

Delayed mortality effects cut the malaria transmission potential of insecticide resistant mosquitoes

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Malaria transmission has been substantially reduced across Africa through the distribution of long-lasting insecticide treated bednets (LLINs). However, the emergence of insecticide resistance within mosquito vectors risks jeopardizing the future efficacy of this control strategy. The severity of this threat is uncertain because the consequences of resistance for mosquito fitness are poorly understood: whilst resistant mosquitoes are no longer immediately killed upon contact with LLINs, their transmission potential may be curtailed because of longer-term fitness costs that persist beyond the first 24 hours after exposure. Here, we used a Bayesian state-space model to quantify the immediate (within 24h of exposure) and delayed (>24h after exposure) impact of insecticides on daily survival and malaria transmission potential of moderately and highly resistant laboratory populations of the major African malaria vector *Anopheles gambiae*. Contact with LLINs reduced the immediate survival of moderately and highly resistant *An. gambiae* strains by 60-100% and 3-61% respectively, and delayed mortality impacts occurring beyond the first 24 hours after exposure further reduced their overall lifespans by nearly half. In total, insecticide exposure was predicted to reduce the lifetime malaria transmission potential of insecticide resistant vectors by two thirds, with delayed effects accounting for at least half of this reduction. The existence of substantial, previously unreported, delayed mortality effects within highly resistant malaria vectors following exposure to insecticides does not diminish the threat of growing resistance, but posits an explanation for the apparent paradox of continued LLIN effectiveness in the presence of high insecticide resistance.

Anopheles gambiae | Insecticide resistance | Delayed mortality | state-space models | transmission potential

Introduction

Insecticides are the most widespread and successful strategy to control and eliminate insect pest populations (1–3). However, their extensive use has inevitably triggered intense selection for insecticide resistance (IR) in targeted populations (4, 5). Consequently, resistance to one or more classes of insecticides has now been documented in over 440 insects and mite species (6). Resistance can spread extremely fast after its initial emergence. For example, the frequency of mutations associated with pyrethroid resistance has increased 50-1000 fold in insects such as aphids and mosquitoes in less than a decade (7, 8).

The challenge of IR is particularly acute in the *Anopheles* mosquitoes that transmit malaria. Malaria remains a leading cause of mortality and morbidity throughout the tropics, where it is estimated to have killed approximately 438,000 people in 2015 alone (9). Historically, disease burden has been highest in sub-Saharan Africa, but great progress has been achieved over the past 15 years with the number of malaria cases being halved (9, 10). The widespread use of long-lasting insecticide-treated bednets (LLINs) has been the major contributor to this decline (10). LLINs provide physical protection from mosquito bites to people sleeping under them, but the main reason for their success is that the insecticides in them kill mosquitoes within a few hours of contact. The addition of insecticides to nets can almost double

the preventive effect of LLINs (11). Only one class of insecticides, the pyrethroids, has World Health Organization (WHO) approval for use on LLINs (12), and their widespread use has led to the rapid emergence and increase of pyrethroid resistance all across Africa (13). With alternative insecticides for LLINs still several years away from being licensed (14), there is great concern that rapidly increasing IR levels will soon erode and reverse current and future malaria control gains.

The WHO classifies mosquitoes as being IR if the population mortality is <90% in the 24 hours following exposure to insecticides in standardized bioassays (15). According to this definition, resistance to at least one class of insecticide has been identified in malaria vectors from 64 countries with ongoing malaria transmission since 2010 (15). Whilst standardized definitions of resistance are of value for surveillance, the reliability of current metrics for predicting the epidemiological consequences of IR are unclear. Specifically, it is unclear how LLINs maintain high levels of efficacy despite increasing levels of IR. We hypothesize that although IR mosquitoes are no longer killed upon immediate contact with insecticides, they may still suffer longer-term consequences from exposure that indirectly reduce their disease transmission potential.

Mosquito survival is the most important biological determinant of malaria transmission intensity (16, 17). This is because only mosquitoes that survive at least 9 further days after consuming infected blood (i.e. the minimum time required for the parasite to complete its extrinsic incubation period (18)) are capable of onward transmission. Malaria vector survival rates are typically low in natural populations, with <20% expected to

Significance

Insecticide resistance poses one of the greatest challenges to the control of malaria and other vector-borne diseases. Quantifying the magnitude of its impact is essential to ensure the sustainability of future control programmes. Mosquito vectors are defined as “resistant” when insecticides are no longer able to kill them on contact. However, they may suffer longer-term impairment following insecticide exposure that reduces their ability to transmit disease. We show that even highly resistant strains of the major malaria vector *Anopheles gambiae* have their lifespan cut by ~50% after exposure to long-lasting insecticide-treated nets (LLINs). These delayed effects are sufficient to reduce their malaria transmission potential by two-thirds and could partially explain why insecticide resistance is not inextricably associated with LLIN failure.

