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The effects of temephos, permethrin and malathion selection on the fitness and fecundity of *Aedes aegypti*

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Abstract. The recent scale-up of insecticide use has led to the rapid spread of insecticide resistance (IR) in mosquito populations across the world. Previous work has suggested that IR mechanisms could influence mosquito life-history traits, leading to alterations in fitness and key physiological functions. This study investigates to what extent mosquito fitness may be affected in a colony of *Aedes aegypti* after selection with temephos, permethrin or malathion insecticides. We measured immature development, sex ratio, adult longevity, energetic reserves under different rearing conditions and time points, ingested bloodmeal volume, mosquito size, male and female reproductive fitness and flight capability in the unexposed offspring of the three selected strains and unselected strain. We found that insecticide selection does have an impact on mosquito fitness traits in both male and female mosquitoes, with our temephos-exposed strain showing the highest immature development rates, improved adult survival, larger females under crowded rearing and increased sperm number in males. In contrast, this strain showed the poorest reproductive success, demonstrating that insecticide selection leads to trade-offs in life-history traits, which have the potential to either enhance or limit disease transmission potential.

Key words. Energetic resources, flight, insecticide resistance, larvicide, life-history parameters, mosquito.

Introduction

Insecticide resistance (IR) in disease vectors is at a crucial tipping point. The recent scale-up of insecticide-based vector control has protected hundreds of millions of people from disease exposure (Bhatt *et al.*, 2016), but has also resulted in the emergence and rapid spread of IR mechanisms across the world (Vontas *et al.*, 2012; Ranson & Lissenden 2016; WHO 2018). Within the major arbovirus vector *Aedes aegypti*, resistance has evolved to the four insecticide classes most commonly used for public health (Ranson *et al.*, 2010; Moyes *et al.*, 2017), with resistance to both larval and adult insecticides well documented in field populations (Montella *et al.*, 2007). This has led to a reduction in the efficacy of current insecticide-based control strategies (Moyes *et al.*, 2017). However, IR is energetically costly and can reduce mosquito fitness in the absence of insecticides, with effects ranging from minimal to highly damaging (Martins *et al.*, 2012; Brito *et al.*, 2013; Belinato & Martins 2016). Resistance mechanisms cause significant changes to key physiological functions in the vector, such as depleting energy resources (Diniz *et al.*, 2015), affecting development time (Martins *et al.*, 2012; Rahim *et al.*, 2017; Ramos *et al.*, 2018) or altering immune functions (Vontas *et al.* 2005), which can lead to changes in disease transmission. Metabolic resistance, caused by elevated enzyme activity, can be energetically costly with resources diverted for sequestration, metabolism and detoxification of insecticides (Saingamsook *et al.*, 2019). Previous studies have shown that metabolic resistance to temephos is associated with a reduction in egg batch size (Martins *et al.*, 2012; Diniz *et al.*, 2015; Viana-Medeiros *et al.*, 2017). Removing insecticide pressures from an environment results in lower frequencies of resistant alleles in mosquito populations, suggesting there is a fitness cost to maintaining these alleles in the absence of insecticide (Coustau *et al.*, 2000; David *et al.*, 2018).

Lipids and glycogen are important energy resources used for processes such as flight, vitellogenesis and immune responses

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(Steele, 1981). Glycogen stores are released from within cells and provide a source of energy for immediate flight, whereas ingested carbohydrates are converted to lipids that are directly involved in oogenesis, moulting and sustained flight (Beenakkers *et al.*, 1981). Resource-based trade-offs have been previously observed in insecticide-resistant mosquito populations, with the over-production of detoxifying enzymes requiring an extensive investment of resources. This can lead to depleted lipid stores, likely because lipids play a vital role in amino acid synthesis, thus leading to a knock-on negative impact on life-history traits, which rely on stored energy reserves (Rivero *et al.*, 2010). If the availability of these resources is altered at either the larval or adult stage then development, reproduction and movement will be affected.

Research into mosquito behaviour, fitness and fecundity tends to focus on measurements of females and their offspring. However, the physiological and behavioural traits observed in females post-mating (egg development, oviposition rates and host-seeking behaviours) are partially attributed to the receipt of male seminal fluid proteins and sperm (Hiss & Fuchs, 1972; Downe, 1975; Adlakha & Pillai, 1976; Klowden, 1993; Villarreal *et al.*, 2018). Both positive and negative associations between resistance and male reproductive success have been demonstrated, with Arnaud *et al.* (2005) reporting that insecticide-resistant beetles have improved reproductive success and are superior sperm competitors, whereas, in resistant mosquitoes, Belinato *et al.* (2012) saw a reduced frequency of female insemination.

While many studies have reported negative effects of IR on fitness and fecundity, a few studies have documented positive effects. Chan & Zairi (2013) demonstrated that permethrin-resistant *Aedes albopictus* survived longer when starved and produced larger females under crowded rearing densities than their susceptible counterparts. If resistant female mosquitoes show increased longevity, they are more likely to survive through a pathogen's extrinsic incubation period, increasing transmission potential (Kramer & Ebel, 2003).

