

ORIGINAL ARTICLE

The effect of size on mate selection, fecundity and survival in *Culex pipiens* mosquitoes

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Abstract

Culex pipiens L. is a medically important mosquito due to its abundance and ability to transmit West Nile virus. Despite being the focus of many mosquito control strategies, very little is known about its mating behaviour. Several control strategies rely on knowing female mate preferences to ensure their efficacy. Therefore, the purpose of this study was to characterize mate selection based on mate quality. Because larger individuals should have more resources available to be used towards mating behaviour, it was hypothesized that larger mates (males or females) would be of higher value and preferentially mated with compared to smaller ones. By manipulating food availability, males and females were reared to be either large or small adults and were mated with either a large or small mate. Through a series of experiments, the effect of size on mate success and post-mating responses was assessed via insemination rates, blood feeding, egg laying, fertility and survival. Despite larger females living longer and being more fecund, males did not preferentially mate with larger females, nor did male size influence female survival or fecundity. Because larval rearing environment had an influence on adult morphology and fitness, it should be taken into consideration in mosquito control programmes.

KEYWORDS

assortative mating, *Cx. pipiens* complex, fertility, insemination rate, mate choice

INTRODUCTION

Culex pipiens L. is an abundant and widely distributed species of mosquito that is also a major transmitter of medically important diseases to human and non-human hosts, including being the primary vector of the West Nile virus (Rochlin et al., 2019). They are well-adapted to anthropogenic spaces, including highly polluted and manufactured larval aquatic habitats (Andreadis, 2012). Due to their adaptability, they tend to be among the most abundant mosquito species in the east-central United States (Molaei et al., 2006). The control of *Cx. pipiens* typically relies on adult surveys and insecticide applications, treatment and removal of larval aquatic habitats and community education and engagement (Bellini et al., 2016). However, there is a growing desire

for sustainable management strategies to include behaviour-based tools to control mosquito populations effectively (Benelli et al., 2016; Bourtzis et al., 2016), such as sterile insect technique and incompatible insect technique (such as *Wolbachia* infection; Cator et al., 2021). Because these techniques can be influenced by female mate selection, we need to know more about what influences *Cx. pipiens* mating behaviour to integrate these behaviour-based strategies effectively.

Mating in mosquitoes typically occurs in flight, with species-specific swarms of males and females forming copulation pairs while in flight, with matings lasting a relatively short period. Some species of *Culex* can mate in cages without forming swarms, including *Cx. pipiens* (Clements, 1999). The behaviours most extensively studied within the *Cx. pipiens* species complex are swarming behaviour and associative

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mating between biotypes (Clements, 1999; Kim et al., 2018), with a few studies characterizing courtship behaviour and mating rate (*Culex quinquefasciatus*: Sebastian & de Meillon, 1967; Williams & Patterson, 1969; *Culex tarsalis*: Reisen et al., 1985; *Cx. pipiens*: Benelli, 2018). Kim et al. (2018) were the first to quantify behavioural differences among the members of the *Cx. pipiens* species complex, which leads to differences in insemination rates between *Cx. pipiens* f. *pipiens* and f. *molestus* parent crosses. Benelli (2018) quantified the courtship behaviour in *Cx. pipiens* and found female rejection of male mating attempts to be common. However, to date, no study has quantified mate selection based on mate quality.

After mating, females undergo extensive physiological and behavioural changes, many of which are energetically expensive (e.g., egg development, altered reproductive and digestive systems, etc.; Clements, 1992). The seminal fluid proteins transferred with sperm during mating affect many female post-mating responses in mosquitoes, such as reducing the remating rate and increasing fecundity (see review by Meuti & Short, 2019). Survival is also known to be contingent on seminal fluid products in several non-mosquito insects (Arnqvist & Nilsson, 2000; South & Lewis, 2011; Wigby & Chapman, 2005) but has not been studied often in mosquitoes. Two studies have directly linked male seminal fluid and female survival. In *Aedes aegypti*, male contributions during mating promote female survival, as seen through females living longer when mated to larger males (Helinski & Harrington, 2011), and by the direct injections of seminal fluid proteins alone promoting female survival (Villarreal et al., 2018). If mate quality affects female survival, then females would benefit by being particular about who they mate with.

Adult size is one factor that can influence a female's post-mating response. How many offspring a female can have is often dictated by life history trade-offs—individuals who invest more in survival might have less energy for reproduction. Alternatively, individuals who can survive longer might be of better quality and able to invest more overall in their reproductive efforts (van Noordwijk & de Jong, 1986). In insects, size is known to influence fertility, lifespan and offspring fitness. For instance, damselfly larvae that were fed more developed into larger adults, lived longer and had a higher mating success than larvae fed poorly, especially the females (De Block & Stoks, 2005). There is considerable evidence that mate selection (both male and female) based on size, with larger individuals preferred, is common in Insecta (e.g., Orthoptera: Bateman, 1998; Phasmatodea: Seely et al., 1991; Hemiptera: Arnqvist et al., 1996; Capone, 1995; Honěk, 2003; Coleoptera: Kalberer & Kölliker, 2017; Omkar & Afaq, 2013; Diptera: Jones & Tomberlin, 2021). Larger male *Ae. aegypti* mosquitoes can inseminate more females, have a greater sperm capacity and transfer more sperm in their ejaculate (Helinski & Harrington, 2011; Ponlawat & Harrington, 2007). In *Aedes* and *Anopheles* mosquitoes, females mating with males with varying ejaculate quality influenced factors related to female fitness (reviewed in Avila et al., 2011; Dottorini et al., 2013; Helinski et al., 2012; Helinski & Harrington, 2011). However, whether mosquitoes mate based on assessing mate size is variable in mosquitoes (Ng'habi et al., 2008), and several studies have found no effect of size (Aldersley et al., 2019; Charlwood et al., 2002; Lang et al., 2018). The

effect of size on mating rate and female post-mating responses has not been described for *Cx. pipiens*.