Reserved for Publication Footnotes

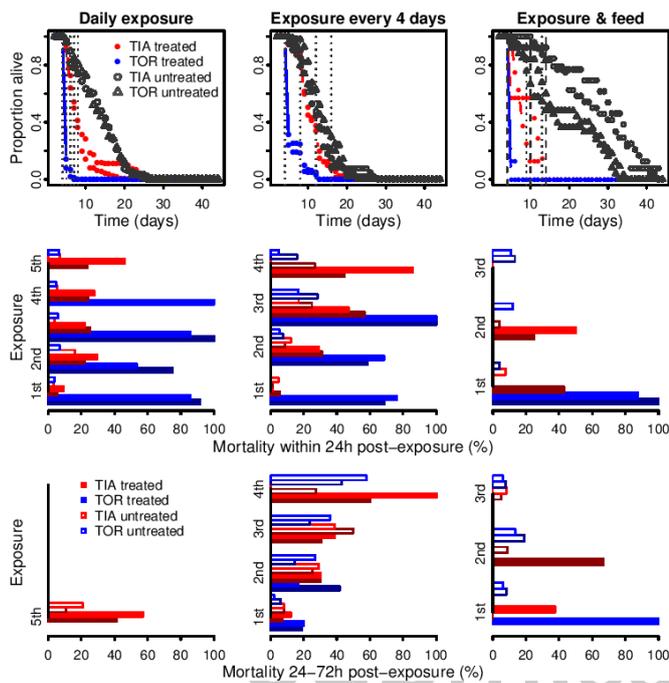


Fig. 1. – Experimental data. Top panels show the observed daily survival curves, i.e. the proportion of mosquitoes from day $x-1$ alive at day x for each exposure regime (across panels), strain (different colours) and treatment (filled vs open symbols) combination. Vertical dotted lines correspond to the time of exposure. Middle panels show the immediate mortality rate of each group, i.e. within 24h of exposure to pyrethroids. Replicates shown with different shades of the same colour. Bottom panels show the delayed mortality rate of each group, i.e. 24 to 72h after exposure to pyrethroids.

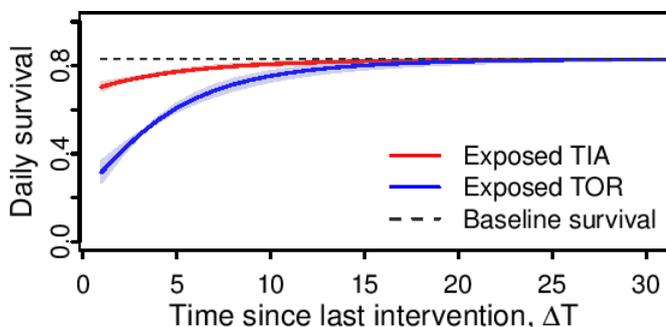


Fig. 2. – Estimated impact of delayed effects of exposure to insecticides on mosquito daily survival of moderately (blue) and highly (red) resistant strains. The dotted line corresponds to the baseline daily survival (and controls) of both strains and the shaded area to the 95% credible interval.

survive long enough to transmit (16, 19). Consequently, even if insecticides have no immediate impact on IR vectors, they could still have a considerable impact on malaria transmission if they reduce the long-term survival of vectors. Additionally, delayed mortality effects of insecticides could effectively slow down the spread of resistance by imposing a cost that prevents resistance genes from going to fixation. Whilst the potential advantages of slow acting insecticides have received theoretical consideration (20), there has been little assessment of whether such effects are already acting within natural vector populations. In this study we test whether reductions in the survival of resistant lines of the major African malaria vector, *Anopheles gambiae*, following repeated insecticide exposures, are evident beyond the first 24 hours after exposure and quantify the associated consequences for their malaria transmission potential. Demonstration of de-

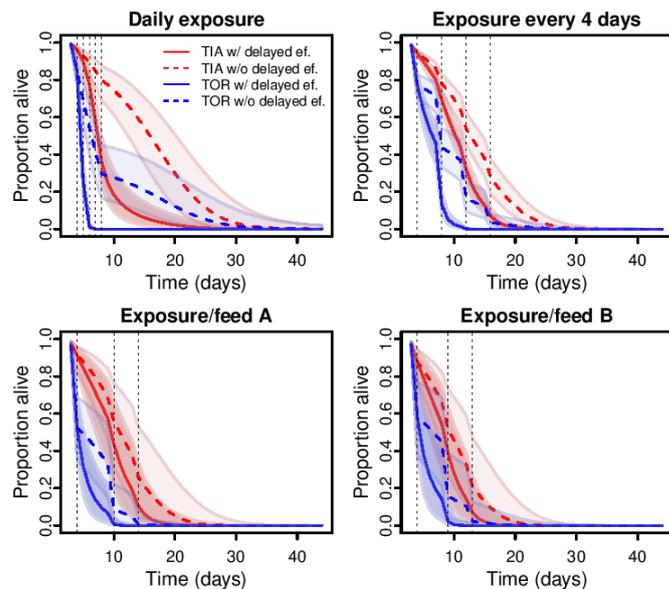


Fig. 3. – Modelled daily survival curves of *An. gambiae* s.s after different exposure regimes to LLINs. Full lines represent the curve estimated from fitting the binomial model to the data, and the dotted lines represent the counterfactual curve predicted with no delayed effects. Lines correspond to the median prediction with shaded 95% credible intervals.

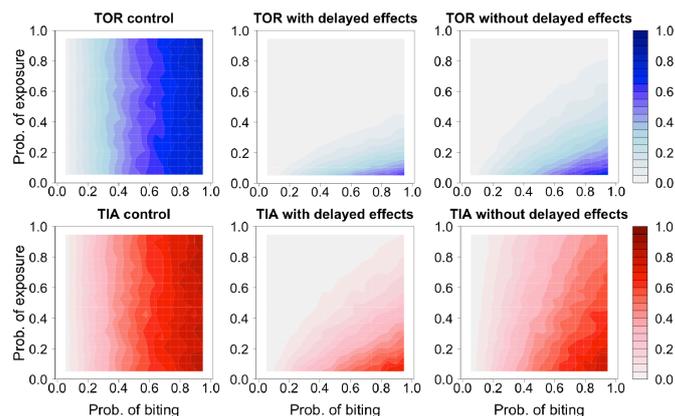


Fig. 4. – Contour plots of the mean number of infectious bites per mosquito of TOR (blue upper panels) and TIA (red bottom panels) strains obtained for mosquitoes exposed to untreated (control) and insecticide-treated nets with and without delayed effects across varying probabilities of biting (x-axis) and exposure (y-axis).

layed mortality impacts from LLIN exposure in resistant malaria vectors could considerably alter prediction of the epidemiological risk posed by IR (16, 17).

