

The impact of sublethal permethrin exposure on susceptible and resistant genotypes of the urban disease vector *Aedes aegypti*

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Abstract

BACKGROUND: In urban environments, some of the most common control tools used against the mosquito disease vector *Aedes aegypti* are pyrethroid insecticides applied as aerosols, fogs or residual sprays. Their efficacy is compromised by patchy deployment, aging residues, and the evolution and invasion of pyrethroid-resistant mosquitoes. A large proportion of mosquitoes in a given environment will therefore receive sublethal doses of insecticide. The potential impact of this sublethal exposure on the behaviour and biology of *Ae. aegypti* carrying commonly reported resistance alleles is poorly documented.

RESULTS: In susceptible insects, sublethal exposure to permethrin resulted in reductions in egg viability (13.9%), blood avidity (16.7%) and male mating success (28.3%). It caused a 70% decrease in the lifespan of exposed susceptible females and a 66% decrease in the insecticide-resistant females from the parental strain. Exposure to the same dose of insecticide in the presence of the isolated *kdr* genotype resulted in a smaller impact on female longevity (a 58% decrease) but a 26% increase in eggs per female and a 37% increase in male mating success. Sublethal permethrin exposure reduced host-location success by 20–30% in all strains.

CONCLUSION: The detrimental effects of exposure on susceptible insects were expected, but resistant insects demonstrated a less predictable range of responses, including negative effects on longevity and host-location but increases in fecundity and mating competitiveness. Overall, sublethal insecticide exposure is expected to increase the competitiveness of resistant phenotypes, acting as a selection pressure for the evolution of permethrin resistance.

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Keywords: *Aedes aegypti*; mating competition; fitness costs; knockdown resistance; sublethal exposure; permethrin

1 INTRODUCTION

Aedes aegypti Linnaeus (Diptera: Culicidae) is the primary vector of dengue, chikungunya and Zika.^{1,2} The management of these diseases remains largely dependent on insecticidal control measures that disrupt the transmission cycle. Pyrethroids remain the most common class of insecticides to be deployed for this purpose and are applied as space sprays and fogs, residual applications and treated materials.³ At field rates against susceptible mosquitoes, these treatments impact mortality and daily survival,^{4,5} but insecticide concentrations will vary substantially depending on the application technique, distance from the spray point, local environment and time since application.^{6,7} Moreover, many insecticides are not applied as part of targeted public health campaigns but by householders⁸ and by peri-urban agricultural activities.³ This results in considerable variation in the spatial and temporal coverage of insecticides and the presence of sublethal insecticide concentrations throughout the treated environment. For *Ae. aegypti*, the lethality of insecticides is further challenged by the selection and invasion of pyrethroid-resistant populations.⁹ As a result of all

these factors, sublethal exposure of mosquitoes is likely to be common.¹⁰

Mechanisms of pyrethroid resistance in *Ae. aegypti* include increased metabolic detoxification of insecticides,^{11,12} changes

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in mosquito behaviour¹³ and point mutations in the voltage-gated sodium channel (VGSC) gene.¹⁴ These point mutations alter how pyrethroids bind to the VGSC¹⁵ and are often termed 'knock-down resistance' (*kdr*) mechanisms. They have been associated with pleiotropic effects in *Ae. aegypti* that reduce female body size,^{16–18} increase larval development times^{18,19} and reduce female fecundity.¹⁷ Sublethal exposure of mosquitoes to insecticides may intensify these pleiotropic effects and result in further changes to physiology and behaviour.²⁰ Such changes are likely to have a major impact on the population dynamics of mosquitoes, competition between strains and resistance selection. There is, however, little research on effects of sublethal pyrethroid exposure on life-history traits and behaviour in *Ae. aegypti*,^{10,21} and none on the impacts on specific resistant genotypes. Exposure of susceptible *Ae. aegypti* to a LD₂₅ dose of residual permethrin and deltamethrin was reported to reduce flight activation time and disrupt orientation,¹⁰ and exposure to transfluthrin at concentrations of LC_{10–30} reduced skip-oviposition behaviour and caused a reduction in egg viability.²¹ Sublethal doses of *d*-Phenothrin, *d*-Allethrin and tetramethrin at \leq LC₅₀ had negative impacts on blood feeding and egg production.²² Sublethal doses of pyrethroids are also noted to have irritant effects on resting mosquitoes.^{23–25}

