Journal Pre-proof

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PII: S2667-114X(23)00009-2

DOI: https://doi.org/10.1016/j.crpvbd.2023.100121

Reference: CRPVBD 100121

To appear in: *Current Research in Parasitology and Vector-Borne Diseases*

Received Date: 6 February 2023

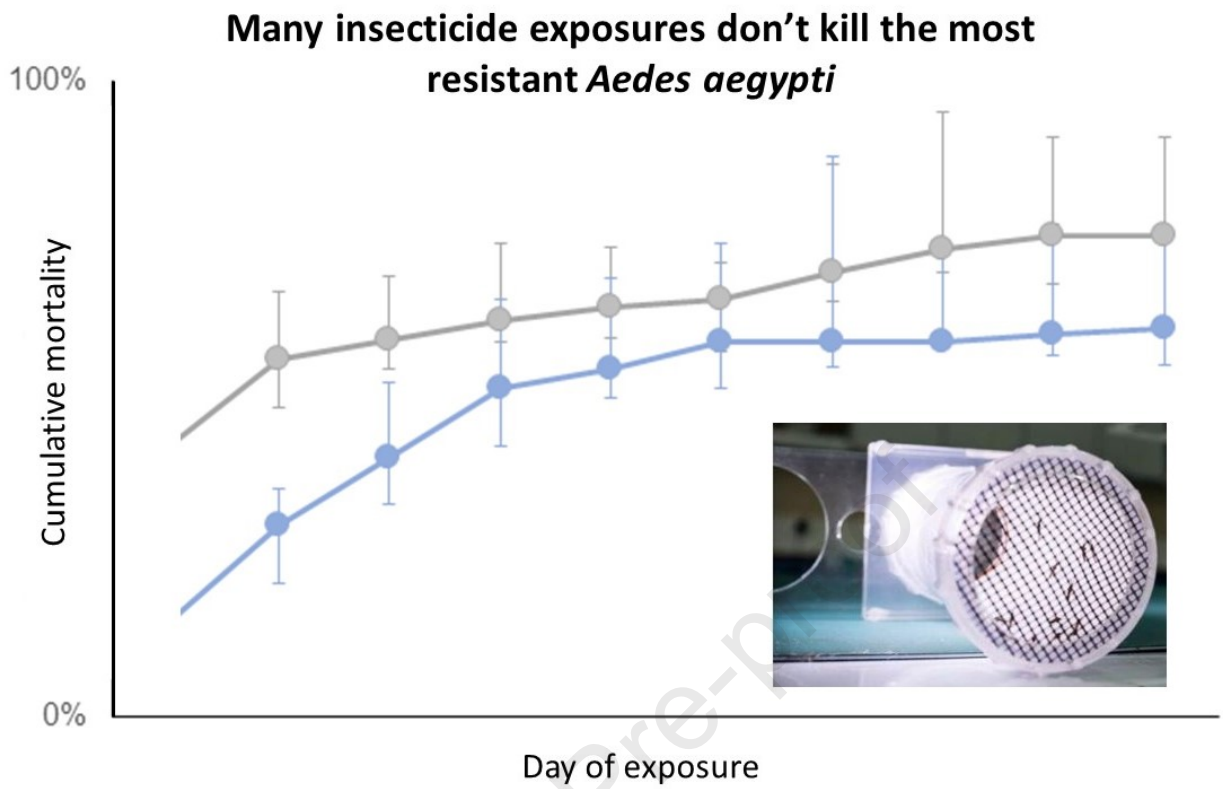
Revised Date: 4 April 2023

Accepted Date: 5 April 2023

Please cite this article as: Al Nazawi, A.M., Weetman, D., Age-dependence of susceptibility to single and repeated deltamethrin exposure in pyrethroid-resistant *Aedes aegypti* strains, *Current Research in Parasitology and Vector-Borne Diseases* (2023), doi: https://doi.org/10.1016/j.crpvbd.2023.100121.

CRediT author statement

Ashwaq Al Nazawi: Conceptualization; Formal analysis; Methodology; Investigation; Resources; Data curation; Funding acquisition; Writing - original draft; Writing - review & editing. David Weetman: Conceptualization; Methodology; Formal analysis; Supervision; Writing - original draft; Writing - review & editing. All authors read and approved the final manuscript.