Results

We investigated the immediate (within 24 hours) and life-long impact of insecticide exposure in two IR strains of *Anopheles gambiae* mosquitoes: i) *Tiassale* (TIA) and ii) *Tororo* (TOR). Both strains are defined as pyrethroid-resistant according to the WHO definition (15) but the exposure duration required to kill 50% of the TIA is 26 times longer than for the TOR strain, indicating that the levels of IR are substantially higher in the former (21). Cohorts of ~100 females of each strain were exposed either to a LLIN coated with the pyrethroid deltamethrin (Permanet 2.0®; LLIN treatment) or to an untreated bednet (control) in WHO standard cone bioassays (15). Over a series of different experiments, the frequency with which mosquitoes were exposed

Table 1. – Estimated (EST; i.e. with delayed effects) and counterfactual (CF; i.e. without delayed effects) mean daily survival over mosquitoes entire lifespan, and mean proportion of mosquitoes alive at day 9 after first exposure, for each treatment (exposed or unexposed to insecticides), strain and exposure regime: A: daily exposure; B: Exposure every 4 days; and C1 and C2: Exposure with simultaneous blood meal. Dash reflect absence of CF value.

Strain	Regime	Mean daily survival		Prop. alive day 9	
		EST	CF	EST	CF
TIA (exposed)	A	0.80	0.90	0.25	0.77
	B	0.74	0.85	0.60	0.74
	C1	0.70	0.79	0.58	0.69
	C2	0.64	0.74	0.39	0.52
TOR (exposed)	A	0.46	0.88	0.00	0.29
	B	0.43	0.81	0.05	0.42
	C1	0.35	0.66	0.07	0.33
	C2	0.38	0.70	0.02	0.16
TIA (unexposed)	A	0.83	-	0.75	-
	B	0.80	-	0.70	-
	C1	0.96	-	0.95	-
	C2	0.96	-	0.96	-
TOR (unexposed)	A	0.83	-	0.75	-
	B	0.82	-	0.74	-
	C1	0.93	-	0.91	-
	C2	0.93	-	0.91	-

to these treatments varied: A) *Daily exposure* for 5 consecutive days; B) *Exposure every 4 days*, for a maximum of 4 exposures over 16 days, and C) *Exposure & feed*, where mosquitoes were exposed every 4 to 6 days for a maximum of 4 exposures, and blood-fed during exposure (in contrast to other regimes where mosquitoes were fed only sugar water; see Methods). These regimes were selected to investigate a range of biologically plausible exposures. Specifically, under natural conditions *An. gambiae* is expected to blood fed once every 2-4 days (22). If a bloodmeal is successfully obtained, the mosquito will refrain from feeding until eggs have been laid (~4 days). Regime A mimics a mosquito that is repeatedly prevented from biting by the presence of a LLIN (thus contacts LLINs on consecutive nights), whereas Regime C corresponds to the scenario where the mosquito is able to bite through the LLIN while simultaneously feeding. Together these regimes cover the likely maximum (daily) and minimum (every 4 days) exposure that *An. gambiae* would expect in areas of high LLIN coverage. In all experiments, mosquitoes were first exposed to insecticides when they were 4-5 days old, and then monitored daily to record mortality until no survivors remained (i.e. maximum of 44 days). Each experiment (A, B & C) was replicated twice per strain, with the exception of the Daily exposure experiment for which there was only one replicate per strain in the control treatment.

Across all experimental regimes, mosquito survival was lower after exposure to insecticides in comparison to the control treatments (Fig. 1 upper plots, black versus coloured lines). Survival was also higher in the more resistant TIA than TOR strain (red vs blue lines), but consistent between replicates of the same experimental treatment and strain combination (lines of same colour). Overall, mortality rates in the 24 hours following exposure to insecticides ranged from 60-100% in the TOR strain, and 3-61% in the TIA strain. The 24-hour mortality of mosquitoes exposed to untreated nets was <20% in both strains (Fig. 1 middle panels). The mortality rate between 24h and 72h (within 1 and 4 days) after last exposure of TIA ranged from 7-100%, which was higher than

that of the controls that ranged 2-57% (Fig. 1, bottom panels). When present this delayed mortality was also higher in the TOR strain (20-100%) than in the controls.

Impact of immediate and delayed effects on survival

Our aim was to test whether reductions in mosquito survival following insecticide exposure persisted beyond the first 24 hours after exposure. To distinguish and quantify these immediate and delayed impacts, we used a Bayesian nonlinear state-space model (SSM) on the cohort data, in which observed daily survival was modelled as a binomial process. Briefly, the model described the daily survival of each strain under the different exposure regimes (A-C) and treatments (exposed or control). Amongst the candidate models tested (i.e. models with varying covariate combinations; see Methods for further details), the one with the highest degree of support incorporated both immediate and delayed impacts of insecticide exposure, and senescence (i.e. increase in baseline mortality rate with age; see Methods and model fit in Fig. S1 in Supplementary Information [SI]). Support for the inclusion of both immediate and delayed impacts of insecticide exposure was particularly strong (see Table S2 and S3 in SI).

The magnitude of insecticide impacts varied between strains (Fig. 2, blue and red lines). For example, the mean daily survival of the TOR strain was 3.7 times lower in the 24 hours following insecticide exposure (at $t=0$ in Fig. 2) than in the unexposed control (Table I), whereas survival in the TIA strain was only 1.2 times lower than the controls over the same period. Similar strain differences were observed in the magnitude of delayed mortality impacts (>24h after exposure; Fig. 2). Although both strains experienced a permanent reduction in survival >24h following LLIN exposure (i.e. the pre-exposure age-independent baseline daily survival levels are never achieved again, Fig. 2 dotted lines); TIA mosquitoes were predicted to require ~7 days to recover their daily survival rate to 95% of the baseline, whereas TOR mosquitoes required ~14 days (i.e. Fig. 2). The delayed mortality effects of TIA disappear faster mainly because the initial impact on TOR survival (i.e. immediate mortality) was much greater, which resulted in a longer period of recovery back (asymptotically) to the baseline daily survival (i.e. control daily survival rate; Fig. 2). After exposure to untreated nets, the daily survival of control mosquitoes from either strain was unaffected by long-term residual impact of insecticides, and remained at baseline levels (Fig. 2, dotted line).