Life-history traits such as longevity and fecundity, and mosquito behaviours including mating, host-seeking and locomotion are critical factors governing the dynamics of mosquito populations in the field.²⁶ If sublethal doses of insecticide affect these traits differentially in susceptible and resistant mosquito populations, this will define the relative fitness of insecticide-resistant genotypes and determine the extent of resistance introgression into a population. This may further threaten the conservation of insecticide susceptibility. Here we assessed the effects of sublethal pyrethroid exposure on the longevity, fecundity, fertility, blood avidity, wing-beat frequency (WBF), host-location and mating success of pyrethroid-susceptible and -resistant *Ae. aegypti* in the presence and absence of a sublethal dose of pyrethroid. We tested the hypothesis that the costs of *kdr* seen in the absence of insecticides is lost during sublethal exposure and that susceptible insects are negatively affected. We were able to document impacts on a specific and ubiquitous resistant genotype by exploiting our access to an *Ae. aegypti* strain with two homozygous *kdr* alleles introgressed into an otherwise susceptible background.

2 MATERIALS AND METHODS

2.1 *Aedes aegypti* strains

S-Cairns is an insecticide-susceptible reference strain of *Ae. aegypti* collected in Cairns, Australia in 2015. R-TL is a pyrethroid-resistant strain that originated from Dili, Timor-Leste in 2009.²⁷ These strains were backcrossed to create R-BC, which carries the homozygous *kdr* alleles V1016G and S989P in an S-Cairns genetic background, as previously described.¹⁸ The characterisation of insecticide resistance of the parental and backcrossed strains to permethrin and other insecticides was conducted as outlined previously¹⁸ using the CDC bottle bioassay procedure.²⁸ The R-TL and R-BC strains exhibit high resistance to permethrin, elevated resistance to lambda-cyhalothrin, low-level resistance to deltamethrin and full susceptibility to malathion and bendiocarb. The S-Cairns strain is susceptible to all of these chemistries and displays 100% mortality at the diagnostic doses,¹⁸ as specified by the CDC bottle bioassay procedure.²⁸

2.2 Mosquito rearing

Larval densities, nutrition and environmental conditions were standardised across all strains. Colonies were established and maintained in the QIMR Berghofer insectary at 27 (\pm 1) °C and 75 (\pm 5)% relative humidity (RH), with a photoperiod of 12 h:12 h light: dark (L:D) cycles. Eggs were hatched by submerging in dechlorinated tap water, and larvae were reared at a density of 250 individuals in 3 L of water. Larvae were provided with Tetra-min fish food (Tetra, Melle, Germany) *ad libitum*. Pupae were transferred to 500 mL plastic containers inside 30 × 30 × 30 cm insect rearing cages (BugDorm, Taichung, Taiwan) where adults emerged and mated freely. Adults were supplied with 10% w/v sugar water *ad libitum* and blood-fed via an artificial membrane feeding system using defibrinated sheep blood (Serum Australis).

2.3 Standard experimental conditions

Adult survival, fecundity, blood avidity, host-location, male mating success and WBF were compared between the three strains (S-Cairns, R-TL and R-BC). All caged comparisons were made in a single controlled temperature room (27 \pm 1 °C, 75 \pm 5% RH, 12:12 L:D cycle). Host-location experiments involved the release of insects into an 18 m³ 'free flight' room maintained at 26 \pm 1 °C, 70% RH.

2.4 Sublethal dose determination

Three replicates of 20 unfed, 2–3-day-old male and female *Ae. aegypti* from the S-Cairns strain were exposed to a dilution series of permethrin (Merck, analytical standard) using CDC bottle bioassay protocols.²⁸ The number of mosquitoes that were scored as 'knocked down' (unable to fly or right themselves when the bottle is gently rotated), appearing as dead or remaining active was recorded after 30 min of exposure (diagnostic time point). After exposure, mosquitoes were transferred to untreated containers with continuous access to 10% sucrose. Mortality was scored 24 h post-exposure. The LD₂₅ for the susceptible S-Cairns strain was determined using nonlinear regression (GraphPad Prism version 7.00) and established as 1 μ g/bottle. In response to 30 min of exposure to that dose, male and female R-BC and R-TL mosquitoes exhibited no knockdown at the diagnostic time point and no mortality 24 h after exposure. Only surviving mosquitoes from this exposure protocol were used in the following comparisons. Mosquitoes used as controls in the following comparisons were treated following the CDC bottle bioassay protocol²⁸ using Wheaton bottles coated with acetone in the absence of insecticide.