Numerous limitations from previous studies likely contribute to poor concordance in study outcomes. Often only one or two fitness-related phenotypes were measured, despite the interdependency between longevity, male and female fecundity and energy resources. Furthermore, there are very few comparable pairs of resistant and susceptible strains, which only differ in resistance phenotype.

Our study aimed to investigate the fitness costs associated with IR by measuring energetic reserves, development, longevity, reproduction and flight in four strains of *A. aegypti* with different histories of insecticide exposure.

Materials and Methods

Establishment and maintenance of four A. aegypti *strains*

An *A. aegypti* colony from Recife, Brazil, was used to create four strains via exposure over 10 generations to either the larval organophosphate temephos (REC-R), adult pyrethroid permethrin (REC-P), adult organophosphate malathion (REC-M), or no insecticide exposure (REC-U) (Thornton *et al.*, 2020). All four strains were established and maintained under standard controlled conditions (27 °C ± 2 °C, and 80% relative humidity, 12:12 light/dark cycle) in an insectary at the Liverpool School of Tropical Medicine. Eggs were obtained by feeding mated adult females on human blood using a Hemotek feeder (Hemotek Ltd, Blackburn, U.K.). To standardize rearing conditions, 200 first instar larvae were counted and placed in plastic larval rearing trays (23.5 × 34.5 × 7.5 cm) containing 1 L of deionized (DI) water and one Brewer's yeast tablet (500 mg). To mimic high larval density rearing, 500 first instar larvae were counted and placed in rearing trays with 1 L of DI water and 1 yeast tablet. For each strain, four larval trays at each density were reared to use for testing and larvae were fed with one yeast tablet every other day. Adults were maintained on 10% sugar solution.

Resistance profiles. Resistance ratios after 1 year of selection, using lethal concentration (LC) 50 and LC95, were previously examined and compared to a fully susceptible New Orleans colony (Thornton *et al.*, 2020). For permethrin, REC-P was five times more resistant than REC-U, REC-M and REC-R. For malathion, REC-R and REC-M were slightly more resistant (~2×) than REC-U or REC-P. With temephos, REC-R, REC-M and REC-P were more resistant (>2×) than REC-U (Table S1).

This study investigated the impact of insecticide selection regimes on four main physiological aspects of mosquito fitness: life-history traits, energy reserves, reproductive fitness and flight capability. The effect of different larval rearing densities and mosquito age were also considered. Figure 1 shows the study design and experimental pathway for each cohort of mosquitoes.

Mosquito life traits

Immature development time. Mosquitoes from each of the four strains, at both rearing densities (standard rearing trays: Rec-R $n = 3$, REC-U $n = 3$, REC-M $n = 2$, REC-P $n = 3$; crowded rearing trays: REC-R $n = 2$, REC-U $n = 2$, REC-M $n = 2$, REC-P $n = 1$), were separated by sex upon pupation into individual male and female holding containers. The number pupating per day was recorded. Mosquito eclosion was recorded for each sex and strain, and adults were retained in separate containers prior to assays.

Longevity

Longevity was recorded for mosquitoes from each strain, at the standard rearing density of 200 larvae/tray. Four cups of females and four cups of males each containing 20 adults were maintained on 10% sugar solution and monitored until all mosquitoes had naturally died. Due to different eclosion dates, each strain had a staggered start date, with the longest experiment lasting for a total of 60 days. The temperature and humidity of the insectary remained constant (27 °C ± 2 °C, and 80% relative humidity) and cup placement rotated daily to ensure standardized conditions. Death was recorded daily.

Objective	Cohort	Outcome	Measured endpoints	Target sample size per strain
Life traits	Standard density	Immature development	Number successfully pupated and time to pupation and sex ratio	3 trays, $n = 200$
			Number successfully eclosed and time to eclosion and sex ratio	
		Adult longevity	Day of death	80 females, 80 males
	Crowded density	Immature development	Time to pupation and sex ratio	2 trays, $n = 500$
			Time to eclosion and sex ratio	
Energy reserves	Standard density	Bloodmeal volume	Haemoglobin content *	10 females
		Reserves (day 2)	Lipid content (ug/mL) *	16 females
			Glycogen content (ug/mL) *	
		Reserves (day 8)	Lipid content (ug/mL) *	16 females
			Glycogen content (ug/mL) *	
	Crowded density	Reserves (day 2)	Lipid content (ug/mL) *	16 females
			Glycogen content (ug/mL) *	
		Reserves (day 8)	Lipid content (ug/mL) *	16 females
			Glycogen content (ug/mL) *	
Reproductive fitness	Male	Fertility	Total sperm count per male *	15 males
			Sperm number per mm of wing length	
		Individual mating success	Number of females inseminated per male	22 males
		Cross mating success	Number of females inseminated per male	10 males
	Female	Female fecundity	Total egg number per female fed to repletion	20 females
			Total L1 per female fed to repletion	
Flight capability	Female	Flight distance	Total distance (m)	33 females
			Average speed (m/s)	
		Flight bursts	Number of bursts over test period	

Fig 1. Study objectives, measured endpoints and target sample sizes. *Wing length measurements were taken for each of the mosquitoes in this assay. The sample size calculation for each primary outcome was based on a pilot study. Statistical modelling of the relationship between measured endpoint and strain indicated that differences between strains explained approximately 10% of variation in the data. Thus, on the assumption of an effect size of 0.1, the R package 'pwr' was used to calculate the minimum sample size under the following assumptions: degrees of freedom for numerator: 5; type I error prop: 0.05; type II error prob: 0.20; effect size: 0.1.