One method of manipulating adult body size is by adjusting the larval environment. For animals with complex life cycles, variation in larval conditions can have strong 'carry-over' effects on adult fitness (De Block & Stoks, 2005; Westby & Juliano, 2017). Mosquitoes raised in a nutrient-poor or high-density environment are smaller than larvae that have not been challenged in this way (Cator et al., 2010; Cator & Zanti, 2016; Reiskind & Lounibos, 2009; Yan et al., 2021). Therefore, we created cohorts of larger or smaller individuals by rearing larval *Cx. pipiens* on high- and low-quality diets. Three sets of experiments were performed to measure the effect of mate size (male or female) on insemination, blood feeding, egg laying, fertility and survival. We hypothesized that mate selection would depend on size, with larger individuals preferred. We predicted that the insemination rate would be higher for larger females than smaller females and that larger females would lay more eggs and live longer. We also predicted that contributions from larger males would elevate female post-mating responses more than contributions from smaller males.

MATERIALS AND METHODS

Origin and maintenance of *Cx. pipiens* colonies

Mosquitoes used in these experiments were the descendants of *Cx. pipiens* egg rafts collected via oviposition traps placed in various locations in Granville, Newark and Columbus, Ohio. Fourth instar larvae from collected rafts were identified to *Cx. pipiens* based on morphological traits (Darsie & Ward, 2005) and were used to supplement the colony each field season using the progeny of at least 10 field-collected egg rafts. Generations used in this experiment were kept in the lab either in a 30 cm³ or a 60 cm³ mesh cage. They were fed a 10% sugar solution and maintained at 27°C with 80% RH and LD 14:10 h conditions in an environmental chamber. Mosquitoes were fed once per month on thawed whole chicken blood in sodium citrate (Pel-Freez Biologicals, Rogers, AR). They were fed via artificial feeders (CG-1836 50 mm glass membrane style feeder, Chemglass Life Sciences, Vineland, NJ) through two layers of pig intestines (The Sausage Maker, Buffalo, NY) and warmed via a circulating water bath set to 37°C. For colony maintenance, the larvae that hatched from collected egg rafts were placed in shallow 2 L plastic containers (25 cm × 18 cm × 6 cm) with 1 L deionized water at a density of 200 larvae per container. They were fed fish pellets (Cichlid Gold, Hikari Food Ind. LTD, Himeji, Japan) ad libitum until pupation. Upon pupation, pupae were transferred into clean cages at a density of approximately 2000 per 30 cm³.

Creating large and small adults

First instar larvae, randomly chosen from within the population of larvae hatch for the colony, were selected for these experiments and

placed in 2 L plastic containers with 1 L deionized water at a density of 200 larvae/L. Creating large and small adults was achieved using methods similar to Cator and Zanti (2016). A total of 10 containers were prepared for each cohort size. To create smaller adults, larvae were fed one small (3.2–3.7 mm) fish pellet each day. To create large adults, larvae were fed three small fish pellets each day. As pupae developed, they were isolated in 5 mL tubes topped with cotton to ensure virginity. Once eclosed, mosquitoes were sexed, placed into separate cages (large male, small male, large female, small female), with 300–500 mosquitoes per cage, and fed 10% sugar ad libitum. Adult size differences were confirmed by measuring wing length as a proxy (Nasci, 1990) as described below.

Experiment 1: Mate size and insemination rate

Mating assay

Once mosquitoes were sexually mature (2–6 days old) they were randomly selected from their eclosion cages to be transferred into clear plastic cages (four per treatment, 30 cm × 17 cm × 23 cm) to create the four treatment groups: large female with large male, large female with small male, small female with large male, small female with small male. Each cage contained 30 males and 30 females. Mosquitoes were allowed to mate for three full days (at 27°C, 35%–45% humidity, and LD 12:12 h), and fed 10% sugar water ad libitum. This procedure was repeated for three biological replicates—cohorts of individuals reared as the result of a single blood feeding event. For the first biological replicate, only two cages were used for treatments involving small females due to females starting to eclose 2 days after large females. Although both size cohorts pupated over approximately 5 days, the small-sized mosquitoes started eclosing roughly 2 days after the large. Therefore, for the second and third biological replicates, treatment larval trays for small individuals were created from early hatching egg rafts, and trays for large individuals were created 2 days later from later hatching eggs. This shift gave the small cohorts enough time to generate similar numbers of similarly aged adults for both treatments in time for mating trials.

Insemination check

After 3 days, all individuals were sacrificed by placing the mating cages into a –20°C freezer for at least half an hour. Each female's insemination status was assessed by carefully dissecting out their sperm storage organs, spermathecae, under a stereo microscope and checking for sperm via 40× magnification using a compound microscope. Females with sperm in at least one of the three spermathecae were scored as inseminated. To confirm mosquito size, a single wing was taken from every mosquito in each treatment and taped to a glass slide via double-sided tape. Images were taken of each wing using an AmScope digital camera (Model MU1000-HS, United Scope LLC, Irvine, CA) mounted to a tri-ocular stereo microscope (SM-1 Series, United Scope LLC). Images were processed using ImageJ (version

1.46r, National Institute of Health, Bethesda, MD) to determine wing length. Wing length measurements were made for all individuals across all treatments for all three biological replicates.

