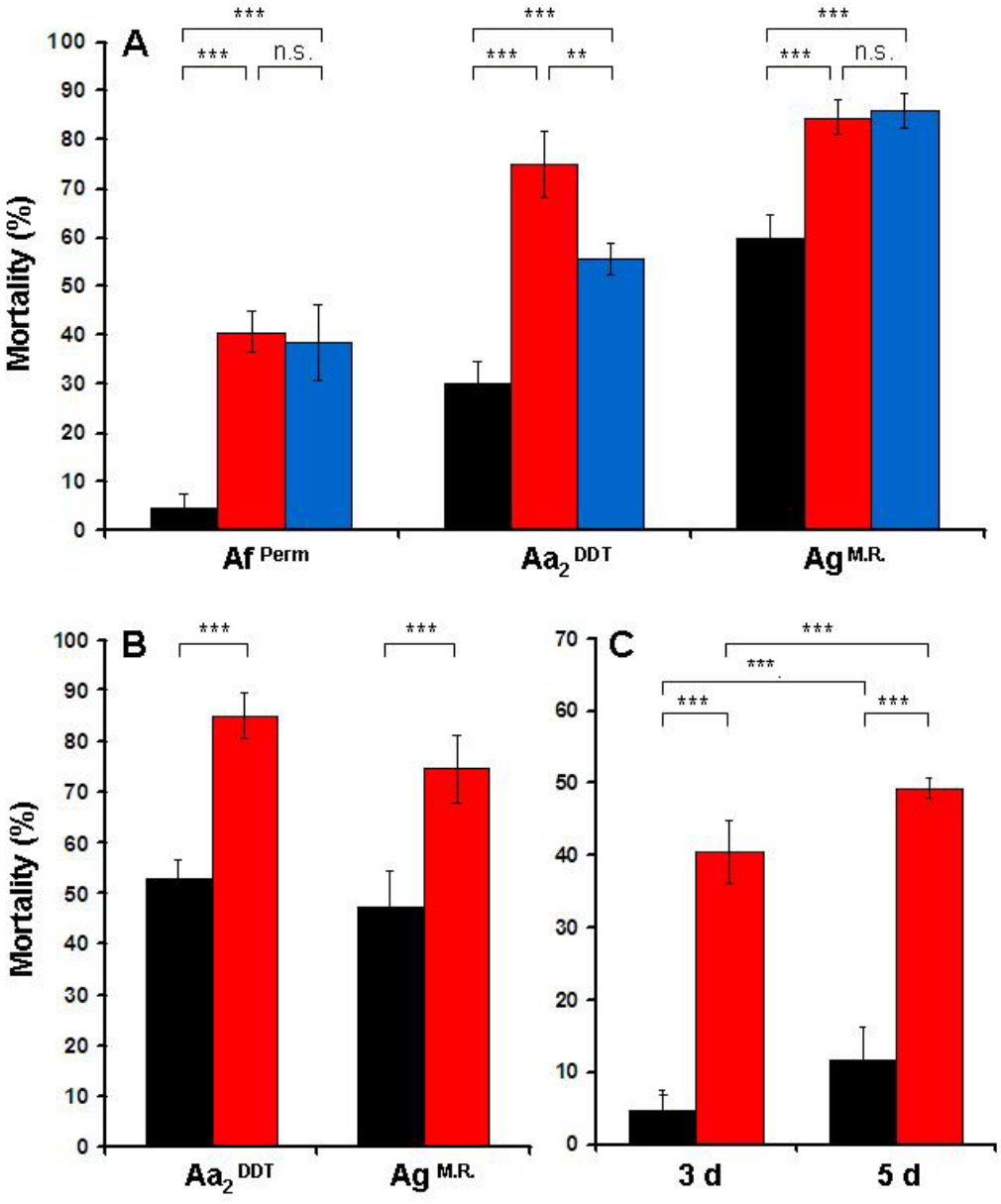

Baseline
Resistant
Af Perm
Aa1 DDT
Aa2 DDT
Ag Bend
100
50
0
0
5
10
15
20
Days post-exposure

Figure 2



Supporting Information

Table S1

Table S1. Details of mosquito species used; abbreviations, colony names, resistance selection and origin.

Species	Abbreviation (used in text)	Colony name		Selected for resistance to	Origin
		Baseline	Resistant		
A. funestus.	Af ^{Perm}	FUMOZ	FUMOZ-R	Permethrin	Southern Mozambique
A. arabiensis	Aa_1 ^{DDT}	MBN	MBN-DDT	DDT	Mamfene, South-Africa
	Aa_2 ^{DDT}	SENN	SENN-DDT	DDT	Sennar, Sudan
A. gambiae s.s.	Ag ^{Bend}	SOG	BENROG	Bendiocarb	Obuasi, Ghana
	Ag ^{MR}	-	GAH	-	Ahafo, Ghana

Table S2

Table S2. Insecticide susceptibility status of the mosquito colonies and species used.

	Pyrethroid		**Carbamate**		**Organo-phosphate**	**Organo-chlorine**	
Colony	0.75% Permethrin	0.05 % Deltamethrin	0.1% Bendiocarb	0.1% Propoxur	5% Malathion	4% DDT	4% Dieldrin
A. funestus							
FUMOZ	R	R	S	R	S	S	S
FUMOZ-R	R	R	S	R	S	S	S
A. arabiensis							
MBN	S	S	S	S	S	S	S
MBN-DDT	R	R	R	S	R	R	S
SENN	R	S	S	S	S	S	R
SENN-DDT	R	R	S	S	R	R	R
A. gambiae s.s.							
SOG	R	R	S	R	S	R	R
BENROG	R	R	R	R	S	R	R
GAH	R	R	R	R	R	R	R

S = insecticide susceptible and R = insecticide resistant. Colonies were tested for their susceptibility to the insecticides listed according to the WHO standard protocol[32]. Those colonies showing mean percentage mortalities >95% were labelled susceptible.