2.5 Adult longevity

Adult mosquitoes (<24 h post-emergence), were exposed to 1 μ g of permethrin/bottle (or bottles coated with acetone only as controls), monitored for 30 min and left to recover for 24 h. Surviving mosquitoes were confined to a 30 × 30 × 30 cm cage, supplied with a 10% sugar solution *ad libitum* and monitored daily until death. Male and female adult mosquitoes were maintained at a density of 40 per cage (as per rearing methods above) to measure adult mosquito longevity. Experiments were completed in three replicates. Survival curves were compared using the Log-rank (Mantel–Cox) test (GraphPad Prism, version 7.00).

2.6 Fecundity

Three pools of 20 recently emerged males and females (<12 h old) were introduced to 30 × 30 × 30 cm cages (BugDorm) for 72 h to allow mating before being offered a blood meal. After another 72 h, female mosquitoes were removed from the cage

and exposed to a sublethal dose of permethrin as detailed above. After 24 h, females were offered a blood meal from the arm of a human volunteer. Fifteen fully engorged females were selected and isolated as individuals in 25 mL containers lined with filter paper and cotton wool. After an additional 24 h, these were moistened with 5 mL of dechlorinated water to encourage oviposition. After 72 h, the number of eggs produced per female was noted. Fecundity experiments were completed in triplicate. A chi-square test was used to test the intra-strain differences in the proportion of females that laid at least one egg. Egg counts per female (nonzero data) were tested for normality using the Anderson–Darling test and differences in egg counts between the insecticide exposed and nonexposed groups were tested using a Mann–Whitney test (GraphPad Prism, version 7.00).

2.7 Fertility

Once the eggs on each filter paper had been counted (see above), the paper was stored dry for 48 h to allow embryonation prior to immersion in water to induce hatching. After 48 h, the eggs were examined under a microscope to determine the displacement of the operculum; an indicator that the egg had hatched.²⁹ The mean percentage egg hatch per female was recorded. Data were tested for normality using the Anderson–Darling test and homogeneity of variances using Levene's test.

As the condition of normality was satisfied, differences in egg viability between groups was tested using Student's *t*-test (GraphPad Prism, version 7.00).

2.8 Blood avidity

We define blood avidity as the proportion of females accepting a blood meal. Pools of 2–3-day-old females from each of the S-Cairns, R-TL and R-BC strains were exposed to a sublethal dose of permethrin as above. After 24 h, female mosquitoes were individually transferred into a rearing cage, allowed to rest for 10 min and offered a blood meal from the right forearm of the same human volunteer for 5 min between 1700 and 1730 h. The number of females showing evidence of a full or partial blood meal was recorded. Three pools of 10 mosquitoes from each strain were used. Differences in avidity were analysed by the Fisher's exact test (GraphPad Prism, version 7.00).

2.9 Host-location behaviour

To assess differences in the success to locate a host (host-seeking efficiency) and time to locate a human host, we conducted 'free-flight' assays in the quarantine facility at QIMRB. Pools of 2–3-day-old female mosquitoes were exposed to a sublethal dose of permethrin, as above. After 24 h, 10 mosquitoes of a single strain were released into a white 18 m³ room (26 ± 1 °C, 70% RH) and allowed to acclimatise for 10 min. A single human observer (maintained for all replicates) then entered and sat in the middle of the room with their lower legs exposed. Successful landing and feeding events by mosquitoes were recorded over the next 10 min. The time to locate the host was recorded and the probing mosquito was removed by aspiration from the leg of the volunteer. After 10 min, all remaining mosquitoes were captured. Each test was conducted in triplicate between 1700 and 1730 h. Differences in host-location success were analysed by Fisher's exact test, while differences in time to locate a host were analysed by a Mann–Whitney test (GraphPad Prism, version 7.00).