Experiment 2: Mate size and female post-mating response

Mating proceeded in the same manner for Experiment 2 as it did in Experiment 1 except instead of sacrificing the mosquitoes after mating, individuals were immediately transferred from the mating cages to 30 cm³ mesh cages by treatment for blood feeding. This was performed by placing the mating cages into a –20°C freezer for 5–8 min and then using an aspirator to transfer all anaesthetized individuals into one of four cages based on treatment. The mosquitoes were then fed thawed chicken blood from artificial feeders as described above. One feeder each was positioned at the top of the mesh cage and mosquitoes were fed freely for 3–4 h, starting late afternoon (2–3 PM). Each individual female was assessed for her blood feeding status during and immediately after feeding and transferred into paper deli cups (16 oz), with a piece of mesh secured over the opening with a rubber band. Inside each cup was a smaller (1 oz) cup filled with DI water and a sprinkle of ground-up fish pellet to serve as an oviposition site. This blood feeding procedure was repeated for three biological replicates. Across all replicates, all females were given access to an oviposition cup regardless of their blood feeding status. They were also assigned a number, and the size of their blood meal was categorized into discrete bins. Females that exhibited a distended red abdomen were deemed fully engorged and females whose abdomens had no trace of blood were termed unfed. Females whose abdomens were visibly reddened, but only partially distended were deemed partially fed. Females were left in these cups until the time of their death.

Fecundity and fertility

Cups from Experiment 2 were checked daily for the presence of egg rafts and when a raft was deposited, the oviposition cup was collected for egg counts. Eggs were counted using a stereo microscope and hand counter. The eggs were then left alone until all the larvae hatched, usually after 1–3 days. Total larvae hatched, regardless of survival, were counted and fertility was calculated by dividing the number of larvae by the number of eggs. These data were collected for all egg laying females across all biological replicates. On three occasions, the number of larvae was slightly higher than the number of eggs counted and therefore the fertility was considered 100%. Eight females (four from the large female, large male treatment group, two from the large female, small male treatment group, and two from the small female, large male treatment group) laid egg rafts that did not produce any larvae. Out of these females, six either did not have sperm in their spermathecae or their insemination status was unknown (poor dissection or escapee). All were excluded from this analysis. Because females were not fed multiple blood meals, only the first egg rafts laid, and their fertility was examined in this experiment.

Survival and insemination

When a female died, the date of her death was noted, and she was dissected in the same manner as described in Experiment 1 to determine insemination status. In all biological replicates females were fed a 10% sugar solution *ad libitum* and every female was monitored up until their natural death.

Experiment 3: Male mate choice

Based on the results of Experiments 1 and 2, a third experiment was performed to look at male mate choice when presented with an option of large and small females. Large and small adult males and females were created in the same manner as described above. Males were given the opportunity to mate with both large and small females by placing 10 males in a 24 cm³ mesh cage with five females from the large treatment group and five females from the small treatment group. Cotton soaked in a 10% sugar solution was added to each cage. This experiment was also run using three biological replicates, with eight cages of 10 males for each treatment group. Matings occurred over 1–2 days and at the end of the experiment, all individuals were sacrificed and male and female wing lengths, as well as female insemination status as a proxy for male mate choice, were recorded for each individual in the same manner as described previously.

Data analysis

Wing length analysis

To confirm size classes, wing lengths from large and small mosquitoes were compared for Experiment 1 and Experiment 3 using generalized linear models (GLM; normal distribution, identity link function), with size, replicate and their interaction as predictors. Separate analyses were performed for males and females in both experiments.

The assumption of normality was validated by analysing the studentized residuals (Shapiro–Wilk test, $p > 0.05$) for Experiment 3 males. For Experiment 1 males and females and 3 females, the reciprocal of wing length was used as the response variable to improve model fit, as indicated by lower AIC_c scores with the transformation, residuals with a mean approximately zero and standard deviation close to one, and consistent residual variance (homoscedasticity). Tukey HSD (honestly significant difference) post hoc tests were performed to identify pair-wise differences between size classes by replicate.

Insemination analysis

The effects of male and female size on female insemination rate for Experiment 1 and Experiment 3 were analysed via GLMs (binomial distribution, Logit link function), with male size, female size, biological

replicate and their interactions as predictors. Residual plots were analysed to assess model fit, and no discernable patterns were found.

Blood feeding analysis

For Experiment 2, the effect of male and female size on whether a female took a blood meal and the size of that blood meal were separately analysed via two GLMs, (binomial distribution, Logit identity link function and parameters estimated using a Firth adjusted maximum likelihood) with replicate, male size, female size, insemination status and all relevant first order interactions. In replicate two, due to an equipment error, treatments with large females only fed for approximately 1 h. Therefore, replicate two was excluded from this analysis due to this error with the blood feeding apparatus. Residual plots were analysed to assess model fit for both analyses and no discernable patterns were found.

Fecundity and fertility analysis

The effect of treatment on egg laying behaviour in Experiment 2 was analysed via a GLM (normal distribution, Identity link function), with biological replicate, male size, female size, blood meal size and their interactions as categorical variables. The assumption of normality was validated by analysing the studentized residuals (Shapiro–Wilk test, $p > 0.05$).

The effect of treatment on fertility was analysed via a GLM (normal distribution, Identity link function), with biological replicate, male size, female size and their interactions as categorical variables. Box Cox transformed fertility data were used as the response variable to improve model fit, as indicated by lower AIC_c scores with the transformation, residuals with a mean approximately zero and standard deviation close to one, and homoscedasticity.

Survival analysis

The effect of treatment on female survival in Experiment 2 was analysed via a Cox proportional hazards model, with biological replicate as a stratified term and with male size, female size and blood feeding status as categorical predictors. No females were censored; all females were tracked until death, and only one female was removed from the analysis due to escaping. Two additional survival analyses were also performed to look for patterns of survival among fed and unfed females separately via two Cox proportional hazards models, with biological replicate as a stratified term and male size, female size and insemination status as predictors. For the blood-fed female analysis, the additional categorical parameter of blood meal size was added to the analysis. Fertility was not considered in these analyses, due to not every female laying eggs and having viable offspring; however, a separate survival analysis with just fertility as the predictor term was run (replicate as a strata term) and was found to be not significant (Cox

TABLE 1 Wing lengths (mm) of adult male and female *Culex pipiens* in Experiment 1 produced by different diets during larval stages (generalized linear models).