To further investigate the magnitude of delayed mortality impacts of insecticide exposure, we used our model to contrast scenarios in which these effects were present (as estimated in data, EST) and in which they were removed (counterfactual, CF). Comparison of the estimated and counterfactual survival estimates (Fig. 3, Table I) indicates that the median lifespan of TOR mosquitoes is reduced by 17-57% in the presence of delayed mortality impacts relative to when they are absent. The median life span in the TIA strain was also estimated to be reduced by 0-40% (depending on exposure regime) in the presence of delayed mortality impacts of insecticides (Fig. 3, Table S4). We investigated how these delayed mortality impacts influenced the proportion of mosquitoes surviving for 9 days after 1st exposure; which is the minimum necessary time for a mosquito to transmit malaria assuming it was infected on first bite (18). The proportion of TIA mosquitoes expected to live at least 9 days following insecticide exposure was predicted to be 25-60% (across different exposure regimes) in the presence of observed levels of delayed mortality, rising to 52-77% when these effects were counterfactually removed (Table I). These differences were even more pronounced within the TOR strain, where <7% were estimated to survive for 9 days following insecticide exposure when delayed mortality impacts were acting (EST), compared to 16-42% when only immediate impacts were assumed (CF, Table I).

The impact of insecticides also differed between insecticide exposure regimes (within each strain). In both strains, mosquito mean daily survival across their lifespan was higher in regime A, with consecutive daily exposures, than in the regime B with similar number but more spaced out exposures (e.g. Table I). However, a smaller proportion of mosquitoes survived until 9 days after first bite in higher frequency daily exposure compared to other treatments (e.g. regime A vs. B and C). For example, no TOR mosquitoes were estimated to be alive at day 9 in the daily exposure regime compared to 2-7% in treatments where exposures were spaced over 4-5 days. Similarly, 25% of TIA mosquitoes were estimated to survive until day 9 under the daily exposure regime, compared to 39-60% when exposures were spaced out (Table I). For regime C, the mean daily survival was ~10% lower in both strains compared to regimes A and B. However, the comparative magnitude of all longevity measures (Table I) between strains was similar with those of regime B, which had similar exposure frequencies. Despite these differences across regimes, the magnitude of delayed insecticide impact was relatively similar. For example, the counterfactual mean daily survival of the TOR strain was approximately 1.9 fold higher than that estimated under each of the three exposure regimes. Similarly, the counterfactual mean daily survival of the TIA strain was approximately 1.2 fold across all exposure regimes (Table I).

Empirically, the delayed effects were higher in Regime C (Fig. 1, bottom panels). To guarantee that the detection of delayed effects was not purely driven by this regime in our models, we re-run the model without regime C. The magnitudes of immediate and delayed effects were slightly smaller but still significant in this analysis, and show clear evidence of delayed effects even with the exclusion of Regime C. These outputs are shown in SI (Table S3).

Implications for malaria transmission potential

Using the observed and counterfactual survival curves, we developed a stochastic individual-based simulation to investigate the potential epidemiological consequences of delayed mortality following insecticide exposure in IR strains of *An. gambiae*. These impacts were quantified in terms of the number of potentially infectious bites a mosquito would be expected to deliver under scenarios where the mortality effects following exposure to insecticides is of a similar magnitude to that detected in our experimental data. Our simulation predicted the probability distribution of the number of infectious bites that a TIA and TOR mosquito could deliver over its lifetime (assuming it was infected on its first bite). Transmission potential (quantified as the mean of this distribution) was simulated under varying levels of insecticide exposure and biting probabilities (detailed in Methods and SI). Predictions were obtained both in the presence of immediate and delayed mortality effects following exposure (as observed in our data), and under the counterfactual scenario where these delayed mortality effects were absent.

Under the control scenarios (exposure to untreated nets), transmission potential was dependent only on biting probability (Fig. 4, left panels) and was relatively high, with 47% of mosquitoes from both strains having potential to deliver at least 1 infectious bite (Fig. 4). Exposure to LLINs was estimated to reduce the overall transmission potential of both TIA and TOR strains by 3.3 and 7.8 times respectively (see reduction of dark blue and red areas across panels in Fig. 4). Notably, there were marked differences between the transmission potential of mosquitoes exposed to insecticides, depending on whether they were assumed to experience immediate mortality impacts, or both immediate and delayed impacts of the magnitude detected in our experiments (Fig. 4). For example, across all combinations of biting and exposure probabilities, the proportion of TIA mosquitoes expected to deliver at least one infectious bite was 33% when only immediate mortality was considered, compared to 14% when delayed impacts were also incorporated. Similarly, for the TOR

strain, the proportion of mosquitoes with potential to deliver one infectious bite fell from 12% to 6% when delayed as well as immediate mortality impacts were included. Thus, incorporation of delayed mortality effects from insecticide exposure is expected to significantly curtail the transmission potential of even technically-defined "resistant" malaria vectors.

Discussion

The cumulative impact of LLIN exposure on the survival of even highly resistant *An. gambiae* mosquitoes was estimated to reduce their expected lifetime transmission by 3-fold, with delayed effects accounting for at least half of this reduction. If delayed mortality effects of similar magnitude occur in natural conditions, estimates of transmission potential of IR mosquitoes should be reduced to ~50% to what would be assumed if insecticides had no impact on their survival.

To our knowledge, delayed mortality effects of a similar magnitude to ours have not been described in malaria vectors or any other insecticide resistant insect. Although the distinction between immediate and delayed mortality has been discussed for other resistant insects (e.g. lesser grain borer which infects maize (23)), the magnitude of the effects from exposure to pesticides has not been accurately quantified. Our results are the first clear evidence that delayed mortality effects occur in IR *Anopheles sp.*, and that they are of sufficient magnitude to have important epidemiological implications for the continued control of malaria.