2.10 Male mating success

Rhodamine B (Rho B; Sigma–Aldrich, St. Louis, MO, USA, 95% dye content) was used to label the seminal fluid of male mosquitoes and determine the successful insemination of female mosquitoes in mating competition assays, as per Johnson *et al.*³⁰ One-day-old males from the S-Cairns, R-TL and R-BC strains were placed in separate cages and provided access to 0.1% Rho B (w/v in 10% sucrose) for 72 h to stain the seminal fluid. A separate group of males from each strain was fed sugar only (control). Rho B/sucrose solutions were replaced every 48 h. After 72 h, male mosquitoes from each of the strains were exposed to a sublethal dose of permethrin, as above. After a further 24 h, mating competition trials between exposed and unexposed males, competing for unexposed females of the same strain, were conducted as described previously.³¹

Briefly, the mating competitiveness of males exposed and unexposed to a sublethal dose of permethrin was tested by introducing 20 virgin females (3–5 days old), 10 Rho B marked virgin males exposed to a sublethal dose of permethrin and 10 unmarked, untreated, virgin males (5–6 days old) to the same cage. After 24 h, female mosquitoes were collected and knocked down by chilling at –20 °C for 30 min. These were dissected in 1% PBS solution to isolate the bursa and spermathecae, which were crushed under a coverslip and examined using a fluorescent microscope (Axioskopp2, Carl Zeiss AG, Oberkochen, Germany) with a fluorescence illuminator (Xcite 120Q, Excelitas Technologies Corp, USA) and a fluorescence filter (BrightLine, Cy3-4040C-000, IDEX Health & Science, USA). The presence of Rho B in these organs demonstrated a successful mating by a permethrin-exposed marked male while the presence of an expanded bursa without Rho B indicated a successful mating event by an untreated, unmarked male. Three replicates were performed for each treatment. Mating success (i.e. proportion of females mated by males from each group) was analysed using chi-square tests (GraphPad Prism, version 7.00) assuming an expected 50% mating success rate for each male group. Each trial was completed in triplicate.

2.11 Wing-beat frequency

The WBF of untethered male and female 3–4-day-old *Ae. aegypti* from each of the strains was recorded as described previously.³² Male and female mosquitoes from the S-Cairns, R-TL and R-BC strains were exposed to sublethal doses of permethrin, as above. After 24 h, single male or female mosquitoes were aspirated from the holding cage into a test vial (60 mL aspirator vial (Bioquip, 2809V, Compton, CA, USA) to record 10 s of uninterrupted flight (i.e. no landing and resting on the sides of the container). Flight sound was recorded as a high-resolution .wav file (32 bit) using a TASCAM portable handheld recorder (DR- 22WL; Montebello, CA, USA). Files were analysed using Audacity software version 2.3.2 (<https://www.audacityteam.org>). The frequency spectrum of each recording was visualised, and the mean WBF determined as the peak of the fundamental frequency. Thirty mosquitoes that were not exposed to insecticide and 30 sublethally exposed mosquitoes from each strain were measured during the WBF experiments. WBF data were analysed using the Mann–Whitney test (GraphPad Prism, version 7.00).

2.12 Ethics

Blood feeding protocols using human volunteers were approved under QIMRB human ethics permit number P2273.

3 RESULTS

3.1 Male and female adult longevity

Female susceptible mosquitoes that survived exposure to the LD₂₅ permethrin suffered a 70% decrease in average longevity when compared to untreated mosquitoes from the same strain. Decreases in average lifespan for the females of R-TL and R-BC mosquitoes were 66% and 58%, respectively. Analysis of their survival curves indicated that these decreases were highly significant (S-Cairns: $\chi^2 = 175.8$, $df = 1$, $P < 0.0001$; R-TL: $\chi^2 = 60.61$, $df = 1$, $P < 0.0001$; R-BC: $\chi^2 = 85.02$, $df = 1$, $P < 0.0001$; Fig. 1(a)). Similar impacts were observed in males (S-Cairns: $\chi^2 = 78.86$, $df = 1$, $P < 0.0001$; R-TL: $\chi^2 = 65.11$, $df = 1$, $P < 0.0001$; R-BC: $\chi^2 = 57.79$, $df = 1$, $P < 0.0001$; Fig. 1(b)), where male susceptible mosquitoes that survived exposure to a sublethal dose of insecticide recorded a 40% decrease in average longevity, R-TL males recorded a 39% decrease and R-BC recorded a 30% decrease in average longevity, when compared to untreated male mosquitoes of the same strain.

3.2 Fecundity and fertility

Sublethal exposure increased the proportion of female mosquitoes that failed to lay eggs. This was not significant for the S-Cairns ($\chi^2 = 2.493$, $df = 1$, $P = 0.1144$) or R-BC ($\chi^2 = 3.103$, $df = 1$,

$P = 0.0781$) strains. In contrast, the proportion of egg-laying females was significantly reduced in females from the R-TL strain ($\chi^2 = 11.28$, $df = 1$, $P = 0.0008$) (Table 1).