Replicate	Size	Female wing lengths		Male wing lengths	
		$\bar{X} \pm \text{SE (N)}$	Significance ^a	$\bar{X} \pm \text{SE (N)}$	Significance ^a
1	Large	3.57 ± 0.01 (217)	b	2.99 ± 0.01 (157)	b
	Small	3.28 ± 0.02 (100)	d	2.71 ± 0.01 (180)	d
2	Large	3.78 ± 0.01 (237)	a	3.04 ± 0.01 (262)	b
	Small	3.42 ± 0.01 (231)	c	2.76 ± 0.01 (239)	c
3	Large	3.57 ± 0.01 (240)	b	2.98 ± 0.01 (239)	a
	Small	3.22 ± 0.01 (235)	d	2.61 ± 0.01 (229)	e

^aLetters indicate significance (Tukey HSD, $p < 0.05$).**TABLE 2** Wing lengths (mm) of adult male and female *Culex pipiens* in Experiment 3 produced by different diets during larval stages (generalized linear models).

Replicate	Size	Female wing lengths		Male wing lengths	
		$\bar{X} \pm \text{SE (N)}$	Significance ^a	$\bar{X} \pm \text{SE (N)}$	Significance ^a
1	Large	3.47 ± 0.17 (80)	a	2.86 ± 0.17 (79)	a
	Small	3.17 ± 0.18 (80)	c	2.64 ± 0.13 (80)	c
2	Large	3.59 ± 0.22 (81)	ab	2.90 ± 0.21 (78)	a
	Small	3.19 ± 0.18 (80)	cd	2.62 ± 0.18 (78)	c
3	Large	3.53 ± 0.16 (79)	b	3.02 ± 0.20 (76)	b
	Small	3.25 ± 0.17 (78)	d	2.63 ± 0.17 (79)	c

^aLetters indicate significance (Tukey HSD, $p < 0.05$).

proportional hazards model, $\chi^2_1 = 1.179$, $p = 0.278$). All survival curves were generated using Kaplan–Meier estimates and pairwise comparisons performed using a Mantel–Cox Log Rank test. For each of the two supplemental survival analyses, two blood-fed and two non-blood-fed females were excluded due to missing insemination data.

All analyses were performed using JMP (version 17, SAS Institute Inc., Cary, NC) excluding the survival analysis. All survival analyses were performed using SPSS Statistics (version 29.0.1.0, IBM Corp., Armonk, NY).

RESULTS

Wing lengths

For every replicate in Experiment 1 there was a significant difference in female wing lengths (Table 1), with larger females having on average 8.8% longer wings than females reared for smaller size (GLM: Size Likelihood Ratio [LR] = 688.35, $p < 0.0001$; Replicate LR = 236.73, $p < 0.0001$; Interaction LR = 3.50, $p = 0.1741$). There was also a significant difference in male wing lengths (Table 1), with larger males having on average 11% longer wings than males reared for smaller size (GLM: Size LR = 828.37, $p < 0.0001$; Replicate LR = 104.43, $p < 0.0001$; Interaction LR = 34.17, $p < 0.0001$). For

every replicate in Experiment 3 both males (GLM: Size LR = 254.08, $p < 0.0001$; Replicate LR = 15.95, $p = 0.0003$; Interaction LR = 18.19, $p = 0.0001$) and females (GLM: Size LR = 282.32, $p < 0.0001$; Replicate LR = 13.84, $p = 0.001$; Interaction LR = 7.57, $p = 0.0227$) showed significant difference in wing length based on rearing environment (Table 2), with larger females having on average 9.6% longer wings than smaller female cohorts, and larger males having on average 10.7% longer wings than smaller male cohorts. These larger and smaller cohorts are within the bounds of the natural variation found with *Cx. pipiens* mosquitoes (females 3.47 mm and males 2.98 mm at the same rearing temperature based on Loetti et al., 2011).

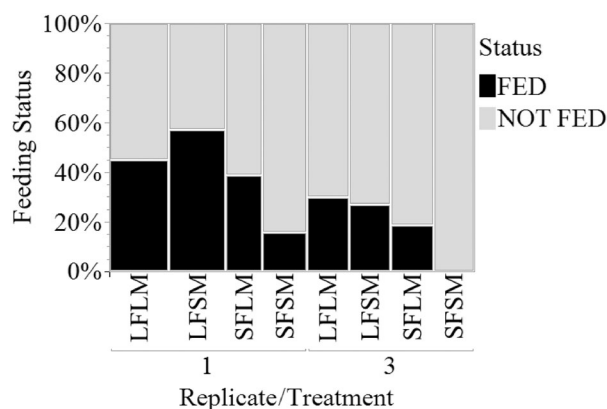
Insemination rate

For Experiment 1, there was no effect of male size (Table S1; GLM: LR: 0.0507, $p = 0.822$), female size (LR: 0.129, $p = 0.719$) or replicate (LR: 0.0944, $p = 0.992$) on insemination rate. Overall, the insemination rate was $70\% \pm 0.14\%$. In Experiment 3, when males were given an option of large and small females to mate with, there was still no difference in insemination status based on male size (Table 3; GLM: LiR: 0.300, $p = 0.584$), female size (LR: 1.63, $p = 0.202$) or replicate (LR: 0.862, $p = 0.650$). The overall insemination rate was $35\% \pm 15\%$. There were no significant interaction terms for either analysis.

TABLE 3 Insemination rate of female *Culex pipiens* in Experiment 3 when caged with either large or small males (generalized linear model).