Quantification of energy resources

Bloodmeal volume. Bloodmeal volume was evaluated by quantifying haemoglobin amount (Briegel *et al.*, 1979), using Drabkin's reagent method. Midguts of blood-fed female mosquitoes were dissected 1 h post bloodmeal and the carcass was stored at −20 °C for subsequent wing measurements. Individual midguts were placed into 1.5-mL Eppendorf tubes containing 500 µL Drabkin's reagent and one metal ball bearing on ice. Samples were agitated in a tissue lyser for 1 min at 15 Hz and another 500 µL Drabkin's reagent was added. Samples were centrifuged at 12770 g for 15 min, before 200 µL of each sample was loaded onto a flat bottomed 96-well plate and read at 540 nm using Gen5 Epoch plate reader. Triplicate readings were recorded for each sample and an average was taken.

Wing length. Wing length was used as an estimate for body size. The right-wing from each female was removed from the thorax and an image was taken using a GXCAM ECLIPSE Wi-Fi microscope camera attached to a GX Stereo microscope. The length of the wing from the axial vein to the distal end of the R1 vein (not including the hairs on the edges of wings) was measured using GXCAM software (GXCAM Ver6.7).

Lipid and glycogen. We determined the lipid and glycogen content of mosquitoes using a standard protocol (*Methods in Anopheles Research*, 2015) with vanillin and anthrone reagents. Mosquitoes from all four strains, at both rearing densities, were split into two separate cohorts to allow energy analysis at two different time points; reserves measured at two days post-emergence (DPE) and reserves measured at eight DPE.

Reproductive fitness

Sperm number

Male and female mosquitoes were separated upon pupation and allowed to emerge in separate holding containers. Fifteen 1-day-old males were removed and individually knocked down on ice before dissection of the testes and seminal vesicles into 50 μL of phosphate-buffered saline (PBS). Samples were torn gently with dissecting pins and pins washed with 150 μL of PBS to obtain a final stock volume of 200 μL. Samples were mixed and 10 μL transferred into multi-well slides (20 individual wells per mosquito). Slides were air-dried, fixed with 70% ethanol and stained with Giemsa dye. Mosquito sperm heads were counted under ×40 magnification. One wing from each male was measured using the method described earlier.

Individual mating success

To determine individual mating success, 22 virgin male mosquitoes of each strain were housed individually in holding cups with three virgin females of the same strain. Males were given four days to mate. On the fourth day, female mosquitoes were knocked down briefly on ice and all three spermatheca were scanned for spermatozoa. Mosquitoes were recorded as either 'positive' or 'negative' for insemination.

Cross mating success. Following the results of strain-specific differences in mating success, REC-M and REC-R strains were further evaluated through a cross mating experiment to determine whether mating success was a male or female trait. The same method was repeated, with 10 virgin males individually housed with three virgin females from either the same strain or the alternate strain, resulting in four different crosses.

Female fecundity

Three mosquito rearing cages (28.5 × 29.5 × 28 cm) for REC-R, REC-U and REC-M, and two rearing cages for REC-P, were prepared with 30 female and 30 male mosquitoes introduced at the same time. Females were given four days to mate and then offered a human bloodmeal using a Hemotek membrane feeding system. All non-fed females were removed from the cage, and an oviposition pot containing damp cottonwool and filter paper was placed into the cage three days later, left overnight and then removed the following day. Multiple parameters were recorded: number of females fed to repletion, number of eggs laid and L1 hatch rate.

Quantification of flight ability

To investigate the effects of IR on mosquito flight ability, we used a tethered insect flight mill (provided by Dr. Jason Lim of Rothamsted Research), housed under standard insectary conditions. Due to low numbers of REC-M at the time of this assay, we only compared females from three strains: REC-R ($n = 33$), REC-U ($n = 66$) and REC-P ($n = 33$). REC-U females were flown at the same time as either REC-R or REC-P females to serve as a comparator.

Then, 2–5-day-old, non-blood-fed, virgin mosquitoes were knocked down briefly on ice before attachment to the tethered flight mill as follows. The rotor arm of the flight mill (radius 4 cm) was dipped into non-solvent glue and held gently onto the upper thorax of the mosquito, avoiding the wings. Mosquitoes on the rotor arm were then placed into one of the eight tethered flight mills, held in place between two opposing magnets to minimize friction, and briefly observed to check flight capability (Fig. S1). After a 30-minute recovery period, mosquitoes could fly freely for one h. The distance covered every five second (to the nearest 10 cm) was recorded using the flight mill software (Flight Mill Version 2).