Age-dependence of susceptibility to single and repeated deltamethrin exposure in pyrethroid-resistant *Aedes aegypti* strains

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ABSTRACT

Monitoring insecticide resistance is crucial in disease-transmitting mosquitoes to allow assessment of viable candidate insecticides to use for control and to provide indication of changes in resistance. Insecticide resistance bioassays are typically performed on young female mosquitoes, yet disease is transmitted by older females, which may also have encountered insecticide multiple times during their adult life. If insecticide mortality rates increase with age directly, or indirectly *via* cumulative toxicity from repeated exposure, the strategy of testing young mosquitoes as the least susceptible cohort would be supported. We tested three hypotheses *via* examination of how age and cumulative exposure impact mortality rates to the pyrethroid deltamethrin in strains of *Aedes aegypti* from Jeddah, Saudi Arabia and the Cayman Islands, which show differences in resistance mechanisms. Females of different ages (5, 7, 10 and 14 days-old) were exposed using WHO tube assays to either a single dose of insecticide, or in a second experiment females (initially 5 days-old) were exposed daily over 10 days. Age only increased mortality in the Jeddah strain at 14 days-old and had no impact on the Cayman strain. This is consistent with greater impact linked to metabolic resistance in the Jeddah strain, though results from qPCR of four candidate genes, failed to provide evidence for a candidate underpinning an age-dependent change in resistance. With repeated exposure, mortality rates of surviving females decreased to very low levels, suggesting that surviving older cohorts of females may exhibit substantially lower susceptibility than young females in single exposure assays. Our results indicate that testing young females with a single insecticide exposure should

capture minimum susceptibility for the majority of the population, but a small fraction of older females may prove particularly unresponsive to pyrethroid-based control measures.

Keywords: Dengue; *Aedes aegypti*; Mosquito; Age; Pyrethroid; Deltamethrin; Saudi Arabia

1. Introduction

Dengue is a viral disease transmitted by the bite of *Aedes* mosquitoes, mainly *Aedes aegypti*, threatening economies and human health in most tropical regions of the world. In Saudi Arabia, dengue has remained endemic since the first case was reported in 1994 in Jeddah (Fakeeh & Zaki, 2001), with subsequent spread to Makkah and Jizan. Insecticide-based control of *Ae. aegypti* remains the main dengue control option in Saudi Arabia as currently there is no preventative or curative medication, and the approved vaccine (Hadinegoro et al., 2015), is not yet available in the Middle Eastern region. With insecticide resistance widespread in *Ae. aegypti* (Moyes et al., 2017), monitoring susceptibility is required to aid optimal insecticide choices for control and identify changes that may be linked to control programmes.

To monitor insecticide resistance, the World Health Organization (WHO) recommends, wherever possible, the use of 3- to 5-day-old, non-blood-fed female mosquitoes, which have not been previously exposed to insecticide in diagnostic dose bioassays (WHO, 2022). This standardisation aims to reduce confounding variables, which can greatly impact test results (Lissenden et al., 2021), and to facilitate comparison among different tests. Standardisation of bioassays is crucial, but single exposure tests performed on young females may miss key aspects of insecticide exposure for wild mosquitoes. Dengue transmission from mosquitoes to humans is unlikely to occur prior to 8 days post-emergence, with 14 or more days probably more typical (Harrington et al., 2001). This period encompasses the extrinsic incubation period (EIP) during which the virus replicates and migrates to the salivary glands for transmission during the next blood-feeding. Moreover, as female mosquitoes age, they are likely to encounter multiple insecticide exposures if earlier encounters have been sub-lethal.

Despite disparity between the standard testing procedure and field realities, surveillance of resistance in young females exposed to a single dose may still represent a good estimation of the maximum probability of resistance if either of two conditions are met: (i) survival of a single insecticide exposure declines with mosquito age, i.e. testing a young cohort captures the maximum possible survivorship expected; and (ii) repeated sublethal exposure experienced by mosquitoes as they age has cumulative injurious effects that result in reduced survival, such that even if survival to any single exposure is not lower in older females, likelihood of repeated

exposure leads to reduced survival. If both conditions apply, enhanced age-dependence of mortality is expected for females experiencing multiple exposures as they age, resulting in a synergistically reduced chance of survival for females old enough to transmit disease. This is important for both assessment of the appropriateness of bioassays to assess resistance, and the potential impacts of resistance on disease transmission.