Male size	Female size	Replicate $\bar{X} \pm SE$		
		1	2	3
Large	Large	0.41 $\pm$ 0.19	0.63 $\pm$ 0.22	0.42 $\pm$ 0.25
Large	Small	0.15 $\pm$ 0.14	0.27 $\pm$ 0.10	0.41 $\pm$ 0.09
Small	Large	0.29 $\pm$ 0.20	0.47 $\pm$ 0.24	0.31 $\pm$ 0.12
Small	Small	0.27 $\pm$ 0.24	0.27 $\pm$ 0.18	0.26 $\pm$ 0.09

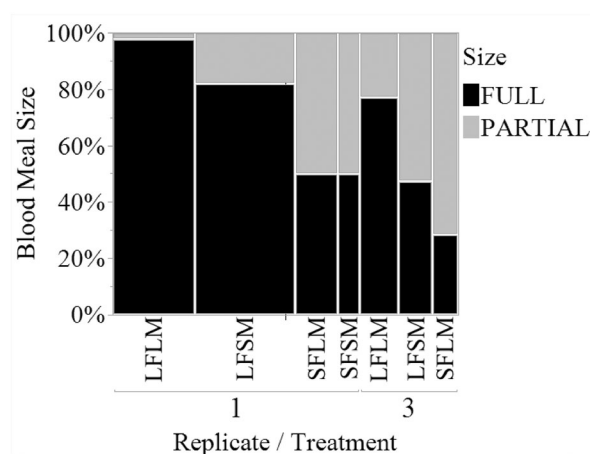
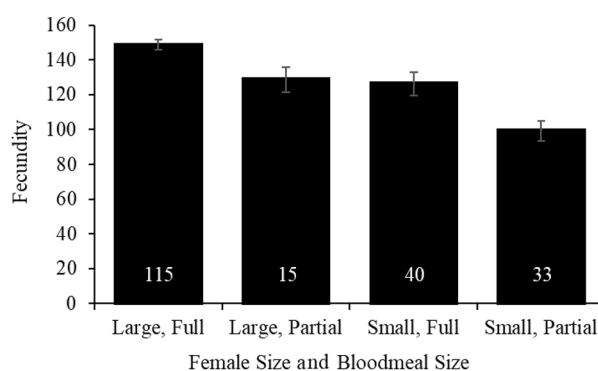
Note: N = 10 females per cage, eight cages per treatment, per replicate.

**FIGURE 1** Female *Culex pipiens* blood feeding status by treatment and replicate in Experiment 2. Females (LF = large females; SF = small females) were allowed to feed freely after mating to either a small (SM) or large (LM) male. Whether or not a female took a blood meal was dependent on several interacting terms (generalized linear model; $p < 0.05$). N = 62–102 per treatment.

Blood feeding

When given the opportunity to blood-feed in Experiment 2, female feeding status was influenced by treatment (Figure 1, Table S2) but not by insemination status (GLM: LR: 3.44, $p = 0.0636$). Overall, females fed more in the first biological replicate (40.8%) than the third (19.2%). There was a significant interaction between replicate and female size (LR: 6.10, $p = 0.0135$). In replicates 1 and 3, 51% and 29%, respectively, of large females were fed compared to 26% and 10% of females that were small. There was also a significant interaction between replicate and male size (LR: 4.83, $p = 0.0279$). In replicates 1 and 3, 43% and 24%, respectively, of females caged with large males were fed compared to 39% and 14% of females caged with small males. There was also an interaction between male and female size (LR: 4.15, $p = 0.0417$). Small females caged with small males were least likely to feed compared to all other treatment groups (contrast testing with Bonferroni correction, $p < 0.0001$). In replicate three, small females caged with small males did not take a single blood meal over the entire time. No other contrasts were significant.

Of the females who fed, female blood meal size was influenced by treatment (Figure 2, Table S3) but not by insemination status

**FIGURE 2** Female *Culex pipiens* blood meal size by treatment (LFLM = large female, large male; LFSM = large female, small male; SFLM = small female large male; SFMS = small female, small male) and replicate in Experiment 2. Of the females that took a blood meal, the size of their meal depended on an interaction between male and female size (generalized linear model; $p = 0.0438$). N = 14–56 per treatment.**FIGURE 3** Female *Culex pipiens* fecundity in Experiment 2 by adult size and blood meal size. Females were allowed to feed freely and the size of their blood meal was qualified into 'Full' and 'Partial' blood meals. Both female size (generalized linear model; $p < 0.0001$) and blood meal size ($p = 0.0059$) had an effect on female fecundity. There was no significant interaction between female size and blood meal size ($p = 0.439$). The data presented are mean $\pm$ SE. Numbers in bars indicate sample size.

(GLM: LR: 0.78, $p = 0.7702$). As before, females in replicate one were more likely to take a larger blood meal (79%) than females in replicate three (54%; LR: 6.56, $p = 0.0104$). Overall, larger females took larger blood meals (82%) than smaller females (44%; LR: 9.57, $p = 0.0020$). While there was no significant main effect of male size (LR: 1.49, $p = 0.2212$), there was a significant interaction between male size and female size (LR: 4.06, $p = 0.0438$). When large females were caged with large males, their blood meals were larger, however, no such difference was found with smaller females (contrast testing with Bonferroni correction, $p < 0.0001$). No other contrasts were significant.

Fecundity and fertility

Both blood meal size and female size influenced egg laying numbers in Experiment 2 (Figure 3; GLM: $F_{7,192} = 8.95$, $p < 0.0001$). Large females laid more eggs on average than small females ($F_{1,192} = 16.21$, $p < 0.0001$) and females that took a larger blood meal laid more eggs ($F_{1,192} = 7.76$, $p = 0.0059$). Male size had no effect on female egg laying ($F_{1,192} = 0.002$, $p = 0.914$), nor did replicate or any interaction term. Fertility was not affected by replicate (GLM: $F_{2,189} = 1.024$, $p = 0.361$), male size ($F_{1,189} = 0.537$, $p = 0.464$) or female size ($F_{1,189} = 2.589$, $p = 0.109$).