When females did lay eggs (egg count ≥ 1), the mean number produced per R-BC female was significantly increased in insecticide-exposed females (Mann-Whitney $U = 262.5$, $P = 0.0178$). No such differences were recorded in the S-Cairns (Mann-Whitney $U = 441$, $P = 0.9911$) or R-TL strains (Mann-Whitney $U = 450.5$, $P = 0.1537$) (Table 1).

Mean egg hatch was 16% lower in insecticide-exposed S-Cairns females than in the unexposed strain ($t_{(28)} = 4.204$, $P = 0.0002$), but there were no differences between the exposed and unexposed R-TL ($t_{(28)} = 0.2536$, $P = 0.8017$) or R-BC strains ($t_{(28)} = 1.593$, $P = 0.1225$) (Table 1).

3.3 Blood avidity

In the absence of insecticide exposure, all females from S-Cairns, R-TL and R-BC strains took a blood meal from the human host except for one individual from the R-BC strain. Exposure to a sublethal dose of permethrin, 24 h prior to being offered a blood meal, reduced blood avidity in the S-Cairns strain by 20% (Fisher's exact test; $P = 0.0237$). Although a reduction in blood

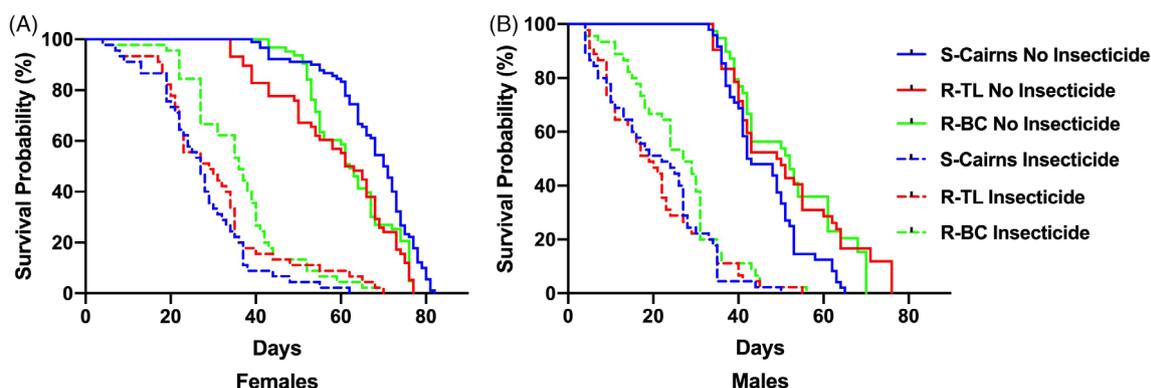


Figure 1. Adult mosquito survival probability. Effects of exposure to insecticide (dotted line) in comparison to no insecticide exposure (solid line) on the survival probability of female (a) and male (b) S-Cairns, R-TL and R-BC strains ($n > 35$).

Table 1. Fecundity and fertility			
	S-Cairns	R-TL	R-BC
% females with zero eggs			
No exposure	24.44 ± 9.7	6.67 ± 3.8	26.67 ± 6.7
Sublethal exposure	40 ± 6.7	35.56 ± 5.9	44.44 ± 2.2
P value	0.1144	0.0008*	0.0781
Mean no. of eggs (nonzero data only)			
No exposure	61.47 ± 3.1 (34)	74.90 ± 2.3 (42)	69.33 ± 2.9 (33)
Sublethal exposure	61.84 ± 3.5 (26)	81.19 ± 3.6 (27)	80.1 ± 3.2 (25)
P value	0.9911	0.1537	0.0178*
% egg hatching success			
No exposure	89.88 ± 1.6	83.22 ± 2.2	86.05 ± 2.1
Sublethal exposure	76 ± 2.2	82.4 ± 1.8	80.22 ± 2.1
P value	0.0002*	0.8017	0.1225

Percentage (mean ± SE) of females that did not lay eggs, the average (mean ± SE) number of eggs per female (number of females included in the analysis) and the percentage (mean ± SE) of eggs that successfully hatched.
*Denotes statistical significance.