The magnitude of delayed mortality effects varied between the two *An. gambiae* strains used here. These differential impacts may be reflective of the mechanisms of resistance within these two strains. Physiological resistance to insecticides can arise through target site mutations that interfere with insecticide binding, metabolic resistance in which insecticides are detoxified by the overproduction of enzymes, and penetration resistance in which the mosquito cuticle is altered in a way that inhibits insecticide uptake (13). The TOR strain exhibits target site resistance through the L1014S *kdr* mutation (24); but has shown no clear evidence for metabolic resistance. In contrast, the TIA strain has both target site resistance arising from a high frequency of 1014F *kdr* allele and metabolic resistance arising from elevated expression of key P450s (25). It is likely that the long-term impacts of LLIN exposure on mosquito survival were minimized in the TIA strain because of its additional capacity to detoxify residual insecticides. If so, the delayed mortality effects could be a transitory feature arising along the evolutionary pathway from full susceptibility to 'complete' resistance (e.g. resistance via multiple mechanisms). For example, delayed mortality impacts may be of most significance in populations where resistance has newly arisen and is conferred by a limited range of target site mutations, but have minimal impact in populations that have developed both multiple resistance mechanisms and compensatory mutations through years of intense selection. Thus even though delayed mortality impacts of insecticides may be reducing the transmission potential of IR mosquitoes under current conditions, this mitigating effect could become eroded by continued, intense selection for resistance in the future.

Our findings may help explain the apparent paradox of increases in the number of malaria cases averted over time that are attributed to LLINs across Africa (10), even in the face of increasing resistance. If IR was causing widespread failure of LLINs, the impact of LLINs on malaria transmission across Africa would be reduced. The available evidence on how IR influences malaria risk is small and shows some discrepancies. For example, parallel studies in Malawi where *An. funestus* is moderately resistant variously reported that LLINs appeared to have little impact (i.e. when the endpoint was prevalence (26)), or were still reducing transmission by 30% (i.e. when the endpoint was incidence (27)). However, recent models suggest that LLINs

continue to be responsible for the vast majority of malaria cases averted in Africa over the last decade (10) even with increasing IR. The presence of these delayed mortality effects, which reduce the impact of IR on transmission, may help explain why a widespread, catastrophic impact of IR has not yet been observed. But because the reduction in malaria transmission potential by mosquitoes exposed to LLINs seems to decrease with increasing intensity of IR (i.e. TOR vs TIA), our findings also serve as a warning that resistance could eventually reduce the public health benefit of pyrethroid-based LLINs.

Some studies have shown that exposure to insecticides alters the behavior of IR arthropods in a way that could indirectly reduce their fitness (e.g. altered dispersal, reduced neurosensory perception and higher risk of predation (13, 28)). For example, exposure to neonicotinoid insecticides at sub-lethal concentration decreases the feeding activity of the grain aphid (23). Similarly, *An. gambiae* exposed to LLINs seem to temporarily lose the ability to host-seek (29). This study did not test for such additional indirect impacts, however preliminary data indicates a reduction in the feeding success of exposed IR mosquitoes. In this and other studies (30, 31) it was observed that the legs of mosquitoes can become detached when trying to feed through nets, which would be one mechanism to explain their subsequent reduction in blood feeding. Further work is needed to quantify this phenomenon and other indirect fitness consequences of LLIN exposure in IR mosquitoes to calculate their combined impact on transmission (13). Alternatively, contact with LLINs could prompt behavioural changes that increase the transmission potential of IR mosquitoes, by for example, changing the time and location of their biting to avoid nets (e.g. "behavioural resistance" (32)). Furthermore, previous studies have suggested that resistance is associated with changes in the susceptibility of mosquitoes to infection (ranging from an enhancement, reduction, or no change (33–35)). IR also drives various physiological modifications that may ultimately impact survival and parasite competence (28). For example, resistant *Anopheles* and other taxa, have an increased capacity to tolerate oxidative stress, which in turn reduces long-term survival (36, 37). Thus whilst results presented here constitute valuable proof-of-principle on delayed mortality impacts from insecticide exposure, consideration of a wider range of indirect consequences is needed to accurately predict the transmission potential of IR mosquitoes.

A previous study tested for a cumulative impact of low dose insecticide exposure in *Anopheles*, but found no evidence of higher mosquito mortality following repeated exposures (33). Similarly, our results show no association between the immediate mortality of mosquitoes following exposure, and the number of times they had been previously exposed. However, we also show that mosquitoes' natural mortality varies with age. Older mosquitoes have been previously shown to be more susceptible to pyrethroids than their younger counterparts (33, 38). Our findings suggest this result may have been driven by changes in the natural mortality of mosquitoes over time (i.e. senescence) rather than increases in susceptibility to insecticide exposure. The ability to estimate additional effects, such as senescence, is one of the advantages of using our modeling approach. The state-space framework used to analyze the survival curves was also critical for the quantification of the non-linear effect of delayed effects of exposure on mosquito mortality, which would not be possible with more commonly used survival analysis.

Our findings highlight the importance of investigating the impacts of resistance beyond immediate mortality. The existence of previously ignored delayed mortality effects presents a hypothesis for why the presence of pyrethroid resistance in African malaria vectors does not appear to have resulted in widespread reductions in LLIN efficacy (10, 27). However, the present study warns that increasing resistance could erode the ability of LLINs

to hold back malaria. As the degree of resistance increases, the magnitude of these delayed mortality impacts may diminish and eventually disappear. This study provides a proof-of-principle for the existence of these delayed mortality effects at a magnitude that could have significant implications for malaria transmission. Ideally the next step would be to validate these findings in wild populations, and assess their relevance to operational control. There are currently several constraints to testing this hypothesis in the field; namely difficulties in aging and determining the history of insecticide exposure of wild mosquitoes and mark-recapture methods for survival estimation have poor efficiency (39). Whilst technology develops, alternatively, this phenomenon could be investigated under semi-field conditions (40) where wild mosquitoes can be exposed to LLINs under realistic but contained conditions. Further empirical studies combined with the modeling framework developed here will be vital for prediction of the impact of insecticide resistance on malaria control.