Survival

Both female size and blood feeding status had a significant effect on female survival (Figure 4; Cox proportional hazards model, $\chi^2_1 = 30.254$, $p < 0.001$ and $\chi^2_1 = 107.917$, $p < 0.001$ respectively) but male size did not ($\chi^2_1 = 0.449$, $p = 0.503$). Therefore, we performed a second survival analysis on this data which included an interaction term between female size and blood-fed status. This interaction was also significant (Cox model, $\chi^2_1 = 20.258$, $p < 0.001$). Post hoc pairwise comparisons indicate that large and small females that were blood-fed generally survived longer than large and small females that were not blood-fed. For the unfed females, large females tended to live longer (Table 4; Log Rank test, $p < 0.05$).

When considering just non-blood-fed females, only female size affected survival (Figure 5, Table 5); neither male size nor insemination status had an effect. For all replicates, larger females lived longer than smaller females (Log Rank test, $p < 0.02$). When considering just blood-fed females, there were no predictors that significantly affected survival (Cox proportional hazards model, $p > 0.05$, $N = 14$ –56 per treatment).

DISCUSSION

Here, we present the first analysis of the effect of larval diet quality on adult size, mating rate and female post-mating responses in *Cx. pipiens* mosquitoes. Because male contributions to females during mating can shift based on adult size (Gary et al., 2009; Helinski & Harrington, 2011; Ponlawat & Harrington, 2007) and larger adult females tend to be more fecund (Armbruster & Hutchinson, 2002; Briegel, 1990; Hawley, 1985; Yan et al., 2021), we predicted that males would mate more often with larger females and females mated to larger males would have higher survival rates and reproductive success. However, only female size affected fecundity and survival, and both these post-mating responses were linked to blood feeding. Male size did not affect any female post-mating responses. Despite a fitness advantage to mating with larger females, and the potential for males to be able to discriminate amongst female sizes based on differences in flight tone (Cator et al., 2010; Villarreal et al., 2017), males appear not to discriminate among females based on size in this experiment.

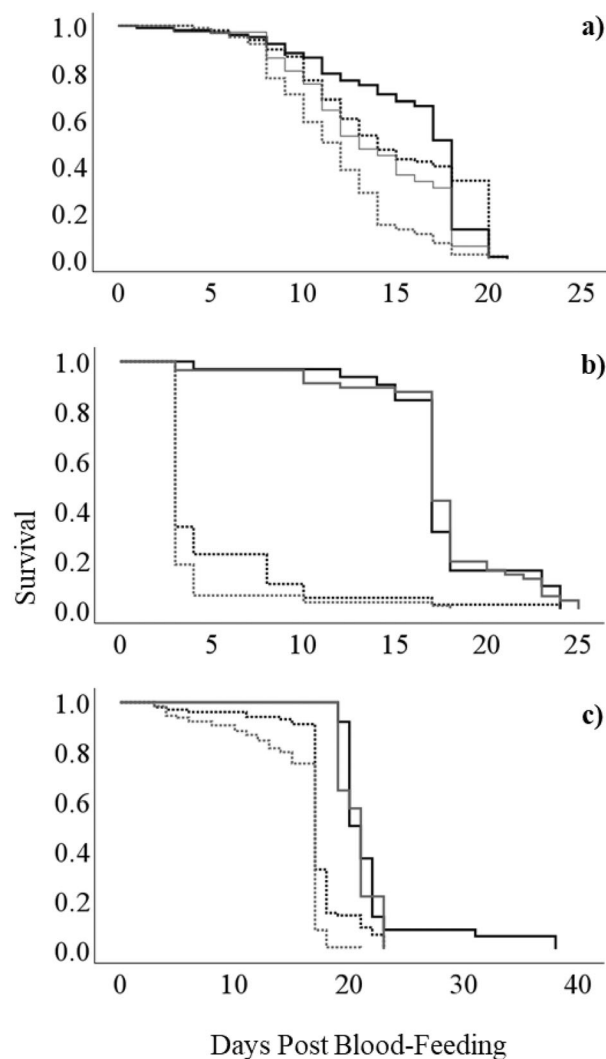


FIGURE 4 Kaplan–Meier estimates of female *Culex pipiens* survival in Experiment 2 for replicates 1 (a), 2 (b) and 3 (c), by female size and feeding status (black, solid line: Large, with blood meal; black, dotted line: Large, without blood meal; grey, solid line: Small, with blood meal; grey, dotted line: Small, without blood meal). There was a significant interaction between female size and feeding status on female survival. $N = 60$ –102 per treatment.

In this study, *Cx. pipiens* male size did not affect insemination rates. Male size can indicate quality, as larger males should have higher energy reserves that can be used towards mating behaviours. Males and females mate while in flight, a highly energetic activity (Maiga et al., 2014). In a study by Yahouédo et al. (2014) when male *Anopheles gambiae* were fed on high- or low-quality diets as larvae, they developed into larger or smaller adults, respectively. However, the larval diet did not affect adult energy reserves. Similarly, male *Ae. aegypti* mosquitoes did not differ in glycogen or sugar reserves when reared for large and small size, although larger males did have a higher lipid content (Aldersley et al., 2019). It could be because of both large and small *Cx. pipiens* males could swarm, they were of high enough quality for the females to be less choosy. Because *Cx. pipiens* tend not to disperse far, compared to other *Aedes/Ochlerotatus* species (Becker

TABLE 4 Female *Culex pipiens* mean survival estimates with SE and 95% confidence intervals (CI) for replicates 1–3 of Experiment 2.