Table 2. Blood avidity

Strain	Treatment	N tested	Fed	Unfed	% Fed	P value
S-Cairns	No exposure	30	30	0	100	0.0237*
	Sublethal exposure	30	24	6	80	
R-TL	No exposure	30	30	0	100	0.4915
	Sublethal exposure	30	28	2	93.34	
R-BC	No exposure	30	29	1	96.7	0.1945
	Sublethal exposure	30	25	5	83.34	

The number/percentage of nonexposed and insecticide-exposed females from each strain successfully taking a human blood meal.
*Denotes statistical significance.

Table 3. Host-location success

Strain	Treatment	N	Probing host	Failure to locate host	% host-seeking success	P value
S-Cairns	No exposure	30	30	0	100	0.0019*
	Sublethal exposure	30	21	9	76.67	
R-TL	No exposure	30	30	0	100	0.0105*
	Sublethal exposure	30	23	7	70	
R-BC	No exposure	30	30	0	100	0.0237*
	Sublethal exposure	30	24	6	80	

The number/percentage of females from each strain successfully locating a human host within the free-flight room.
*Denotes statistical significance.

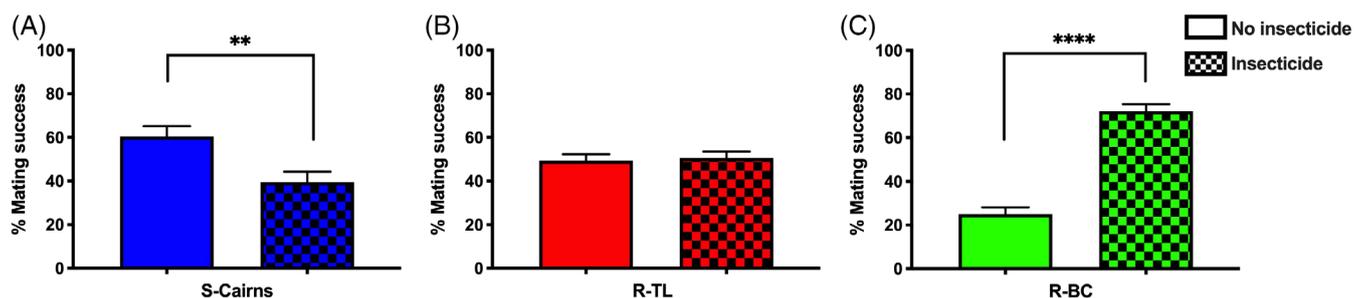


Figure 2. Percentage (mean \pm SE) male mating success of insecticide exposed and unexposed males in the presence of unexposed females of the same strain: (a) S-Cairns (b) R-TL (c) R-BC. ** $P < 0.01$, **** $P < 0.0001$.

avidity was recorded in the R-TL and R-BC strains post-exposure (7% and 13%, respectively) these reductions were not significant (R-TL: Fisher's exact test; $P = 0.4915$, R-BC: Fisher's exact test; $P = 0.1945$) (Table 2).

3.4 Host-locating behaviour

All unexposed females, regardless of strain, located a human host within 10 min (Table 3). When exposed to a sublethal dose of permethrin, host-location was significantly reduced by 20–30% in all strains (S-Cairns: Fisher's exact test; $P = 0.0019$; R-TL: Fisher's exact test; $P = 0.0105$, R-BC: Fisher's exact test; $P = 0.0237$).

3.4.1 Time taken to locate the host

For those females that located a host, the mean time taken for female mosquitoes to find a human host in the free-flight room did not differ significantly between permethrin exposed or unexposed females of any strain (S-Cairns: Mann-Whitney $U = 242$,

$P = 0.1608$; R-TL: Mann-Whitney $U = 263.5$, $P = 0.3252$; R-BC: Mann-Whitney $U = 262$, $P = 0.2690$).

3.5 Male mating success

The mating success of S-Cairns males was significantly reduced post-exposure to a sublethal dose of permethrin when competing against nonexposed S-Cairns males ($\chi^2 = 9.524$, $df = 1$, $P = 0.002$; Fig. 2(a)). In contrast, sublethal insecticide exposure significantly increased male mating success in mosquitoes from the R-BC strain when competing against unexposed males ($\chi^2 = 33.23$, $df = 1$, $P < 0.0001$; Fig. 2(c)). No significant difference in male mating success was observed between males from the R-TL strain exposed and nonexposed to a sublethal dose of permethrin ($\chi^2 = 0.0229$, $df = 1$, $P = 0.8795$; Fig. 2(b)).