Previous studies on *Aedes* spp. and *Anopheles* spp. have typically shown that susceptibility to insecticides increases with chronological age (Lines & Nassor, 1991; Rajatileka et al., 2011; Chouaibou et al., 2012; Jones et al., 2012; Sikulu et al., 2014; Mbepera et al., 2017; Knecht et al., 2018). However, it remains unclear whether age-dependence of survival may depend on resistance mechanisms expressed by the strains examined (Lissenden et al., 2021), e.g. metabolic enzymes such as P450s, *versus* target site mutations, both of which are commonly associated with resistance in *Ae. aegypti* (Moyes et al., 2017). Results for the effect of repeated exposure are more ambiguous. Viana et al. (2016) exposed two resistant *An. gambiae* colonies to pyrethroid-treated bednets either daily or at 4-day intervals. Daily exposures did not show consistently increasing mortality, and whilst mortality at the longer intervals did increase with exposure number, the mosquitoes were also four days older each time. Predictions for the effect of repeated exposure are potentially complex because initially sublethal but injurious insecticidal effects might be offset by induction of detoxification enzymes by insecticide exposure leading to a net protective effect (Poupardin et al., 2008). However, such overexpression is expected to be costly, and may be less sustainable for older females.

In this study we aimed to determine the impact of both age and repeated exposure on two *Ae. aegypti* strains, Jeddah and Cayman, which exhibit contrasting dependence on metabolic detoxification mechanisms. Using these two strains we sought to test the hypotheses that: (i) mortality will increase with age for a single exposure; (ii) age dependence will be stronger in the strain showing evidence of metabolic resistance, with survival linked to overexpression of candidate resistance genes; and (iii) repeated exposure will have cumulative effects which reduce age-specific survivorship further, acting to progressively elevate mortality rates with increasing exposures (and age).

2. Materials and methods

2.1. Mosquito strains

Field-collected Saudi Arabian *Ae. aegypti* originated from larval collected from several breeding sites from the dengue-endemic city of Jeddah (21°60'3.97N; 39°27'2.49E), and the first

laboratory generation was used for experiments. Standardised WHO bioassays show that *Ae. aegypti* from Jeddah are resistant to the pyrethroids, permethrin and deltamethrin, underpinned by target site (*kdr*) mutations (S989P, V1016G and F1534C) and cytochrome P450-based metabolic mechanisms (Al Nazawi et al., 2017). The Cayman strain also exhibits pyrethroid resistance based on a different trio of *kdr* mutations (V410L, V1016I, F1534C), but in contrast to Jeddah strain, synergist assays and selection experiments have detected little additional contribution from metabolic resistance mechanisms (Harris et al., 2010; Thornton et al., 2020). Each laboratory strain was raised under the same standardised conditions (Al Nazawi et al., 2017).

2.2. Bioassays

Jeddah and Cayman non-blood-fed females of different ages were exposed in WHO bioassay tubes to 0.05% deltamethrin papers. Note that this is slightly higher than recommended (0.03%) for *Aedes* spp. by the WHO (WHO, 2022), but this difference was unimportant for the purposes of this study. In the first experiment in which females were exposed only once (single exposure assay), 2–4 replicates (mean = 3.4) of 13–25 (mean = 20.7) mosquitoes per replicate aged 5, 7, 10 or 14 days were exposed to 0.05% deltamethrin or WHO control papers (pyrethroid absent) for 1 h. After the exposure, females were transferred to recovery tubes and provided with 10% sucrose. After a 24-h recovery period, the final mortality was recorded. Single exposure bioassay data are provided in Supplementary Table S1. In the multiple exposure experiment female Cayman and Jeddah females (starting at 5 days-old) were exposed to 0.05% deltamethrin for 1 h. After a recovery period of approximately 23 h (reduced from the standard 24 h to prevent assay time-of-day becoming progressively later), mortality was recorded, dead females removed, and all survivors re-exposed to the same deltamethrin dosage. Exposures were continued for 10 days at which point the surviving mosquitoes were 14 days-old. To reduce handling, both control and test mosquitoes remained in the same holding tubes throughout the experiment. Owing to higher expected mortality in the Jeddah than Cayman strain (see *Section 3.1*), more replicate bioassay tubes were run for Jeddah (n = 8) than Cayman (n = 4) strain. Multiple exposure bioassay data are provided in Supplementary Table S2. Jeddah control females from the single exposure experiment, as well as final survivors from Jeddah at the end of the multiple exposure experiment, and age-matched controls were preserved in RNALater (Thermo Fisher Scientific, Delaware, USA) and stored at -20 °C.