Methods

Experimental design

Two strains of *An. gambiae* mosquitoes differing in their IR levels were used in this study: Tiassale (TIA) which originates from Southern Cote d'Ivoire, and Tororo (TOR) from Uganda. Details of their resistance profile can be found in (21) and references therein. A fully susceptible strain was not included in this study as all mosquitoes die within 24h and hence delayed mortality cannot be measured. Cohorts of ~100 mosquitoes of each strain were exposed to Permanet 2.0 LLINs containing 50mg/m² deltamethrin (Vestergaard-Frandsen), the standard dose to mimic field exposures, or to an insecticide-free bednet for 3 minutes using the WHO cone bioassay (15). Details of the experimental design, such as sample sizes and frequency of exposure are detailed in Table S1 in SI. Three alternative exposure regimes were used A) Daily exposure; B) Exposure every 4 days; and C) Exposure & feed; and two replicates were carried out for each regime and strain combination. The mosquitoes for the replicates were taken from different colony cohorts apart from those in regime A, which were from the same colony cohort (hence only 1 replicate was available for A). Mortality was recorded daily starting 24 hours after the first exposure and all surviving mosquitoes were held with access to sugar solution *ad libitum*. For the exposure regime C, mosquitoes were starved of sugar water 12 hours prior to exposure and mosquitoes were aspirated into two containers, one covered with a Permanet 2.0 and the second with an untreated net. Mosquitoes were provided access to a blood meal for twenty minutes via a volunteer's arm rested on the netting of each container. Unfed mosquitoes were then counted and discarded. Mortality was recorded daily starting 24 hours after the first exposure. At the end of the bioassay, daily mortality was available for a total of 1497 mosquitoes, from 22 different experimental groups (3 exposure regimes, 2 strains, 2 treatments i.e. exposed and non-exposed to insecticide, and 2 replicates).

Bayesian survival model

A Bayesian state-space model (SSM) was constructed to quantify the impact of the different insecticide exposure regimes on *An. gambiae* survival, and disentangle the impacts of immediate (i.e. within 24 hours of exposure) and long-term cumulative mortality. The observed number of mosquitoes alive, $N_{i,t}$, in each experimental replicate i (22 in total), at time t , was modelled as a binomial variable: $N_{i,t} \sim \text{Binomial}(S_{i,t}, N_{i,t-1})$; where $N_{i,t-1}$ is the total number of mosquitoes alive in group i at time $t-1$ and $S_{i,t}$ is the probability of daily survival described with a logit link to its non-linear predictor ($\bar{S}_{i,t}$):

$$\bar{S}_{i,t} = \beta_0 + \beta_1 t + \beta_2 t^2 - \beta_{3,x,s} E_{i,t} + u_i \quad (1)$$

Here, β_0 corresponds to the intercept and the coefficients β_1 and β_2 were used to incorporate natural mortality (i.e. senescence) over time (or age, t). The short-term 'immediate' impact of exposure to a (treated or untreated) bednet, on mosquito daily survival was represented by the coefficient β_3 , which was allowed to have a different value for each treatment x (i.e. exposed or unexposed to insecticides) and strain s (i.e. TIA or TOR) combination. Biologically, $\beta_{3,x,s}$ corresponds to the magnitude (in the predictor scale) of the reduction in daily survival occurring after exposure. Exposure is treated as the non-linear covariate E and was introduced to quantify the postulated delayed effects of insecticide, which was constructed as the superposition of multiple, time-decaying effects corresponding to the multiple exposure regimes:

$$E_{i,t} = \sum e^{-\beta_{4,x,s} \Delta T_{i,t}} \quad (2)$$

where, β_4 quantifies the decay rate of the delayed mosquito mortality risk after exposure, and is specific to each treatment x and strain s ; and ΔT the time since last exposure in each replicate i at time t . The coefficient u was incorporated into the model as a Gaussian random effect that accounts for

681 other unattributed differences between replicates. Further details, including
682 prior distributions and model code are provided in SI.

683 Model selection

684 An initial set of 11 candidate models representing differing, biologically
685 plausible permutations of our predefined coefficients: i.e. senescence (as a
686 linear or quadratic effect), immediate effects of exposure, delayed effects
687 of exposure and random effect of replicate; were constructed (see Table S2
688 in SI). After assessing convergence, model goodness-of-fit and the Deviance
689 Information Criterion (DIC) of all candidate models (41), we chose the best
690 model (described in equation 1). All models were fit using Monte Carlo
691 Markov Chain methods within software JAGS (42) via interface with R (R
692 Development Core Team). Further details can be found in SI.

693 Prediction of the impact of delayed effects

694 The survival curves $S_{i,t}$ for each replicate were estimated as a function
695 of the predicted coefficients obtained from equation 1. The relative impact
696 of delayed effects was quantified by comparing these survival curves, which
697 incorporated delayed effects of the magnitude detected in experimental re-
698 sults, with "counterfactual" scenarios in which their effect had been removed
699 after model fitting. This was done during the refit of the model by setting
700 the decay rate coefficient of delayed effects ($\beta_{4,x,s}$) to the very high value
701 of 10000 (i.e. delayed effects do not exist and only immediate mortality can
702 impact mosquito survival).

703 Transmission potential (T_p)

704 A stochastic individual-based simulation was used to investigate the
705 potential epidemiological consequences (i.e. transmission potential, T_p) of
706 delayed mortality following insecticide exposure in resistant strains of *An.*
707 *gambiae*. These impacts were quantified in terms of the number of poten-
708 tially infectious bites a mosquito would be expected to deliver under
709 scenarios when exposure to insecticides is of a similar magnitude as detected
710 in our experimental data.