3.6 Wing-beat frequency

Impacts of permethrin exposure of WBF did not follow any specific pattern. There was no effect on the WBF of R-BC females

Table 4. Wing-beat frequency of permethrin exposed and unexposed strains (mean \pm SE, ** $P < 0.01$, *** $P < 0.001$)

	S-Cairns	R-TL	R-BC
Females			
No exposure	511 \pm 6.7	513 \pm 4.8	501 \pm 4.7
Sublethal exposure	529 \pm 6.2	536 \pm 5.3	500 \pm 4.6
Change in WBF	18	23**	-1
Males			
No exposure	779 \pm 8.4	807 \pm 9.1	757 \pm 7.1
Sublethal exposure	813 \pm 6.6	773 \pm 6.3	767 \pm 4.6
Change in WBF	34**	-34***	10

(Mann–Whitney $U = 403$, $P = 0.204$), R-BC males (Mann–Whitney $U = 445.5$, $P = 0.492$) or S-Cairns females (Mann–Whitney $U = 398.5$, $P = 0.135$). Exposure increased the WBF of S-Cairns males (Mann–Whitney $U = 270$, $P = 0.003$) and R-TL females (Mann–Whitney $U = 254.5$, $P = 0.002$), but decreased WBF in R-TL males (Mann–Whitney $U = 240$, $P = 0.0005$) (Table 4).

4 DISCUSSION

The current paper documents the impact of sublethal permethrin exposure on parameters related to survival, fecundity, fertility, blood feeding, host-location and mating success. We demonstrate, for the first time, some specific, negative impacts of sublethal exposure on susceptible *Ae. aegypti* mosquitoes, but we also note that detrimental effects on longevity and host-location are also evident in highly resistant insects. There are also less predictable, positive impacts of exposure on insects carrying the *kdr* genotype. These include increases in fecundity and mating success. When considered in their entirety, sublethal insecticide exposure has a largely negative impact on susceptible mosquitoes, but strongly resistant mosquitoes (homozygous for the *kdr* genotype) are not fully protected. On balance, it is predicted that resistant insects will out-compete susceptible populations in sublethally treated environments.

Sublethal pyrethroid exposure had negative consequences on adult mosquito longevity in all strains in this study. In terms of reproductive potential, there were negative effects in the fully susceptible strain on egg hatch but not on the number of eggs laid. This was in contrast to the responses of R-BC females, where exposure was uniquely associated with an increase in the number of eggs produced. While exposure reduced blood-feeding success in susceptible insects, resistant phenotypes were unaffected. Host-location ability was reduced in all strains. Most strikingly, sublethal permethrin exposure increased mating success for males with the introgressed *kdr* genotype.

These results have implications for the competitiveness of resistant phenotypes and genotypes in the presence of sublethal doses of pyrethroid, which are likely to be far more common in the urban environment than the recommended lethal field rates. The obvious emphasis on the lethal impact of insecticides on mosquitoes means that the effects of sublethal exposure on -insecticide-susceptible and -resistant insects has been largely ignored, particularly in the context of mosquito vectors of disease. Studies on sublethal insecticide exposure in *Ae. aegypti* tend to be conducted on susceptible insects and focus on a very small number of traits.^{10,21–23,33} Given the ubiquity of pyrethroid resistance in *Ae. aegypti* populations around the globe, this limits our ability

to understand the impacts of sublethal exposure in the field and the extent to which they will favour further selection for resistance in *Ae. aegypti*.

In the absence of insecticide selection, the evolution of resistance is often accompanied by pleiotropic fitness costs (reduced longevity, body size and increased larval development times),^{34,35} supporting the idea that reductions in selection pressure can help conserve susceptible genotypes.^{18,36} An understanding of how fitness is modulated by the presence of sublethal doses of insecticide will help predict the success of that strategy. For example, the reductions in longevity associated with the pleiotropic effects of *kdr* genotypes in the absence of insecticides^{17–19} disappear in the presence of sublethal doses of insecticides and any competitive advantage of the susceptible genotype is lost. One potential advantage of a universal impact on survival is that this might affect the vectorial capacity of both susceptible and resistant populations (e.g. reduce daily survival to the extent that the vector can no longer sustain virus transmission³⁷) but that cannot be directly derived from our laboratory-based observations.