2.3. Analysis of candidate gene expression

Total RNA was extracted from the pools of female mosquitoes in each strain using the Ambion RNAqueous Kit (LifeTechnologies, Paisley, UK), according to manufacturer's instructions, with the quantity of RNA yields assessed using a Nanodrop ND-1000 (Thermo Fisher Scientific, Delaware, USA). Synthesis of cDNA used Superscript III (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's guidelines, and cDNA was purified using a QIAquick spin column (QIAuick PCR Purification Kit, Qiagen, Manchester, UK). Four candidate genes, linked to deltamethrin resistance in the Jeddah strain (Al Nazawi, 2019) were assayed for their relationship with age (Experiment 1) or differential expression between survivors and controls at the end of Experiment 2 using quantitative real time PCR: the cytochrome P450s CYP9J7, CYP9J26, CYP9J27, and a histone methyltransferase AAEL006013. The qRTPCR reactions were performed in a volume of 10 µl with 5 µl of SYBR® Green (Applied Biosystems, Texas, USA), 0.4 µl forward and reverse primer (10 µM), 3.2 µl ddH_2O, and 1 µl of cDNA (approximately 2 ng), under the following conditions: 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 10 s. The relative expression level of each candidate gene relative to the susceptible strains was calculated using the ΔΔCT method (Schmittgen & Livak, 2008) after normalisation with two housekeeping genes, RPS3 (ribosomal protein S3) and 60S ribosomal protein L8. Results for qRT PCR are provided in Supplementary Table S3, and primers are shown in Supplementary Table S4. Primers were designed using NCBI Primer-Blast, except for CYP9J27 for which primers were from Ishak et al. (2017).

2.4. Statistical analysis

The effect of age (Experiment 1) on mortality was analysed using a generalised linear model (GLM) with binomial link function in SPSS v. 26, with strain and age as fixed effects and the number of mosquitoes tested per bioassay tube as a covariate. The effect of repeated exposure (Experiment 2) was analysed using a generalized estimating equation (GEE), owing to the repeated testing design, with test day as a within subjects covariable, strain as a between-subjects effect and the number of mosquitoes tested per bioassay tube as a covariate. In each model, interaction terms were included, and the model simplified to its minimal version by progression removal of non-significant terms, starting with higher-order terms. Partial rank correlation was used to further evaluate the relationship between test day, proportionate bioassay mortality and the number of mosquitoes tested in Experiment 2; rank correlation was used to avoid assumption of any particular data distribution. Results for the gene expression analyses were analysed in SPSS using Kruskal-Wallis tests to compare ΔΔCT-values (relative to the

susceptible colony samples) among the different ages tested for each gene in the Jeddah control samples, and *t*-tests to compare the ΔΔCT-values between Jeddah survivors of all exposures with age-matched controls. A single outlier - when analysed across all data for each gene separately - was detected for gene Cyp9J7 in one replicate of the single exposure experiment pools at day 14 *via* Grubb's test (at $P < 0.01$) and was removed prior to analysis (Supplementary Table S3). Throughout, *P*-values of < 0.05 were regarded as significant.

3. Results

3.1 Impacts of age and multiple exposures on deltamethrin-induced mortality

In Experiment 1 females of different ages from the Jeddah and Cayman colonies were exposed in a single bioassay to deltamethrin. Across all ages, mortality for the Jeddah strain was 56% (95% CI: 49–63%) and for the Cayman strain 39% (95% CI: 34–45%). This apparent difference between strains was not consistent across ages though, as reflected by the non-significant strain effect in the GLM, the significant difference in mortality only for the 14-day time point and the significant strain × age interaction term for 14 days (Table 1). This inconsistency is clearly illustrated in Fig. 1, with no trend in mortality rate evident across ages for the Cayman strain, and a significant increase only at the oldest age tested for Jeddah. Mortality in control tubes, which lacked insecticide exposure, at each age in each strain did not exceed 5% (Supplementary Table S1) and the relatively modest variation in numbers tested per tube (*Section 2.2*; Supplementary Table S1) did not significantly affect mortality (Table 1).