Statistical analysis

Statistical analysis was carried out using IBM SPSS Statistics (Version 24) or in RStudio (R version 3.6.0). To evaluate differences between strains in number of mosquitoes successfully pupating and eclosing, *t* tests were performed in SPSS, with differences in sex ratio for both pupae and adults analysed using chi-square test. Differences in the longevity of female and male mosquitoes from each strain were investigated using Kaplan–Meier survival curves and compared using Logrank (Mantel–Cox).

To determine if bloodmeal volume, wing length or energy content differed between strains, we used generalized linear mixed models (GLMMs) using the 'lme4' package in R. GLMMs for energy resources were fit with a Gaussian distribution. To account for variation in body size between individual mosquitoes, wing length was included in the GLMM as a random effect. Stepwise regression was used for model selection. All explanatory variables and two-way interactions were fit, and their significance was tested using log-likelihood ratio

tests by comparison to a null model with only an intercept. Pairwise comparisons between categories were conducted using Tukey range tests ('lsmeans' package Version 2.30-0), with the p value significance threshold adjusted using the Bonferroni correction method. To investigate male fecundity, we analysed sperm number per mm of wing length for each strain. For individual mating and cross mating, we investigated the associations between the proportion of females successfully inseminated and strain using GLMMs fit with a binomial distribution, following the same method as previously described. Statistical significance of female fecundity was investigated using t tests.

Flight ability parameters (average speed, maximum speed, number of flight bursts and flight burst length) were analysed using RStudio prior to further analysis using SPSS. Individuals, which flew less than 50 m, were not included in analysis to rule out the possibility that attachment to the flight mill may have compromised their flight. Then, t tests were carried out using SPSS.

Results

Mosquito life traits

Immature development time. At standard rearing density, REC-R and REC-U had the highest pupation and eclosion rates, and at the crowded rearing density, REC-R had the highest pupation and eclosion rate (Table 1). Female-to-male ratios also differed between strains for both pupae and adult mosquitoes (Table 1). For all strains, the time to 50% pupation and eclosion was slower in the higher density trays.

Longevity. With a mean female survival of 28.07 days [95% confidence intervals (95% CI) 25.23–30.91], REC-R had greater longevity than REC-U (20.49 days, 95% CI 18.74–22.25, $p < 0.001$), REC-M (22.68 days, 95% CI 20.99–24.37, $p < 0.001$) and REC-P (21.45 days, 95% CI 20.24–22.67, $p < 0.001$).

With a mean male survival of 35.13 days (95% CI 32.52–37.73), REC-R had greater longevity than REC-U (25.86 days, 95% CI 22.81–28.91, $p < 0.001$) and REC-M (27.09 days, 95% CI 24.67–29.52, $p < 0.001$). REC-P had a mean survival of 36.80 days (95% CI 34.51–39.09), also surviving significantly longer than REC-U ($p < 0.001$) and REC-M ($p < 0.001$) (Fig. 2).

Energy resources. To determine whether energetic resources differed between strains, we first explored adult body size, followed by the relationship between body size and blood volume consumed.

At the standard rearing density REC-R, REC-U and REC-P female mosquitoes were all significantly larger than REC-M

Table 1. Mosquito pupation, eclosion and sex ratios by strain and rearing density.

Density	Strain	Mean number pupated and time to 50% pupation: Female	Mean number pupated and time to 50% pupation: Male	% Pupated	Pupae sex ratio (F:M)	Mean number eclosed and time to 50% eclosed: Female	Mean number eclosed and time to 50% eclosed: Male	% Eclosed	Adult sex ratio (F:M)
200 larvae/tray	REC-R	96.0 (SD ± 2.4) 4 days	110.3 (SD ± 6.3) 3 days	100.0	1:1.15	80.3 (SD ± 9.2) 7 days	98.7 (SD ± 1.7) 5 days	89.5	1:1.23
	REC-U	92.0 (SD ± 7.8) 4 days	115.0 (SD ± 0) 3 days	100.0	1:1.23	87.0 (SD ± 7.9) 7 days	98.7 (SD ± 2.9) 5 days	92.8	1:1.13
	REC-M	75.5 (SD ± 13.5) 4 days	75.5 (SD ± 11.5) 2 days	75.5*	1:1	54.0 (SD ± 10) 5 days	54.5 (SD ± 2.5) 5 days	54.25*	1:1
	REC-P	76.7 (SD ± 10.2) 3 days	83.3 (SD ± 18.4) 2 days	80.0*	1:1.09	59.7 (SD ± 8.3) 6 days	63 (SD ± 13.4) 5 days	61.0*	1:1.07
500 larvae/tray	REC-R	213.0 (SD ± 6.0) 8 days	256.5 (SD ± 2.5) 4 days	93.9*	1:1.20	155.0 (SD ± 4) 10 days	217.4 (SD ± 1.5) 6 days	74.5*	1:1.40
	REC-U	118.5 (SD ± 6.5) 6 days	149.5 (SD ± 3.5) 4 days	53.6	1:1.26	88.5 (SD ± 1.5) 8 days	117 (SD ± 5) 6 days	41.1	1:1.32
	REC-M	111.5 (SD ± 2.5) 5 days	195.0 (SD ± 19.0) 3 days	61.3	1:1.75	79.5 (SD ± 0.5) 8 days	145.5 (SD ± 19.5) 6 days	45.0	1:1.83
	REC-P	217.0 (SD ± 0) 6 days	260.0 (SD ± 0) 4 days	47.6	1:1.19	160 (SD ± 0) 8 days	214 (SD ± 0) 7 days	37.4	1:1.34

*Significant difference when compared to REC-U ($p < 0.05$).