Fecundity, fertility and behaviours such as host-location and blood-feeding are also important indicators of the success of mosquitoes in the field.^{26,38} In the current study, we sought to determine whether sublethal exposure to permethrin would affect these traits and, potentially, the population dynamics of the strains under investigation. The introgressed *kdr* genotype did not impact the proportion of females that can produce eggs in the presence or absence of sublethal permethrin doses, although it was associated with an increase in the number of eggs produced in the presence of permethrin. There was no impact on avidity for a blood meal. In susceptible insects, there were significant decreases in egg hatch and avidity, both representing a clear disadvantage against the resistant genotype. The increases in egg production seen in the R-BC strain may result from hormesis, a phenomenon where many insects display an increase in measures of fecundity or fertility in relation to low-dose exposures to toxins,³⁹ but the phenomenon remains unexplored in mosquitoes. Our observations of reduced host-location in exposed females of all strains support a previous study which demonstrated that mosquitoes treated with sublethal levels of pyrethroids are less responsive to attractants than controls at 24 h post-insecticide exposure.¹⁰ These observations indicate that flight activation is impeded by exposure to permethrin. This impedance appears to occur regardless of permethrin resistance, demonstrating that a resistant phenotype does not confer protection against all sublethal impacts.

Sublethal exposure considerably reduced the capacity of susceptible insects to compete with unexposed males for conspecific females but had no effect on interactions involving the most resistant strain (R-TL). However, exposed males with the introgressed *kdr* genotype (R-BC) were far more successful at mating with untreated conspecifics. This suggests that males with the *kdr* genotype will outcompete their susceptible counterparts in the presence of sublethal doses of pyrethroid and further reduce the frequency of susceptible alleles in the population. Previously, the *kdr* genotype has been shown to have negative impacts on the mating success of male mosquitoes when in competition with insecticide-susceptible males in the absence of insecticides,^{31,40,41} but here we demonstrate that this disadvantage is reversed with sublethal exposure.

The mechanism behind this enhanced mating success is unknown. In a prior study,³¹ a pleiotropic association between

the *kdr* genotype and a reduction in male WBF was flagged as a potential cause of reduced mating success in the absence of insecticides. Females may actively choose males based on their ability to modulate their acoustic signal. However, in the current study, the WBF of males with the introgressed *kdr* genotype did not change with sublethal exposure, so we can rule out acoustic signaling as the likely mechanism of increased mating success. Again, increased fitness in sexually dimorphic characters in insects has been associated with hormesis in response to insecticide exposure⁴² and this may have played a role in our observations.

Pyrethroid resistance threatens our most affordable and widely deployed defences against *Aedes*-borne diseases. This study shows that sublethal exposure may exacerbate that threat. Insecticide resistance management (IRM) strategies are urgently required to preserve and sustain these tools and conserve insecticide susceptibility. This is crucial because there are few molecules available for vector control, and few under development.⁴³ IRM strategies all aim to reduce selection pressure, often in the assumption that reductions in insecticide coverage, or the replacement of one molecule with another in space (mosaics) or time (rotations), will allow the immigration of susceptible insects or the reduction in the frequency of resistant alleles due to a costly phenotype in the absence of insecticide.^{9,43–45} We now show that these costs are largely negated by the presence of sublethal insecticides and that susceptible insects will be at a distinct competitive disadvantage. Given the ubiquity and persistence of pyrethroids in the urban environment,⁴⁶ resulting from a combination of peri-urban agriculture, household insecticide use and public health campaigns, the practicality of using untreated refugia or mosaics to relieve selection pressures requires careful assessment.

5 CONCLUSION

It is important to understand the fitness costs and behavioural changes that result from the evolution of insecticide resistance under a range of environmental conditions. Our results provide clear evidence that exposure to sublethal doses of permethrin adversely affects a range of biological and behavioural traits in insecticide-susceptible *Ae. aegypti* while actively increasing the competitiveness of strains harbouring the V1016G/S989P *kdr* genotype. Vector control programs that utilise mosaics or rotations of insecticides with different modes of action, in the expectation that resistance-related fitness costs will help to reduce the frequency of resistance alleles, are likely to be compromised by the ubiquitous presence of poorly targeted pyrethroids in the environment. This study has highlighted the threat of sublethal insecticide exposure to the conservation of insecticide susceptibility in mosquito populations.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests. The opinions expressed herein are those of the authors and do not necessarily reflect those of the Australian Defence Force and/or extant Defence Force Policy.

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