711 We simulated transmission potential for the full range of combinations
712 for the probabilities of biting and exposure, although some of the combina-
713 tions in this space of scenarios are unlikely (e.g. it is near-impossible that with
714 an exposure probability of 1 implying an intact LLIN, biting probability can
715 ever approach 1). We explored the space of exposure and biting probabilities
716 through 400 distinct combination scenarios (20x20 values) and each scenario
717 was simulated 1500 times to obtain a frequency distribution for the number
718 of infectious bites. The simulation used the following assumptions: (i) adult
719 female mosquitoes began their life on day zero, and were given their first

720 opportunity to blood-feed on day 2; (ii) all mosquitoes became infected with
721 malaria upon their first blood meal; after feeding, surviving mosquitoes had
722 the opportunity to blood feed again every 3 days; (iii) Feeding success was
723 determined as a binomial distribution based on the probability of biting
724 achieved for each draw; (iv) mosquitoes become infectious after an average
725 of 12 days after becoming infected; This incubation period was drawn from a
726 normal distribution with mean 12 and standard deviation of 1.5, which
727 resulted in a range between 9 days and 23 days (values known to occur at
728 temperatures between 30° and 20°C (18)).

729 Based on these assumptions and the generated probabilities of exposure
730 and biting, a binomial process was simulated to determine when a mosquito
731 was exposed to insecticides and when it was successful at biting, during their
732 lifetime (i.e. from day 1 to day 50). The daily survival of each mosquito was
733 based on the estimated posterior distributions of the SSM implemented to
734 our experimental data (i.e. equation 1). For each mosquito of each strain (TIA
735 and TOR) and treatment (exposed to insecticide treated nets and control),
736 the survival curves (equation 1) were re-estimated using the exposure over
737 time (i.e. across the 50 days when exposures occurred) obtained from the
738 exposure-biting relationship, and independent draws from the posterior
739 distributions of the coefficients obtained from the SSM for the respective
740 observed and counterfactual (without delayed effects) survival curves. The
741 use of the posterior distributions, as opposed to a mean coefficient, ensured
742 that all uncertainty was correctly propagated through to the estimates of
743 transmission potential. The survival state of a mosquito at day t (alive or
744 dead from day 1 to 50) was also defined through a binomial process with a
745 probability of daily survival.

746 Finally, the total number of infectious bites expected to be delivered by
747 a mosquito, or transmission potential (T_p) of each mosquito, was obtained:

$$748 T_p = \sum_t S_t B_t I_t \quad (3)$$

749 Where S_t is the survival state on day t (i.e. alive or dead), B_t is the number of
750 bites on day t and I_t is the infectious state on day t. The T_p of each mosquito
751 were finally used to generate a heatmap of transmission potential across the
752 varying exposure and biting probabilities, for each strain, with and without
753 delayed effects.