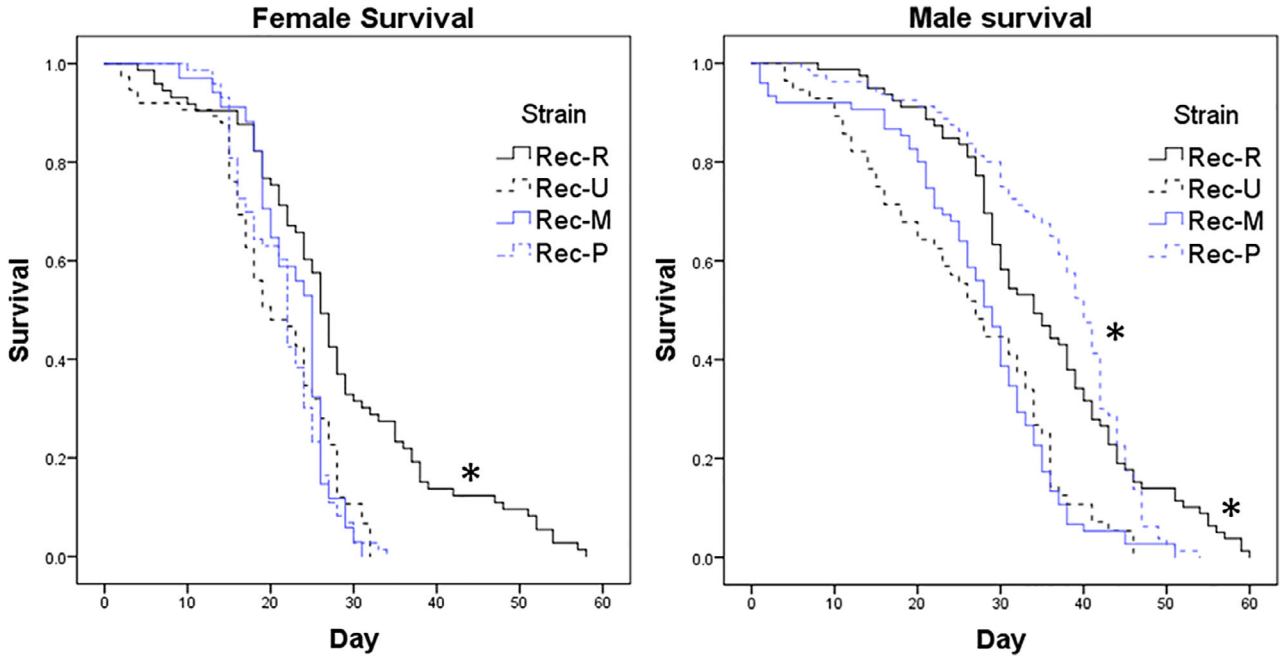

Fig 2. (A) Kaplan–Meier survival curves of REC-R ($n = 71$), REC-U ($n = 73$), REC-M ($n = 34$) and REC-P ($n = 76$) female mosquitoes and (B) Kaplan–Meier survival curves of REC-R ($n = 77$), REC-U ($n = 54$), REC-M ($n = 74$) and REC-P (n = 77) male mosquitoes. *$p < 0.05$.

(Fig. 3) (Table S2 and Fig. S2). At the crowded rearing density, there was a significant difference in size between all strains of mosquito.

There was a positive correlation ($R^2 = 0.27$) between bloodmeal volume and wing length ($\chi^2 = 15.599$, df = 1, $p < 0.001$), with no difference in this relationship between strains ($\chi^2 = 1.111$, df = 3, $p = 0.57$).

Lipid. The fixed effects of 'strain', 'density' and 'age' each contributed significantly to the explanatory power of the best fit model of lipid content (Table S3).

There was a significant interaction between 'strain' and 'density' ($\chi^2 = 34.138$, df = 3, $p < 0.001$). When reared at standard density there were no differences between any combinations of strains, however, at high-density lipid content for both REC-R and REC-U was significantly higher than REC-P [REC-P – REC-R ($p = < 0.001$, 95% CI −49.24 to −16.42), REC-P – REC-U ($p = 0.008$, 95% CI −51.27 to 12.347); Table S4].

The best fit model for lipid content also reported a significant interaction between 'strain' and 'age' ($\chi^2 = 50.503$, df = 3, $p < 0.001$; Fig. S3). At two DPE lipid content for REC-R was significantly higher than REC-M and REC-P [REC-M – REC-R ($p = < 0.001$, 95% CI −55.78 to −21.79), REC-P – REC-R ($p = < 0.001$, 95% CI −57.01 to −25.07)]. All other pairwise comparisons at two DPE were not significantly different. At eight DPE, REC-M lipids were significantly higher than REC-P with no difference between all other pairwise comparisons [REC-M – REC-P ($p = < 0.001$, 95% CI 17.73–54.70); Table S5].