In Experiment 2 females from each strain were exposed to deltamethrin for 10 consecutive days beginning at an age of 5 days (the same age as the youngest in Experiment 1). Overall mortality was higher for the Jeddah strain (76%; 95% CI: 68–82%) than the Cayman strain (61%; 95% CI: 51–71%). Though the largest difference was evident in the first test day (Fig. 2), the strain effect overall was highly significant in the GEE model, as was test day, with the negative beta value indicating a decline in mortality rate with exposure, whilst the interaction between strain and test day was non-significant (Table 2), indicating comparability of trends. Owing to retention of females within their original tubes to reduce handling (see *Section 2.2*), the number of females tested per tube inevitably declined over time, and this has a significant effect on mortality, with more females tested associated with higher mortality (Table 2). However, the final GEE model incorporates both the number tested per tube and test day as terms and indicates significant but opposing effects of each. This is further illustrated by a strongly significant partial rank correlation analysis between mortality rate and test day, controlling for the effects of test

day (r_s = -0.30, df = 117, P < 0.001). The change in mortality rates over time in each strain is evident in cumulative mortality plots, in which there is a noticeable plateau in the latter stages of the experiment (Fig. 3). Indeed, the mortality rates in the Jeddah and Cayman strains reduced to 0% and 6.3% on day 10, respectively, and were also dramatically lower in 10-day-old females subjected to six consecutive repeated exposures, when compared to a single 6 h exposure (Supplementary Figure S1).

3.2 Age- and multiple exposure dependence of expression of candidate genes

In the Jeddah strain, which shows evidence of metabolic resistance to pyrethroids, the expression levels of candidate genes were compared to the susceptible strain, and among females of different ages. Each of the P450 genes exhibited significantly higher expression compared to the susceptible colonies at age 5 days, with some evidence of age-dependent change evident for Cyp9J27 and especially Cyp9J26 (Fig. 4); however, none of the trends across age were significant (Table 3). The final gene (histone methyltransferase, AAEL006953) did exhibit significant evidence of age-dependence (Table 3) but was not significantly overexpressed in the Jeddah females compared to controls (Fig. 4). Survivors from 10 days of repeated exposure were compared to age-matched controls from the same experiment to investigate whether the candidate genes might have contributed to survival. Here, none of the candidate genes showed a difference in expression between the two groups.

4. Discussion

Bioassays are a crucial tool for monitoring emergence and increases in resistance in all programmes using insecticide for control. This is especially true for disease vectors where the impact of resistance on disease outcomes is typically difficult to measure (Kleinschmidt et al., 2018). Resistance monitoring can provide an early warning of oncoming threats to effectiveness of insecticide-based control provided they are as relevant as possible to the insect cohort causing disease. Prior findings of age-dependent reductions in mortality (see *Section 1*), coupled with disease transmission only by older female mosquitoes has led to questions over whether the impact of resistance on disease may be overestimated by bioassays performed on young mosquitoes (Jones et al., 2012), which is recommended by the WHO (WHO, 2022) and constitute almost all bioassay records (Praulins et al., 2022). Moreover, whilst bioassays are occasionally performed on adult mosquitoes caught directly from the wild, which may include older females, this is viewed as a last resort, in part because of possible effects of prior sublethal

exposures (WHO, 2022). Yet given the early warning motivation for testing, it is preferable to conservatively avoid underestimating resistance in a targeted population by testing the least susceptible cohort, which led us to propose and test three hypotheses.