Replicate	Status	Estimate	SE	95% CI		Significance ^a
1	Large, fed	15.61	0.42	14.79	16.42	a
	Large, unfed	14.60	0.48	13.66	15.55	a
	Small, fed	13.47	0.70	12.09	14.85	b
	Small, unfed	11.56	0.34	10.90	12.22	c
2	Large, fed	17.41	0.66	16.12	18.70	a
	Large, unfed	4.88	0.39	4.12	5.64	b
	Small, fed	17.35	0.55	16.28	18.42	a
	Small, unfed	3.72	0.31	3.12	4.33	c
3	Large, fed	21.92	0.70	20.55	23.29	a
	Large, unfed	17.20	0.34	16.54	17.85	b
	Small, fed	20.64	0.41	19.83	21.45	a
	Small, unfed	15.34	0.34	14.68	16.00	c

Note: Letters indicate significance (pairwise comparisons, $p < 0.05$).

^aLog rank (Mantel–Cox) pairwise comparisons for each replicate.

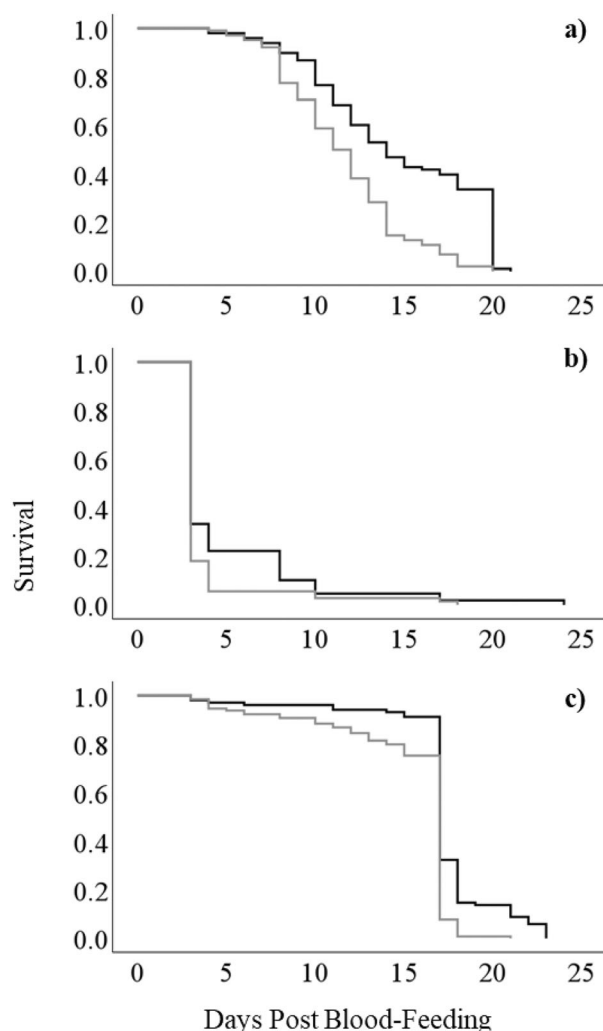


FIGURE 5 Kaplan–Meier estimates of non-blood-fed female *Culex pipiens* survival in Experiment 2 for replicates 1 (a), 2 (b) and 3 (c), by female size (black, solid line: Large, grey, solid line: Small). Larger females live significantly longer than smaller females (Cox proportional hazards model, $p = 0.002$). $N = 33$ –69 per treatment.

et al., 2010), male quality as it relates to size may not impact swarming behaviour. Male size and swarming behaviour were studied in *An. gambiae*, although results are inconsistent regarding whether larger males have an advantage (Charlwood et al., 2002; Ng'habi et al., 2008). Similar studies have not been performed for *Cx. pipiens*.

Although larval diet did not affect insemination rates, adult diet likely plays a role in mating decisions. Adult diet has been shown to affect insemination rates, with male *Anopheles coluzzii* inseminated rate changing depending on whether they were fed a mango or papaya puree (Nignan et al., 2020). In *Cx. pipiens*, males fed on a lower-quality sugar diet had smaller male reproductive accessory glands, and females mated to these males laid fewer eggs and had lower larval survival (Huck et al., 2021). In a separate *Cx. pipiens* experiment in which adults were fed high- and low-quality diets (Villarreal, unpublished data), males fed high-quality diets as adults had a higher insemination rate than males fed a low-quality adult diet. This phenomenon was also recorded in *An. gambiae* (Gary et al., 2009). Further exploration is needed to understand how larval and adult diet influence *Cx. pipiens* male quality and female mate selection.

Male size did not affect the fecundity and fertility of females. In *Cx. pipiens*, only blood feeding and female size influenced fecundity, and both variables have been linked to fecundity in several other mosquito species (Briegleb, 1990; Hawley, 1985; Lyimo & Takken, 1993; Packer & Corbet, 1989; Renshaw et al., 1994). Based on several studies, male size differences created through larval crowding and nutrient deprivation are expected to affect the components of his ejaculate, with smaller males suffering greater sperm depletion and changes to the amounts of specific seminal proteins transferred (as reviewed in Meuti & Short, 2019). Similar to our study, male size also did not affect female fecundity in the geometrid moth *Epirrita autumnata* despite female size affecting fecundity. The authors attributed this to the small ejaculate size in this species (Tammaru et al., 1996). Although we were unable to record ejaculate size, the lack of fecundity and fertility difference between male size classes would indicate

TABLE 5 Cox proportional hazards model parameter estimates and hazard ratios for non-blood-fed females.