Glycogen. The fixed effects of 'strain', 'density' and 'age' each contributed significantly to the explanatory power of the best fit model for glycogen content (Table S6).

There was a significant interaction between 'strain' and 'density', indicating that the relationship between strain and glycogen content was dependent on density at the larval stage ($\chi^2 = 22.241$, df = 3, $p < 0.001$). Pairwise comparisons showed that at standard density the mean glycogen content for REC-R was higher than both REC-P and REC-U, all other combinations were not significantly different [Rec-R – REC-P ($p = 0.003$, 95% CI 7.35 – 25.85), REC-R – REC-U ($p = < 0.001$, 95% CI 8.83–26.71); Table S7]. However, when reared at high density there was no difference in glycogen contents between any combinations of strains.

The interaction between 'strain' and 'age' also contributed to the model of glycogen content, indicating that the relationship between strain and glycogen content varied depending on the DPE ($\chi^2 = 24.985$, df = 3, $p < 0.001$). At two DPE, glycogen content for REC-R was significantly higher than REC-M, REC-P and REC-U, with no significant difference between any combination of these other strains [REC-M – REC-R ($p = 0.005$, 95% CI −26.74 to −7.02), REC-P – REC-R ($p = < 0.001$, 95% CI −29.25 to − 10.47), REC-R – REC-U ($p = < 0.001$, 95% CI 12.56–31.73); Table S8 and Fig. S3]. At eight DPE, there was no difference between any combinations of strains.

Reproductive fitness

Sperm number. REC-R contained a significantly higher number of sperm per mm of wing length than all other strains

Table 2. Mean sperm number, wing length and sperm number per mm of wing length for each of the four strains.

Strain	*N*	Sperm number (95% CI)	Wing length (mm) (95% CI)	Sperm number/mm wing length (95% CI)
REC-R	14	3806.14 (2222.24–5390.05)	2.60 (2.55–2.66)	1475.22* (851.17–2099.28)
REC-U	15	1779.07 (1033.09–2525.04)	2.62 (2.57–2.68)	681 (394.14–969.01)
REC-M	15	1318.27 (629.16–2007.37)	2.57 (2.53–2.61)	511.20 (244.88–777.53)
REC-P	14	1719.86 (1182.61–2257.10)	2.61 (2.56–2.65)	657.12 (448.64–865.60)

*Significant difference compared to all other strains $p < 0.05$.

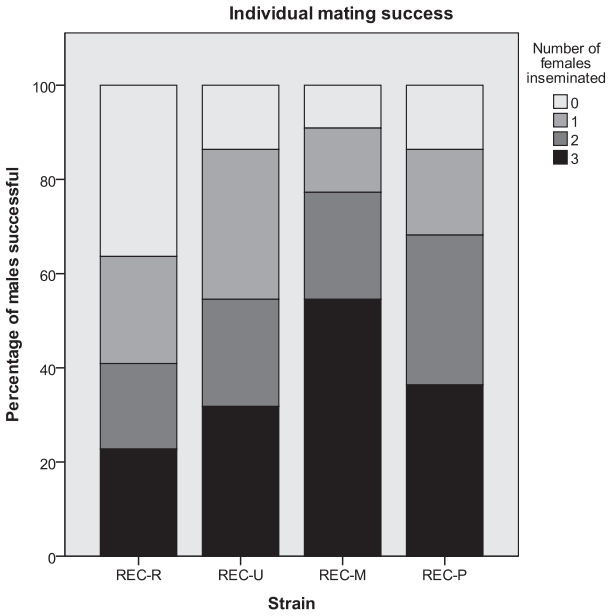

Fig 3. Wing length of four strains of *Aedes aegypti*, reared at standard 200/tray (REC-R $n = 36$, REC-U $n = 38$, REC-M $n = 35$, REC-P $n = 32$) and crowded 500/tray (REC-R $n = 32$, REC-U $n = 32$, REC-M $n = 35$, REC-P $n = 32$) larval densities. Different letters indicate statistically significant differences between strains ($p < 0.05$) per density, with 95% confidence intervals.