Hypothesis 1 proposed that mortality would increase with age. Results from Experiment 1 only partially supported this hypothesis, with mortality only increasing at the oldest age tested in the Jeddah strain and showing no difference among ages for the Cayman strain. This contrasts with findings from highly resistant *An. coluzzii* mosquitoes from Tiassalé, Côte d'Ivoire, tested either from the field (Chouaibou et al., 2012) or as a colonised laboratory strain (Praulins et al., 2022), which showed stepwise increases in resistance moving through younger to older test ages to a maximum of 10 days-old. Though intermediates were not tested, a very large increase comparable to the change we detected at 14 days for Jeddah, was found when comparing young (3–5 day-old) and old (17–19 day-old) *An. gambiae* from Burkina Faso (Jones et al., 2012). More variable results have also been reported, with magnitude of change dependent on the strain of *Ae. aegypti* or *Anopheles* spp. tested (Rajatileka et al., 2011) or the specific pyrethroid tested for *An. arabiensis* (*s.l.*) (Mbepera et al., 2017). Nevertheless, the overall picture from our results and other studies is that whilst the age at which an increase occurs, or indeed whether an increase occurs at all, may vary depending on the mosquito strain and potentially also pyrethroid insecticides tested, bioassay mortality never appears to *decrease* with age for mosquitoes.

The causes of variability between strains motivated our second hypothesis, which was that strains more dependent on, potentially more costly, overexpression of enzymes for resistance may show greater age-dependence in mortality. Our results are consistent with this prediction in that whilst both strains we tested have three linked *kdr* mutations, in Cayman, selection for *kdr* mutants alone achieved the same level of resistance as multi-generational strong selection with permethrin (Thornton et al., 2020) suggesting their sufficiency for resistance. The synergist PBO has no impact on pyrethroid mortality in the Cayman strain (Harris et al., 2010), but causes significantly elevated mortality in Jeddah (Al Nazawi et al., 2017). Our study involved comparison of only two strains, and differences in their age-related susceptibility profiles could have other explanations beyond the mechanistic prediction we proposed. Furthermore, our qPCR results failed to implicate any convincing candidate genes which might be involved in Jeddah, with the gene showing age-dependence (AAEL006953), the only one not overexpressed compared to the susceptible colonies. However, it is perhaps notable that the *Anopheles* spp. tested in the above studies from Côte d'Ivoire and Burkina Faso, which showed marked age-dependence of mortality, are strongly dependent on overexpression of P450 enzymes for pyrethroid resistance, rather than *kdr* mutations (Edi et al., 2012, 2014; Toé et al., 2015; Williams et al., 2019). In *Ae. aegypti*, dependence of a substantial proportion of pyrethroid

resistance on interacting *kdr* mutations is common (Moyes et al., 2017), suggesting perhaps a lesser likelihood of strong age-dependence in mortality for *Aedes* than *Anopheles* disease vectors. Future testing might exploit lines genetically modified to include *kdr* mutants (Grigoraki et al., 2021) or overexpress metabolic enzymes (Adolfi et al., 2019) to further explore this hypothesis.

The third hypothesis was that repeated exposure to pyrethroid, which might often be expected for female mosquitoes surviving to older ages in the wild, would cause increasingly injurious effects and lead to an increasing mortality rate. This would be expected to elevate age-dependence of mortality. Results did not support this hypothesis in either strain, with mortality rates declining over repeated exposures to near-negligible levels, leading to distinctive plateauing of cumulative mortality curves. Even after 10 daily exposures left almost 25% and 40% alive of the then 14-day-old females from Jeddah and Cayman, respectively, suggesting selection for a cohort no longer impacted by exposures, at least at this dosage level. Our experiment could not distinguish whether this represents selection *per se* or phenotypically plastic induction of protective enzymes, but the findings potentially contradict the implication that maximum resistance in a population will be captured by testing a young cohort.

There are two caveats which should be noted. The first is that the number of mosquitoes tested per tube declined with time in the experiment. Recent work has shown that whilst testing 15–30 mosquitoes per WHO bioassay tube produces fairly stable results, mortality is significantly reduced if smaller numbers are tested (Praulins et al., 2022). It is likely that the reduction, and thus the total number surviving at the end would have been lower had numbers per tube been standardised throughout. Conversely the repetitive handling which would have been required to achieve this seems likely to have elevated mortality, so did not represent a satisfactory solution. Instead, our analyses accounted for this significant variation attributable to variation in tubes and still demonstrated a significant decline in mortality rate over time in both strains (evident by an absence of a significant strain × test day term in the GEE model), but we do not discount that this had a quantitative impact. The second factor is that in the wild it is unlikely that females would repeatedly encounter insecticides over such a prolonged period without successfully blood-feeding, which was not part of the experiment. Studies which have addressed the effect of blood-feeding on insecticide resistance typically find reduced mortality following a blood meal (Spillings et al., 2008; Oliver & Brooke, 2014; Machani et al., 2019), although more variable results across multiple strains and insecticides have also been documented (Rajatileka et al., 2011). Whilst we cannot rule out that blood-feeding would affect results, the balance of evidence suggests it would not qualitatively change the findings.