Parameter	B ± SE	Exp(B)	95% CI	Wald	p-Value
Male size (large vs. small)	0.002 ± 0.08	1.00	(0.85, 1.18)	0.001	0.977
Female size (large vs. small)	0.57 ± 0.19	1.77	(1.23, 2.55)	9.326	0.002
Insemination (no vs. yes)	0.002 ± 0.16	1.00	(0.73, 1.37)	0	0.99
Female size × Insemination	−0.10 ± 0.21	0.91	(0.60, 1.36)	0.228	0.633

Abbreviation: CI, confidence interval.

that even if ejaculate volume did vary, it did not affect female post-mating responses. It could be that variation in ejaculates transferred is either less important in *Cx. pipiens* than *Ae. aegypti* or that the size differences created in this experiment were not large enough to influence the components of the seminal fluid. Despite the different-sized cohorts being statistically distinct, both are within the natural variation of *Cx. pipiens* (Loetti et al., 2011). Moreover, components of the seminal fluid are known to vary between species (Meuti & Short, 2019). Further manipulations of ejaculate size are needed to confirm the role of non-sperm male contributions to female fitness in *Culex* mosquitoes.

Larger female *Cx. pipiens* lived longer regardless of male mate size. Blood feeding, sugar feeding and larval diet are all known to affect female mosquito survival (*An. gambiae*: Gary et al., 2009; *Ae. aegypti*: Joy et al., 2010; Yan et al., 2021; *An. coluzzii*: Nignan et al., 2020). Whether large or small females were found to live longer varies by study and by species in mosquitoes (i.e., larger living longer: Reiskind & Lounibos, 2009; smaller living longer: Joy et al., 2010; no effect of size: Paton et al., 2013; Westby & Juliano, 2017). Our data indicate that large female *Cx. pipiens* can survive longer and invest more in reproduction. They did not experience a life history trade-off in survival and fecundity like seen with smaller females. Therefore, male *Cx. pipiens* should preferentially choose to mate with larger females over smaller ones. However, when given the option of mating with females that varied in size, males exhibited no size-based mate selection in this experiment. Body size has been linked to flight tone in several species of mosquitoes (Cator et al., 2010; Cator & Zanti, 2016; Villarreal et al., 2017; Wekesa et al., 1998), therefore, males should have sufficient information to discern large from small females. It is possible that males could mate several times before sperm and seminal fluid get depleted, as seen with *Ae. aegypti* (Helinski et al., 2012; Helinski & Harrington, 2011), and therefore may mate with any female encountered. Whether males show increased mate preference with decreased quality or increased sperm depletion is currently unknown in *Cx. pipiens* and requires further exploration.

The only potential effect male size had on female post-mating responses was in blood feeding rates. Larger females were more likely to take a blood meal regardless of treatment. They did tend to take a larger blood meal when caged with larger males, even though this was not driven by insemination rates. Smaller females took similarly sized meals regardless of treatment but were more likely to take a blood meal after being caged with large males. Although blood feeding varied by replicate, the overall trends remained the same between

replicates, indicating other extraneous uncontrolled factors that influenced overall feeding behaviour between replicates, such as seasonality and variable abiotic lab conditions. For other mosquito species, whether mating or male quality affects female blood feeding behaviour is highly variable (as reviewed in Klowden, 1995; League et al., 2021). Overall, female insemination status did not impact blood feeding propensity, which is similar to what is found in *Aedes* and *Anopheles* mosquitoes (Dahalan et al., 2019; League et al., 2021). However, Villarreal et al. (2018) found female *Ae. aegypti* mated or injected with male seminal fluid proteins did express a different pattern of blood feeding behaviour. And in *Cx. pipiens*, mated females took a larger blood meal than virgin females (Adlakha & Pillai, 1976). Because there was variability between replicates in blood feeding status and blood meal size, further manipulations are needed to determine the interplay between mate selection, blood feeding rate and fecundity in *Cx. pipiens*.

CONCLUSION

Here, we present evidence of female *Cx. pipiens* size, determined by larval diet, as the primary driver of fecundity and survival, with negligible effects of male size on female post-mating responses. Although the influence of male size on female fitness was minimal, future studies are needed to disentangle the interplay between diet, ejaculate quality and male size, independent of larval conditions. These studies would require the challenging task of creating genetically distinct strains of *Cx. pipiens* differing in body size.

Our findings highlight the significant influence of larval rearing environments on female fitness, not only in fecundity but also immunity (Suwanchaichinda & Paskewitz, 1998; Telang et al., 2012) and virus transmission (Alto et al., 2008; Vantaux et al., 2016). Manipulating these environments offers a promising strategy to reduce population size and vector capacity. Given that *Cx. pipiens* is the main transmitter of West Nile virus, these results have important implications for *Cx. pipiens* vector control programmes.

AUTHOR CONTRIBUTIONS

Susan Villarreal: Conceptualization; investigation; funding acquisition; writing – original draft; methodology; validation; visualization; writing – review and editing; data curation; formal analysis; supervision; project administration; resources. **Amelia Senior:** Data curation; investigation. **Matthew Price:** Data curation; investigation.

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ETHICS STATEMENT

This research was conducted in accordance with best practices for humane handling of mosquitoes.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data supporting the findings in this study are available in the Men-deley Data repository at <https://doi.org/10.17632/5r496x7w7f.2>.

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SUPPORTING INFORMATION

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

Table S1. Insemination rate (fraction of females with sperm confirmed in their spermathecae) of female *Culex pipiens* in Experiment 1 (generalized linear model).

Table S2. Parameter estimates from the generalized linear model of blood feeding status by male size, female size, insemination status, replicate and their interactions. Object in parentheses of main effects indicates the reference level for the estimate. The same reference level applies to all interactions.

Table S3. Parameter estimates from the generalized linear model of blood feeding size by male size, female size, insemination status, replicate and their interactions. Object in parentheses of main effects indicates the reference level for the estimate. The same reference level applies to all interactions.

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