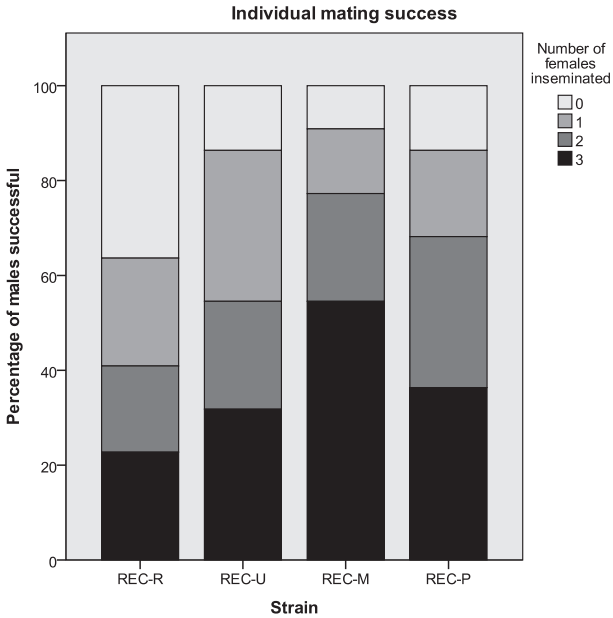

Fig 4. Individual mating success of one male mosquito ($n = 22$ per strain) with three female mosquitoes ($n = 66$ per strain).

[REC-U $t(27) = 2.5487$, $p = 0.017$; REC-M $t(27) = 3.1404$, $p = 0.004$; REC-P $t(26) = 2.6862$, $p = 0.012$] (Table 2).

Individual mating success. Binomial regression analysis showed that overall strain was a statistically significant factor for individual mating success over the 3-day period ($\chi^2 = 14.675$, df = 3, $p = 0.002$).

A significant difference in mating success was observed between REC-M and REC-R ($p = 0.002$, 95% CI 0.188) (Fig. 4 and Table S9). All other pairwise comparisons were not significantly different.

Cross mating. Mating success was explored further through cross mating of the poorest performing strain (REC-R) and the highest performing strain (REC-M). Results show that mating success is a male trait and again that strain is a significant factor ($\chi^2 = 15.372$, df = 3, $p = 0.002$). REC-M males were more successful at inseminating both REC-M females ($p = 0.033$, 95% CI 11.976) and REC-R females ($p = 0.066$, 95% CI 6.345), than REC-R males were (Table S10).

Female fecundity. REC-U females produced a larger mean egg batch per female (35.02 eggs/female) than REC-R (18.03 eggs/female) and REC-M (22.60 eggs/female); however, neither comparison was statistically significant (REC-R $p = 0.122$, 95% CI −40.137 to 6.964; REC-M $p = 0.289$, 95% CI −40.176 to 15.642; Table 3). REC-U also had a higher larval hatch rate per female (26.6 larvae/female) than REC-R (13.2 larvae/female), REC-M (9.9 larvae/female) and REC-P (16.1 larvae/female); however, no comparisons were significantly different (REC-R $p = 0.205$, 95% CI −847.97 to249.97; REC-M $p = 0.143$, 95% CI −952.18 to 198.84; REC-P $p = 0.353$, 95% CI −1147.32 to 559.65).

Quantification of flight ability

A total of 99 mosquitoes were flown on the tethered insect flight mill. REC-P flew a longer distance within an hour than REC-R; however, neither strain was statistically significant compared to REC-U (Table 4) [REC-P $t(69) = 0.2792$, $p = 0.7809$;

Table 3. Fecundity of females fed to repletion.

Strain	*N*	Mean eggs	Mean L1	% Hatch
REC-R	63	18.03	13.2	73.0
REC-U	65	35.02	26.6	75.8
REC-M	60	22.6	9.9	44.0
REC-P	35	41.9	16.1	38.4

REC-R $t(71) = 0.8975$, $p = 0.3725$]. REC-P also showed more sustained flight when compared to REC-U, with less than half of the number of flight bursts of REC-R [REC-P $t(69) = 1.2982$, $p = 0.1985$; REC-R $t(71) = 0.5759$, $p = 0.5665$]; however, this was not statistically significant.

These results show that insecticide selection does have an impact on the life-history traits of both female and male mosquitoes. Compared to all other strains, REC-R had the highest pupation and eclosion rates at both rearing densities, female and male adults survived longer, females were larger at the crowded rearing density and males produced more sperm per mm of wing length. However, REC-R males and females had the poorest reproductive fitness with males inseminating the fewest females and females laying the fewest eggs. In comparison, REC-M had the smallest females at both rearing densities, but the highest individual female insemination success rate.

Discussion

Throughout this study, the temephos exposed REC-R strain has shown the most noticeable differences in fitness and fecundity when compared to the other exposed and unexposed. With higher pupation numbers at both rearing densities, males and females surviving longer, increased energy resources under certain conditions and highest sperm number, our results suggest a fitness advantage due to sustained temephos selection pressure. However, despite the increased sperm number seen in REC-R, there appears to be a net fecundity cost due to poor male mating success and lower mean egg numbers.

One possible explanation for why REC-R males had the highest sperm count but lowest insemination success is that this strain produces a larger ejaculate but at less frequent intervals. This result is mirrored in work by Belinato *et al.* (2012) who saw that mating efficacy was inversely proportional to temephos resistance ratio, and in work by Diniz *et al.* (2015) who showed that resistance status impacts male mating success. Body size is a well-documented factor in male mating success, with previous studies (Ponlawat & Harrington, 2007, 2009) reporting that *A. aegypti* body size was correlated with sperm number. However, our study confirmed that the significant differences in sperm number between strains were not attributable to differences in body size.