5. Conclusions

Taken together, our results and those from other studies suggest that the strategy of testing young mosquitoes should capture the minimum susceptibility exhibited by the vast majority of a mosquito population, but a small proportion of older females may persist that have survived multiple insecticide exposures. Given the much-elevated potential of these females to be of disease-transmitting age, this relatively small fraction may be of non-negligible importance in highly resistant populations such as those from Saudi Arabia. Such females may have the capacity to repeatedly bypass pyrethroid treated materials to feed and transmit infection. How to efficiently capture the resistance profiles of this cohort is less clear. Laboratory studies will inevitably have caveats, and whilst currently discouraged as a general strategy, complimentary testing of wild-caught females alongside standard methodologies which target young females is worth considering, if possible combined with methods for age-estimation (Siria et al., 2022).

Funding

The project was supported by a PhD Studentship from the Saudi Cultural Bureau to Ashwaq M Al Nazawi. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Ethical approval

Not applicable.

CRediT author statement

Ashwaq Al Nazawi: Conceptualization; Formal analysis; Methodology; Investigation; Resources; Data curation; Funding acquisition; Writing - original draft; Writing - review & editing. David Weetman: Conceptualization; Methodology; Formal analysis; Supervision; Writing - original draft; Writing - review & editing. All authors read and approved the final manuscript.

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary files.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Authors are grateful to all the municipal staff (administrators and laboratory technicians in the vector control programme) of Makkah and Jeddah in the Saudi Arabia. We also thank all staff in the vector control centre of Makkah, who participated in the mosquito collection.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://

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Figure legends

Fig. 1 Mortalities of females of different ages from each strain in the single exposure experiment.

Fig. 2 Daily mortalities exhibited by each strain over the 10-day multiple exposure experiment.

Fig. 3 Cumulative mortalities exhibited by each strain over the 10-day multiple exposure experiment. Starting numbers of 5-day-old females were 160 for Jeddah and 93 for Cayman.

Fig. 4 Results from qPCR comparing expression of candidate pyrethroid resistance genes at different ages. Bars show mean ddCt values compared to susceptible colonies. Asterisks indicate significant overexpression in 5-day-old females compared to colonies: ***$P < 0.001$; **$P < 0.01$; NS, no significant difference.

Table 1

Generalized linear model for the effects of strain and age on deltamethrin-induced mortality of *Ae. aegypti* females.

Source	β	SE	Wald χ^2	*df*	Probability
Intercept	0.244	0.248	0.965	1	0.326
Strain (Cayman)	0.641	0.340	3.551	1	0.059
Strain (Jeddah)	Reference				
Age (14 days)	-1.791	0.428	17.552	1	< 0.0001
Age (10 days)	0.162	0.407	0.158	1	0.691
Age (7 days)	-0.459	0.366	1.573	1	0.210
Age (5 days)	Reference				
Strain × Age (14 days)	1.348	0.532	6.426	1	0.011
Strain × Age (10 days)	-0.958	0.514	3.469	1	0.063
Strain × Age (7 days)	-0.080	0.497	0.026	1	0.872
Strain × Age (5 days)	Reference				

Note: Minimal model shown: number tested in tube and associated interaction terms removed as not significant.

Abbreviations: df, degrees of freedom; SE, standard error.

Table 2

Generalized estimating equation for effects of strain, test day of multiple exposure and the number of mosquitoes in the test tube on mortality of *Ae. aegypti* females.

Source	β	SE	Wald χ^2	*df*	Probability
Intercept	-1.411	0.5415	6.786	1	0.009
Strain (Cayman)	-1.004	0.2044	24.133	1	< 0.001
Strain (Jeddah)	Reference				
Test_day	-0.223	0.072	9.666	1	0.002
Number tested in tube	0.053	0.020	7.365	1	0.007

Note: Minimal model shown: interaction terms removed as not significant.