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- 756 1. Aktar W, Sengupta D, Chowdhury A (2009) Impact of pesticides use in agriculture: their
757 benefits and hazards. *Interdiscip Toxicol* 2(1):1–12.
- 758 2. Dedyryer C-A, Le Ralec A, Fabre F (2010) The conflicting relationships between aphids and
759 men: A review of aphid damage and control strategies. *C R Biol* 333(6–7):539–553.
- 760 3. Hemingway J (2014) The role of vector control in stopping the transmission of malaria:
761 threats and opportunities. *Philos Trans R Soc London B Biol Sci* 369(1645).
- 762 4. Melander AL (1914) Can insects become resistant to sprays? *J Econ Entomol* 15:400–404.
- 763 5. Whalon ME, Mota-Sanchez D (2008) *Global pesticide resistance in arthropods* (CABI,
764 Wallingford) doi:10.1007/s13398-014-0173-7.2.
- 765 6. Roush RT, Tabashnik BE (2002) *Pesticide Resistance in Arthropods* (Springer New York, New
766 York [etc.]) doi:10.1007/978-1-4684-6429-0.
- 767 7. Foster SP, et al. (2014) A mutation (L1014F) in the voltage-gated sodium channel of the grain
768 aphid, *Sitobion avenae*, is associated with resistance to pyrethroid insecticides. *Pest Manag Sci*
769 70(8):1249–1253.
- 770 8. Toé KH, et al. (2014) Increased Pyrethroid Resistance in Malaria Vectors and Decreased Bed
771 Net Effectiveness, Burkina Faso. *Emerg Infect Dis* 20(10):1691–1696.
- 772 9. WHO (2015) *World Malaria Report 2015* doi:978 92 4 156515 8.
- 773 10. Bhatt S, et al. (2015) The effect of malaria control on *Plasmodium falciparum* in Africa
774 between 2000 and 2015. *Nature* 526(7572):207–211.
- 775 11. Russell TL, et al. (2010) Impact of promoting longer-lasting insecticide treatment of bed
776 nets upon malaria transmission in a rural Tanzanian setting with pre-existing high coverage
777 of untreated nets. *Malar J* 9:187.
- 778 12. Kelly-Hope L, Ranson H, Hemingway J (2008) Lessons from the past: managing insecticide
779 resistance in malaria control and eradication programmes. *Lancet Infect Dis* 8(6):387–389.
- 780 13. Ranson H, et al. (2011) Pyrethroid resistance in African anopheline mosquitoes: what are
781 the implications for malaria control? *Trends Parasitol* 27(2):91–98.
- 782 14. Ranson H, Lissenden (2016) Insecticide resistance in African Anopheles mosquitos: A
783 worsening situation that needs urgent action to maintain malaria control. *Trends Parasitol*
784 In press.
- 785 15. WHO (2013) *Test procedures for insecticide resistance monitoring in malaria vector mosquitoes*.
- 786 16. Garret-Jones C, Shidrawi GR (1969) Malaria vectorial capacity of a population of Anopheles
787 gambiae. *WHO Bull* 40:531–545.
- 788 17. Ferguson HM, et al. (2012) Selection of mosquito life-histories: a hidden weapon against
789 malaria? *Malar J* 11:106.
- 790 18. Beier JC (1998) Malaria parasite development in mosquitoes. *Annu Rev Entomol* 43:519–543.
- 791 19. Noden BH, Kent MD, Beier JC (1995) The impact of variations in temperature on early
792 *Plasmodium falciparum* development in Anopheles stephensi. *Parasitology* 111(05):539–545.
- 793 20. Read AF, Lynch PA, Thomas MB (2009) How to Make Evolution-Proof Insecticides for
794 Malaria Control. *PLoS Biol* 7(4):e1000058.
- 795 21. Bagi J, et al. (2015) When a discriminating dose assay is not enough: measuring the intensity
796 of insecticide resistance in malaria vectors. *Malar J* 14:210.
- 797 22. Gillies MT (1953) The duration of the gonotrophic cycle in Anopheles gambiae and Anopheles
798 funestus, with a note on the efficiency of hand catching. *East Afr Med J* 30(4):129–135.
- 799 23. Iversen Getchell A, Subramanyam B (2008) Immediate and Delayed Mortality of Rhyzopertha
800 dominica (Coleoptera: Bostrichidae) and Sitophilus oryzae (Coleoptera: Curculion-
801 idae) Adults Exposed to Spinosad-Treated Commodities. *J Econ Entomol* 101(3):1022–1027.
- 802 24. Ramphul U, et al. (2009) Insecticide resistance and its association with target-site mutations
803 in natural populations of Anopheles gambiae from eastern Uganda. *Trans R Soc Trop Med Hyg*
804 103 (11):1121–1126.
- 805 25. Edi CVA, Koudou BG, Jones CM, Weetman D, Ranson H (2012) Multiple-Insecticide
806 Resistance in Anopheles gambiae Mosquitoes, Southern Côte d'Ivoire. *Emerg Infect Dis*
807 18(9):1508–1511.
- 808 26. Mathanga DP, et al. (2015) The effectiveness of long-lasting, insecticide-treated nets in a
809 setting of pyrethroid resistance: a case-control study among febrile children 6 to 59 months
810 of age in Machinga District, Malawi. *Malar J* 14(1):457.
- 811 27. Lindblade KA, et al. (2015) A cohort study of the effectiveness of insecticide-treated bed nets
812 to prevent malaria in an area of moderate pyrethroid resistance, Malawi. *Malar J* 14:31.
- 813 28. Rivero A, Vézilier J, Weill M, Read AF, Gandon S (2010) Insecticide Control of Vector-
814 Borne Diseases: When Is Insecticide Resistance a Problem? *PLoS Pathog* 6(8):e1001000.
- 815 29. Siegart PY, Walker E, Miller JR (2009) Differential behavioral responses of Anopheles
816 gambiae (Diptera: Culicidae) modulate mortality caused by pyrethroid-treated bednets. *J
817 Econ Entomol* 102(6):2061–71.
- 818 30. Alexander B, et al. (2009) Susceptibility to chemical insecticides of two Brazilian populations
819 of the visceral leishmaniasis vector Lutzomyia longipalpis (Diptera: Psychodidae). *Trop Med
820 Int Health* 14(10):1272–1277.
- 821 31. Norris LC, Norris DE (2011) Insecticide resistance in Culex quinquefasciatus mosquitoes
822 after the introduction of insecticide-treated bed nets in Macha, Zambia. *J Vector Ecol*
823 36(2):411–420.
- 824 32. Parker JEA, et al. (2015) Infrared video tracking of Anopheles gambiae at insecticide-treated
825 bed nets reveals rapid decisive impact after brief localised net contact. *Sci Rep* 5:13392.
- 826 33. Glunt KD, Thomas MB, Read AF (2011) The Effects of Age, Exposure History and
827 Malaria Infection on the Susceptibility of Anopheles Mosquitoes to Low Concentrations of
828 Pyrethroid. *PLoS One* 6(9):e24968.
- 829 34. Vontas J, et al. (2007) Transcriptional analysis of insecticide resistance in Anopheles stephensi
830 using cross-species microarray hybridization. *Insect Mol Biol* 16(3):315–324.
- 831 35. Alout H, Berthomieu A, Hadjivassilis A, Weill M (2007) A new amino-acid substitution
832 in acetylcholinesterase 1 confers insecticide resistance to Culex pipiens mosquitoes from
833 Cyprus. *Insect Biochem Mol Biol* 37(1):41–47.
- 834 36. Vontas JG, Small GJ, Hemingway J (2001) Glutathione S-transferases as antioxidant defence
835 agents confer pyrethroid resistance in Nilaparvata lugens. *Biochem J* 357(Pt 1):65–72.
- 836 37. Oliver S V, Brooke BD (2016) The Role of Oxidative Stress in the Longevity and Insecticide
837 Resistance Phenotype of the Major Malaria Vectors Anopheles arabiensis and Anopheles
838 funestus. *PLoS One* 11(3):e0151049.
- 839 38. Jones CM, et al. (2012) Aging partially restores the efficacy of malaria vector control in
840 insecticide-resistant populations of Anopheles gambiae s.l. from Burkina Faso. *Malar J* 11:24.
- 841 39. Buonaccorsi JP, Harrington LC, Edman JD (2003) Estimation and Comparison of Mosquito

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Survival Rates with Release-Recapture-Removal Data. *J Med Entomol* 40(1):6–17.
40. Ferguson HM, et al. (2008) Establishment of a large semi-field system for experimental study of African malaria vector ecology and control in Tanzania. *Malar J* 7(1):158.
41. Spiegelhalter DJ, Best NG, Carlin BP, van der Linde A (2002) Bayesian measures of model complexity and fit. *J R Stat Soc Ser B (Statistical Methodol)* 64(4):583–639.

42. Plummer M (2003) JAGS: A program for analysis of Bayesian graphical models using Gibbs sampling. doi:<http://www.ci.tuwien.ac.at/Conferences/DSC.html>.

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