Our results on female fecundity are again similar to Belinato *et al.* (2012), who showed females from a highly resistant temephos field strain laid fewer eggs than the susceptible counterpart. One limitation of our study is we were unable to measure fecundity throughout the female's lifespan due to an unavoidable change in blood source after the first gonotrophic cycle.

While reduced fecundity in resistant strains could lead to lower mosquito densities, adult female longevity is a crucial factor in the vectorial capacity of wild mosquito populations. REC-R female and male mosquitoes survived for significantly longer than other strains in this study; however, previous work using a different *A. albopictus* reported that temephos resistant field strains had a shorter lifespan than their susceptible counterpart (Rahim *et al.*, 2017). There are important differences between our study design and the one followed by Rahim *et al.* (2017), most notably, we tested laboratory mosquitoes with an extended history of insecticide pressure, in contrast to a progeny originating from only one round of larval temephos exposure. We also did not offer a bloodmeal to females during the longevity assay and instead provided continued access to sucrose solution.

Results from energy content analysis show that teneral energy reserves do not explain the stark differences in fitness traits for REC-R. There was no significant difference in lipid or glycogen content observed between strains, instead differences were only observed between the two larval rearing densities and mosquito age. Energy content cannot, therefore, explain reductions in egg batch size, improved immature development or increased longevity. With lipids and glycogen being important for use in flight, we were not surprised to observe no difference in flight duration or flight burst number between strains.

It is important to note that while the strains used all originated from the same parental colony, these fitness experiments were carried out under laboratory-controlled conditions. The Recife colony used for selection had a background of previous temephos exposure and each strain underwent differential selection with exposure to insecticides using concentrations at 50% lethal dose (LD) over a period of 12 months. The physiological costs of resistance are often underestimated within a laboratory setting due to a lack of stress factors that are experienced in the field. In this study, however, we took the stress of larval crowding into consideration when assessing life-history traits.

Interestingly, our data suggest that continued selection to the organophosphate temephos at larval stages leads to shorter developmental time and increased longevity but reduced fecundity in the unexposed offspring. However, switching to selection with the organophosphate malathion in adult stage leads to better reproductive fitness but at the cost of longevity. With spermatogenesis thought to peak at the pupal stage, one explanation is that exposure during larval development can only lead to resource allocation that benefits longevity rather than reproduction. Conversely, improved fecundity in strains historically exposed during the adult life stage suggests that resources are diverted to offspring production rather than adult survival. These results have worrying implications for vector control programmes that target larval stages with insecticides, as longevity of the vector population is a key determinant of disease transmission potential.

Table 4. Mean flight distance and number of flight bursts over 1 h.

Strain	*N*	Distance (m) (95% CI)	Ratio*	Number flight bursts (95% CI)	Ratio*
REC-R	23	751.93 (387.39–1116.47)	0.80	21.22 (12.63–29.80)	1.20
REC-P	21	1012.57 (508.92–1519.22)	1.07	9.81 (2.87–16.75)	0.55
REC-U	50	944.64 (701.27–1188.01)	–	17.70 (10.32–25.08)	–

*Ratio compared to REC-U mosquitoes flown at the same time.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. The set-up of the tethered insect flight mill used to assess the flight capability of mosquitoes. Mosquitoes fly around a radius measuring 4 cm, causing the light encoder to periodically break a laser beam, which measures distance. One full rotation of the flight mill rotor arm = 25.13 cm. Image taken from (Somerville *et al.*, 2019).

Fig. S2. Bloodmeal volume relationship. Relationship between wing length and bloodmeal volume is not statistically distinguishable between strains. Shaded areas show upper and lower CIs for the line of best fit as predicted by the model. CIs overlap at all points in range, so all strains follow the same linear relationship.

Fig. S3. Predicted mean energy content for each *Aedes aegypti* strain reared at two different larval densities; lipid content at two days post-emergence (DPE) (A), lipid content at eight DPE (B), glycogen content at two DPE (C) and glycogen content at eight DPE (D).

Table S1. Lethal concentrations and resistance ratios of Recife strains for three insecticides (i.e. permethrin, malathion and temephos). Taken from Thornton *et al.* (2020).

Table S2. Mean wing length comparisons of four strains of *Aedes aegypti* reared at two different larval densities.

Table S3. GLMM lipid model statistics.

Table S4. The effects of strain and density on lipid content.

Table S5. The effects of strain and age on lipid content.

Table S6. GLMM glycogen model statistics.

Table S7. The effects of strain and density on glycogen content.

Table S8. The effects of strain and age on glycogen content.

Table S9. Differences in individual mating success between all four strains of *Aedes aegypti*.

Table S10. Cross mating success between REC-M and REC-R males when given the opportunity to mate with REC-M and REC-R females.

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All other authors declare no conflict of interest.

Author contributions

LJR and KG conceived and designed the study, KG collected the data, KG and FM analysed the data, KG wrote the first draft of the manuscript. All authors contributed to the final draft of the manuscript.

Date availability statement

The data that support the findings of this study are openly available in Open Science Framework at https://osf.io/crsmu/ (DOI 10.17605/OSF.IO/CRSMU)

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