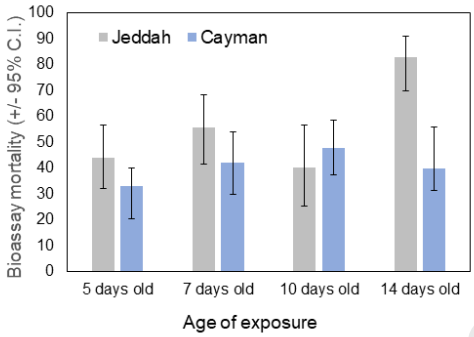
Abbreviations: df, degrees of freedom; SE, standard error.

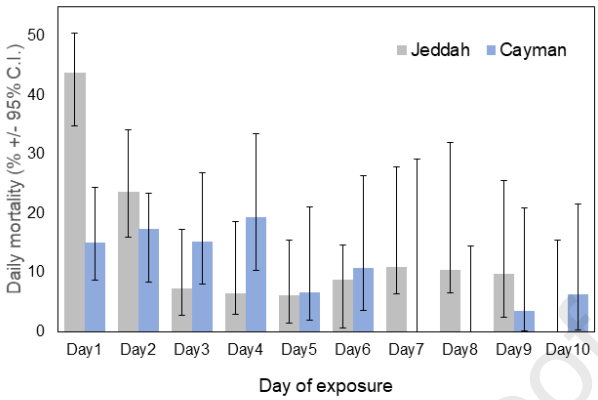
Table 3

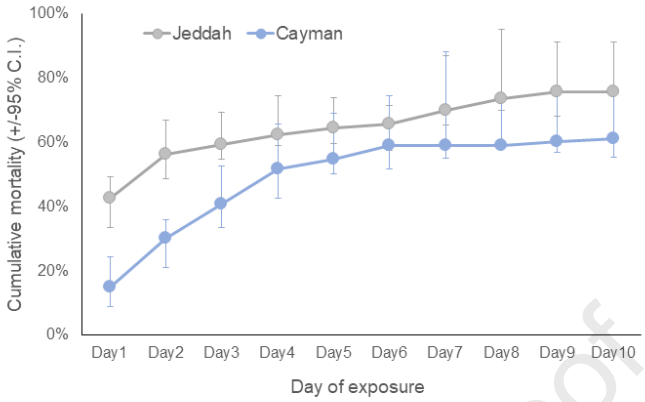
Analysis of gene expression in Jeddah samples aged 5, 7, 10 and 14 days relative to the three susceptible strains using Kruskal-Wallis tests, and between survivors at day 10 of repeated exposures *vs* age matched controls using *t*-tests.

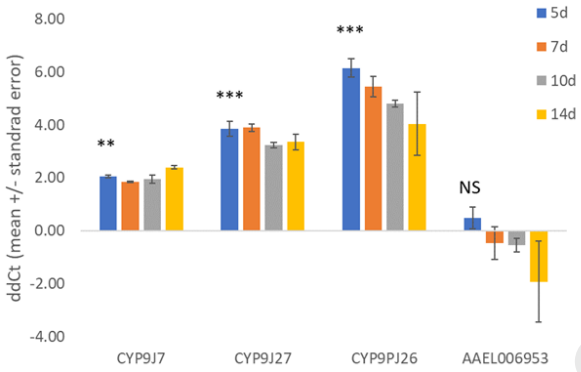
Gene	Comparison among ages			10-day survivors *vs* controls		
	χ^2	*df*	*P*-value	*t*	*df*	*P*-value
CYP9J7	2.301	3	0.512	1.211	4	0.293
CYP9J27	4.351	3	0.226	1.997	4	0.116
CYP9J26	5.312	3	0.150	0.975	4	0.385
AAEL006953	9.064	3	0.028	0.212	4	0.842

Abbreviation: df, degrees of freedom.









Highlights

- Insecticide resistance testing targets young mosquitoes expected to be least susceptible, but below transmission age.
- Age-dependent mortality differed between resistant *Aedes aegypti* strains potentially due to different mechanisms.
- Mortality rates declined with repeated exposure leaving some highly resistant females of disease-transmission age.
- Additional methods are required to track resistance in older wild females if insecticide encounters are common.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: