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The effects of female age on blood-feeding, insemination, sperm storage, and fertility in the dengue vector mosquito *Aedes aegypti* (Diptera: Culicidae)

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ABSTRACT

Mating induces behavioral and physiological changes in female insects—collectively referred to as the female post-mating response (PMR)—that facilitate the production of progeny. PMRs are elicited by transfer of male-derived seminal components during mating, but are altered by other factors, including adult age. Increased female age is often accompanied by declines in fertility. However, mating shortly after emergence also impacts fertility in the insect model *Drosophila melanogaster*. Here, we determined the age post-emergence when females of the vector mosquito *Aedes aegypti* can be inseminated and blood-feed. We next examined fecundity, fertility, and the storage of sperm in the female reproductive tract in "young" (30-41 hours-old) and "old" (2- and 3-week-old) females, finding that blood-feeding began at 14 hours, and mating at ~24 hours post-emergence. Although young females consumed smaller blood quantities and stored fewer sperm, they were similarly fertile to 4-day-old controls. Old females, however, suffered significant declines in fecundity by 2 weeks of age. Our results show that female *Ae. aegypti* start to become sexually receptive 1 day after their emergence, but can ingest blood much sooner, suggesting that mating is not a prerequisite to blood-feeding, and that females can ingest an arbovirus infected blood-meal shortly after emergence.

1. Introduction

Female insects undergo a series of physiological and behavioral changes upon insemination—collectively referred to as the female post-mating response (PMR)—that primarily serve to promote the generation of progeny (Avila et al., 2011; Hopkins et al., 2018). Female PMRs typically include increased rates of egg development and ovulation, the inhibition of re-mating in the short- or long-term, structural changes in the female reproductive tract, alteration of female lifespan, and changes in gene expression from female reproductive tissues (Avila et al., 2011; Hopkins et al., 2018; Meuti and Short, 2019; Nanfack-Minkeu and Sirot, 2022). Seminal fluid proteins transferred along with sperm during mating elicit female PMRs in several species (Avila et al., 2011; Hopkins et al., 2018), although non-proteinaceous molecules, such as 20-hydroxyecdysone or juvenile hormone, can induce (Gabrieli et al., 2014; Peng et al., 2022) or influence (Clifton et al., 2014) female PMRs. In addition to male-specific molecules, female proteins secreted from the sperm storage organs are also required for fertility (Allen and Spradling, 2008), playing roles in ovulation, egg-laying, and sperm storage (Schnakenberg et al., 2011; Sun and Spradling, 2013) in mated females.

Although sex-specific molecules are required for the fertility of a mating pair, other factors can influence individual PMRs, including adult age. Age-related effects on fertility are well documented in insects, with male or female age each influencing reproductive outcomes (Grotewiel et al., 2005; Miller et al., 2014). In *Drosophila melanogaster,* old males do not induce optimal sperm storage and egg laying levels in their mates (Ruhmann et al., 2018). In female *D. melanogaster*, fecundity peaks when mated at 3–4 days post-emergence and declines with age

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thereafter (David et al., 1975; Fricke et al., 2013). Males transfer less sperm to old females, although old females store normal quantities of sperm (Manier et al., 2011). While increased age is hypothesized to affect several processes (Miller et al., 2014), declines in fecundity appear to primarily result from decreased germline stem cell proliferation that occurs as females age (Zhao et al., 2008). While old females suffer declines in fertility, mating shortly after emergence can also impact fertility. Female insects are often refractory to mating after their emergence from pupae. Female *D. melanogaster* become receptive to mating 24–48 h post-emergence (Fricke et al., 2013; Manning, 1967). However, females that mate 24 h post-emergence are less fertile than females mated a few days later (Fricke et al., 2013), suggesting that females require additional time to complete sexual maturation and reach optimal fertility.

Female *Aedes aegypti*, the primary vector of the dengue (Guzman and Harris, 2015), Zika (Alfonso-Parra and Avila, 2018), chikungunya (Vega-Rúa et al., 2014), and yellow fever viruses (Chippaux and Chippaux, 2018), are unreceptive to mating after emergence. In field and laboratory containers, male and female *Ae. aegypti* eclose together over the course of one or two days. Males are unable to mate until their abdominal terminalia rotate, which takes ~24 h (Roth, 1948). Once females initiate flight, sexually mature males will attempt to copulate by engaging their genitalia (Roth, 1948). However, reports differ on when insemination occurs, ranging from within 24 h of emergence to 3 days post-emergence (Gunathilaka et al., 2023; Gwadz et al., 1971a; Gwadz and Craig, 1968; Spielman et al., 1969). Although female *Ae. aegypti* require a bloodmeal to develop their eggs, the age when they first blood feed is also unclear. Early studies suggest that blood-feeding can occur as shortly as 18 h post-emergence, while others suggest that females require several days (Christophers, 1960). Reports are also mixed on whether females mate before or after blood-feeding (Facchinelli et al., 2023).

Female *Ae. aegypti* undergo similar PMRs observed in other insects, including increases in egg-laying (Villarreal et al., 2018) and longevity (Helinski and Harrington, 2011; Villarreal et al., 2018), which can increase vectorial capacity and influence mosquito population size. Mating also results in decreased sexual receptivity (Degner and Harrington, 2016a; Helinski et al., 2012) and changes in gene expression from female reproductive tissues (Alfonso-Parra et al., 2016), including the spermathecae (Camargo et al., 2020; Pascini et al., 2020)—the sites of long-term sperm storage (Degner and Harrington, 2016b)—which produce products essential for optimal mosquito fertility (Pascini et al., 2020; Shaw et al., 2014). However, the effects of age on female fertility have not been fully described to date, which is of relevance as *Ae. aegypti* females typically mate only once in their lifetime (Degner and Harrington, 2016a). Further, the post-emergence timing of blood-feeding has direct implications for disease transmission by this species.

Here, we examined the quantity of sperm stored in the spermathecae, fecundity and fertility of "young" (mated 30–41 h post-emergence) and "old" (mated 2- and 3-weeks post-emergence) female *Ae. aegypti*. To do so, we first determined the age post-emergence when females 1) can be inseminated by sexually mature males and 2) consume their first blood meal. We found that although females were refractory to mating for ~24 h post-emergence, blood-feeding occurred sooner, at 14–17 h. Although we detected differences in blood-meal size consumed by young females and in the quantity of sperm stored compared to controls, young females in three age groups (30–33-, 34–37-, and 38–41-h post-emergence) were similarly fertile to 4-day-old controls. Although 2- and 3-week-old females mated in similar proportions and consumed similar quantities of blood as control females, we observed a significant decline in fecundity by 2 weeks of age. Our results show that female *Ae. aegypti* are fully fertile once they can be inseminated. However, females suffer similar declines in fecundity with increased age, a common effect observed in other insects.

2. Materials and methods

2.1. Mosquitoes

Thai strain *Ae. aegypti* (Alfonso-Parra et al., 2014) were used in our assays. Eggs were hatched under vacuum pressure (−50 kPa) for 30 min and larvae were reared at a density of 200/L in type II H_2O and supplemented with four large (7.2–8.2 mm) Hikari Gold Cichlid food pellets (Hikari, Himeji, Japan). Pupae were transferred to individual 5 ml tubes to ensure virginity. Larvae and adults were kept in incubators at 27 °C, 70% relative humidity and a 12:12 h light:dark photoperiod. Forty-eight hours after pupation, tubes containing a single pupa were checked every 4 h for adults and the time of emergence was noted. Females from the young age groups (10–41 h) were maintained in their respective 5 ml tube until ~10 min prior to our assays (i.e., they never had access to sugar). Control and "old" females (2- and 3-weeks old) were placed into 8 L cages and had access to 10% sucrose *ad libitum*; sugar was removed 24 h prior to our assays as a sugar meal can impede blood-feeding (League et al., 2021). All females in the fertility assays were given 10% sucrose once mating and blood-feeding had occurred. Males had constant access to 10% sucrose. Biological replicates were performed using males and females from independently hatched cohorts. Four-day-old females were used as controls in our assays. To ensure that males had completed genital rotation (Roth, 1948), females of all age groups were mated to 4–6-day-old males.

2.2. Determination of insemination status

Females of each age group (~30) were placed into 8 L cages ~10 min prior to the start of our assays. At the appropriate time, males were aspirated into the cage to a 2:1 male:female ratio, and the cage was placed into an incubator. Females were given 2 h to mate. At the end of the mating period, cages were placed into a −20 °C freezer, and females were subsequently collected and stored at −80 °C until dissections commenced. To determine if insemination occurred, female reproductive tracts were dissected in 1X PBS to determine the presence of seminal fluid in the bursa, the organ where males deposit sperm (Degner and Harrington, 2016b), which expands to accommodate the ejaculate. The number of biological replicates performed for the young age groups are as follows: 10–13 h (1), 14–17 h (2), 18–21 h (5), 22–25 h (5), 26–29 h (4), 30–33 h (4), 34–37 (4), 38–41 h (3), and 4 d (5). Three biological replicates were performed for 2-week and 3-week-old females and their controls. A subset of inseminated females in each group was further processed to determine sperm quantity in the female spermathecae (see below).

2.3. Blood-feeding assays and blood-meal size measurements

Nine-to-twelve virgin females were placed into a 0.5 L cup ~10 min prior to our assays. At the appropriate time, females were offered a human forearm for 10 min; three biological replicates were performed for all age groups. Blood feeding on human subjects was approved by the Comité de Bioética Sede de Investigación Universitaria (Universidad de Antioquia) and all volunteers signed a consent form. At the end of the feeding period, cups were placed at −20 °C for 10 min. Females were then checked for presence of blood using a ZEISS Stemi 508 stereo microscope (ZEISS, Oberkochen, Germany). Blood-fed females were subsequently weighed using an AND HR-202i microbalance (A&D Company, Tokyo, Japan) to determine their weight in mg. To quantify blood-meal size, 45–50 non-blood-fed females from each of the young (10–41 h), 4-day-old control, 2-week and 3-week-old age groups were weighed to determine their average non-blood-fed (NBF) weight. The average female NBF weight from each group was subtracted from the blood-fed female weight of the corresponding group as in (Harrington et al., 2001; Villarreal et al., 2018). Blood-fed and non-blood-fed females were frozen simultaneously and weighed within 1 h of the termination

of the assay.

2.4. Fecundity and fertility assays

Females were placed into 8 L cages ~10 min prior to mating; each cage contained ~30 females. At the appropriate time, males were aspirated into the cage in a 2:1 male:female ratio and the cage placed into an incubator. Females were given 2 h to mate. Although a proportion of females remate when mass mated, re-insemination does not influence fecundity (Agudelo et al., 2021) or the quantity of sperm stored (Agudelo et al., 2022) in Thai strain *Ae. aegypti*. After 2 h, females were blood-fed on the arm of a volunteer and males were removed. Non-engorged females were discarded. Four days after blood-feeding, females were individually aspirated into 50 ml conical tubes with a 13 × 4 cm paper towel strip and 6 ml of type II H_2O. The paper strip was removed 48 h later, and the eggs were counted using a ZEISS Stemi 508 stereo microscope (ZEISS, Oberkochen, Germany). Eggs were partially dried and stored in an incubator until they were hatched 5–7 days later. Reproductive tracts of females from the young female age groups (30–33, 34–37, and 38–41-hours-old) were dissected after egg-laying to identify inseminated females by determining the presence/absence of sperm in the spermathecae (Parsana et al., 2022). Eggs laid by mated females were hatched by placing the paper strip into a 40 ml cup, filling it with type II H_2O, supplementing it with a pinch of active yeast, and placing it under a vacuum for 30 min. Resulting larvae were counted 4–6 days later. Hatch percentage was calculated as the number of larvae/number of eggs; females that laid zero eggs were omitted from the analysis. Three biological replicates were performed for each age group examined.

2.5. Sperm quantification

To assess quantity of sperm stored in the spermathecae of mated females, we utilized a subset of mated females from our insemination assays as sperm enter the spermathecae ~30 s after insemination and sperm storage is completed minutes later (Degner and Harrington, 2016b); for each age group, a similar number of mated females were obtained from 3 biological replicates. Sperm were quantified using a modified protocol reported in (Ponlawat and Harrington, 2009). Briefly, spermathecae were dissected in 1X PBS, placed into a 250 μL chamber containing 100 μL of 1X PBS, and ruptured with minutiae pins to release sperm. Samples were then mixed by pipetting up and down. An additional 100 μL of PBS was added and the solution was re-mixed. Ten 5 μL aliquots were placed onto a glass slide and dried for 5 min at 50 °C. Sperm were fixed in 70% ethanol and sperm nuclei stained with Giemsa dye (Merck, Kenilworth, USA). Sperm heads in each drop were counted under brightfield illumination at 200X magnification. This subsample was used to calculate total sperm (Hatala et al., 2018).

2.6. Statistical analysis

The response variables were compared between the age ranges separately for young females (10–41 h-old) and old females (2–3 weeks old). For the proportion of mated females, the proportion of females that blood-fed, and the proportion of emerged larvae (i.e., hatch percentage), a Generalized Linear Mixed Model (GLMM) was used with a binomial or quasibinomial distribution to correct for overdispersion. A Deviance test was requested from the GLMM's to evaluate the effect of age, and replicate was used as a random effect.

For the other variables—blood meal size, the quantity of sperm stored, and fecundity (i.e., the number of eggs laid)—an analysis of variance (ANOVA) was used after validation of the assumptions of normality and homogeneity of variances. In all ANOVA models, age was included as a fixed effect. Data for individuals within each replicate and age group were averaged and ANOVA models were run using replicate as a random factor to test for the effect of age. Means with 95% confidence intervals were calculated for all models.

When a significant effect of age was found ($p < 0.05$), multiple comparison tests were requested adjusting the p value according to the false discovery rate (FDR) (Benjamini and Hochberg, 1995). All analyses and graphs were carried out with R software (R Core Team, 2022). ANOVA models were executed with the lm command stats package and the GLMMs were performed with the glm stats package and glmer (lme4 stats package) (Bates et al., 2015). Means, confidence intervals and multiple comparisons were obtained with the emmeans package (Lenth, 2023).

3. Results

3.1. The post-emergence age when female Aedes aegypti can be inseminated

We first determined the time post-emergence that females of our laboratory *Ae. aegypti* strain could be successfully inseminated by sexually mature males. We grouped females into 4-hour blocks based on the time of their emergence. As Thai females can actively fly at 10 h post-emergence, the earliest age group used in our study was 10–13 h post-emergence, followed by 14–17 h, 18–21 h, 22–25 h, 26–29 h, 30–33 h, 34–37, and 38–41 h. We also examined two groups of "old" females: 2- and 3-weeks-old. Both young and old females were compared to 4-day-old females.

We observed a significant association between post-emergence age when mating was initiated and the proportion of females that were inseminated during the 2 h mating interval (Deviation test GLMM: $X^2 = 552.129$, df = 5, $p < 0.001$; Fig. 1A). Nearly all females were refractory to mating for one day post-emergence: insemination was first detected in females of the 22–25-hour age group, but only 3.4% were successfully inseminated at this time (Fig. 1A; Table S1). The proportion of females that were inseminated steadily increased after this time, with > 50% of females inseminated in the 34–37 h age group, reaching 82% in 38–41 h age group (Fig. 1A; Table S1). Nearly all control females (99%) were inseminated in our assays (Fig. 1A, B; Table S1 and S2). The proportion of 2- and 3-week-old females that were successfully inseminated did not significantly differ from control females (Deviation test GLMM: $X^2 = 5.348$, df = 2, $p = 0.069$; Fig. 1B): 96% and 94% of 2-week and 3-week-old females, respectively, were successfully inseminated in our assays (Fig. 1B; Table S2).

3.2. The post-emergence age when female Aedes aegypti consume their first blood-meal

We next determined the time post-emergence when female *Ae. aegypti* would take their first blood-meal, as a blood-meal is required for egg development in this species. Virgin females were grouped by age as in our insemination assays and were given 10 min to blood-feed.

We observed a significant association between post-emergence age and the proportion of females that blood-fed (Deviation test GLMM: $X^2 = 400.705$, df = 7, $p < 0.001$; Fig. 1C). Blood-feeding occurred sooner than insemination: females were first observed to blood-feed at 14–17 h post-emergence, but only 10% of females blood-fed at this time (Fig. 1C; Table S3). The proportion of females that blood-fed increased with time post-emergence, with > 50% of females taking a blood-meal by 22–25 h, reaching 84% in the 38–41 h age group (Fig. 1C; Table S3). Nearly all control females (99%) took a blood-meal (Fig. 1C, D; Table S3 and S4). Old females did not significantly differ from control females (Deviation test GLMM: $X^2 = 3.717$, df = 2, $p = 0.156$; Fig. 1D), with 96% and 95% of 2-week-old and 3-week-old females, respectively, consuming a blood meal (Fig. 1D; Table S4).

3.3. Quantity of blood consumed by young and old females

We next examined the quantity of blood consumed by females in our

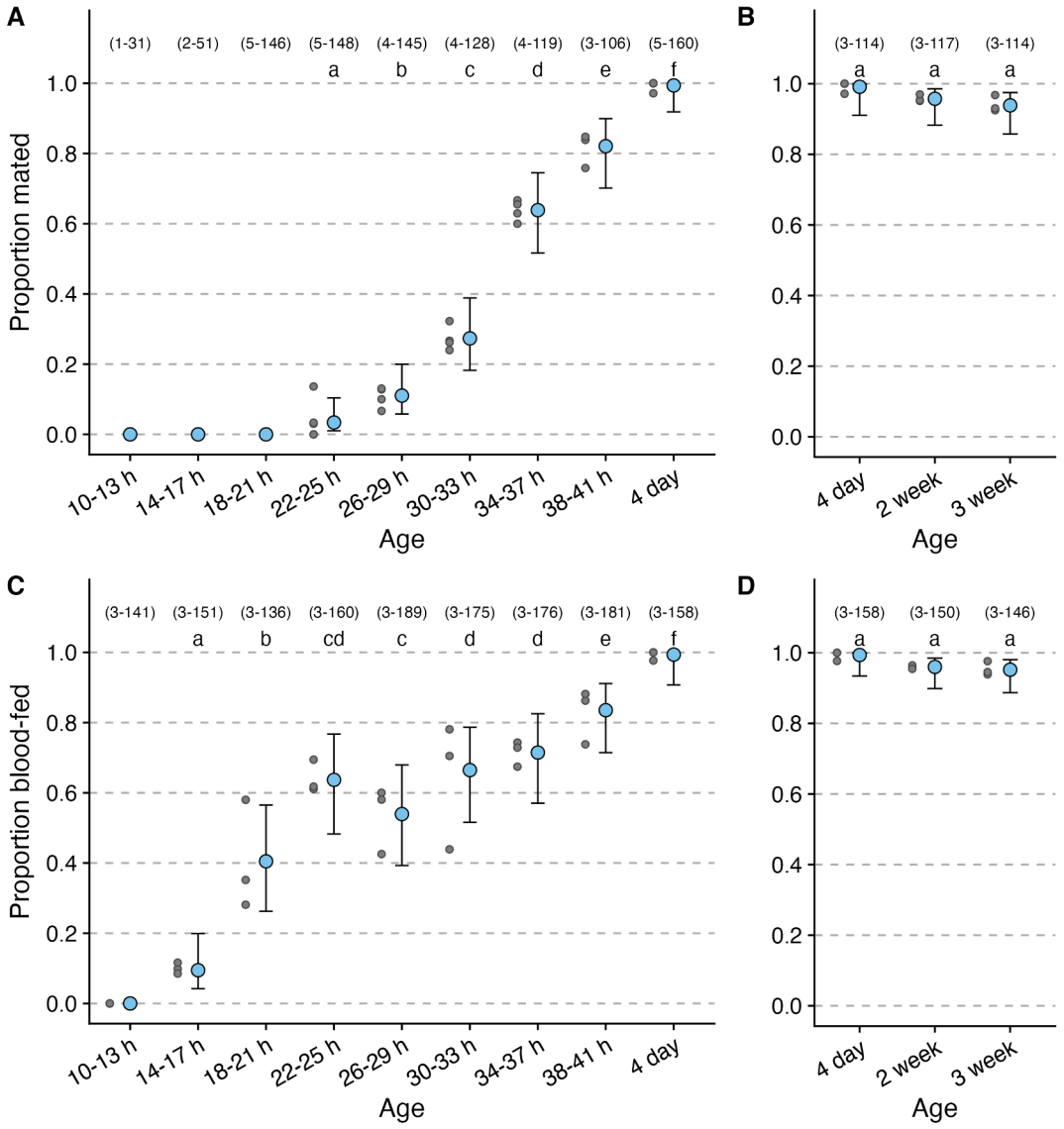

Fig. 1. The proportion of females in the young (**A**) and old (**B**) age groups that mated with sexually mature males during the 2 h mating interval. The proportion of females in the young (**C**) and old (**D**) age groups that blood-fed during the 10 min feeding interval. Means for all replicates are shown in blue, with error bars representing the 95% confidence intervals. Means for individual replicates are shown as grey dots next to each bar. The number of replicates performed and the total sample size for each group are shown above the bars. Different letters above the bars represent significant differences ($p < 0.05$) between female age groups according to multiple comparison tests with an FDR adjustment.

blood-feeding assays. We observed a significant association between female age and the quantity of blood consumed (F-test ANOVA: $F_{7,16} = 19.548$, $p < 0.001$; Fig. 2A). The two earliest age groups, 10–13 h and 14–17 h, consumed the smallest quantity of blood (Fig. 2A), with blood-meal size significantly increasing in the 22–25 h age group (Fig. 2A; Supplemental File 1); females in the subsequent young age groups consumed similar quantities of blood (Fig. 2A; Supplemental File 1). Blood-meal size was largest in 4-day-old control females, which differed significantly from all young female age groups (Fig. 2A; Supplemental File 1). Blood-meal size consumed by 2-week- and 3-week-old females did not significantly differ from control females (F-test Anova: $F_{2,6} = 0.681$, $p = 0.542$; Fig. 2B).

3.4. Sperm quantities stored by young and old females

We next examined the quantity of sperm stored in the spermathecae by young and old females. Females were mated to 4–6-day old Thai males, which transfer 1000–2000 sperm to females during an initial mating (Agudelo et al., 2022), of which ~400 are stored in the spermathecae of the female reproductive tract (Agudelo et al., 2022). We observed a significant association between the quantity of sperm stored in the spermathecae and the age post-emergence when mating was initiated (F-test ANOVA: $F_{4,10} = 11.649$, $p < 0.001$; Fig. 3A). Females in the earliest age group (26–29 h) stored the lowest quantity of sperm (Fig. 3A). A slight increase in sperm quantity was observed after this time, with females in the 38–41 h age groups storing similar quantities of sperm as control females (Fig. 3A; Supplemental File 1). Females in 2-week age group stored a similar number of sperm as did control females (Fig. 3B; Supplemental File 1). However, 3-week-old females stored significantly fewer sperm compared to 2-week-old and 4-day-old control females (Fig. 3B; Supplemental File 1).

3.5. Fecundity and fertility of young and old females

Finally, we examined the fecundity and fertility of recently emerged females in 3 age groups: 30–33 h, 34–37 h, and 38–41 h. These age

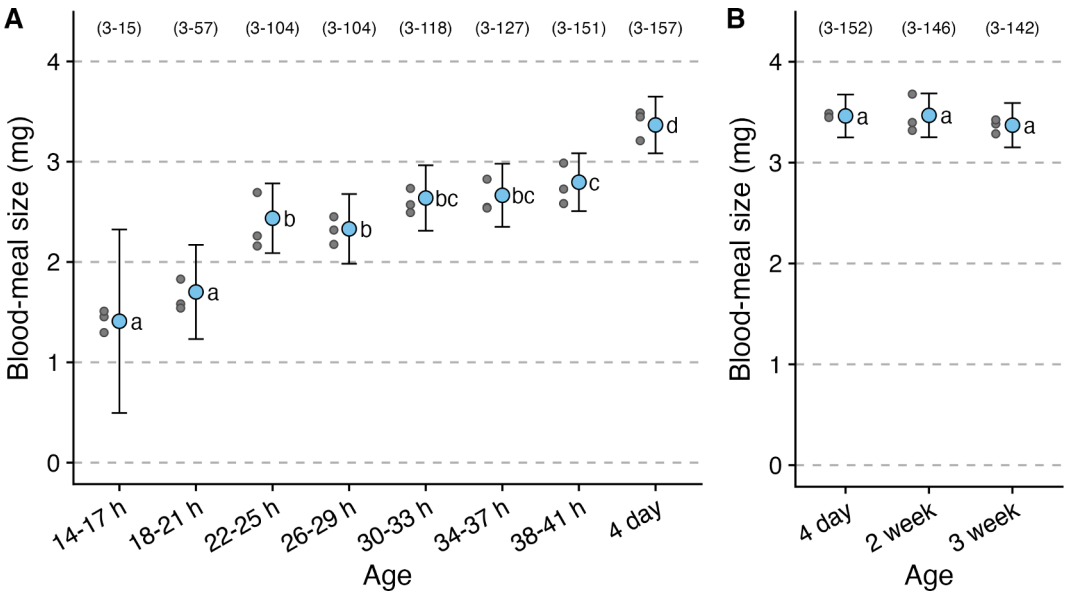

Fig. 2. Mean blood-meal sizes of females in the young (**A**) and old (**B**) age groups. Means for all replicates are shown in blue, with error bars representing the 95% confidence intervals. Means for individual replicates are shown as grey dots next to each bar. The number of replicates performed and the total sample size for each group are shown above the bars. Different letters next to the bars represent significant differences ($p < 0.05$) between female age groups according to multiple comparison tests with an FDR adjustment.

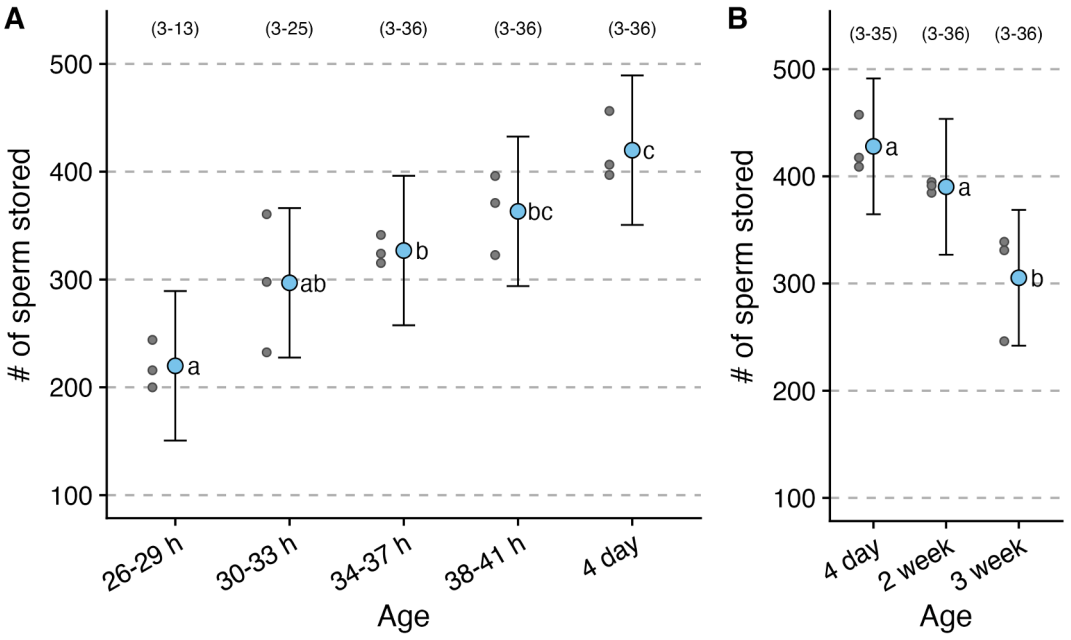

Fig. 3. The quantity of sperm stored in the spermathecae of females in the young (**A**) and old (**B**) age groups. Means for all replicates are shown in blue, with error bars representing the 95% confidence intervals. Means for individual replicates are shown as grey dots next to each bar. Different letters next to the bars represent significant differences ($p < 0.05$) between female age groups according to multiple comparison tests with an FDR adjustment.

groups were chosen as a sufficient proportion of females of these ages would be inseminated and consume a blood-meal (Fig. 1A, C). We found that females in the young age groups laid a similar number of eggs (F-test ANOVA: $F_{3,8} = 0.501$, $p = 0.692$; Fig. 4A) and were similarly fertile as control females (Deviation test GLMM: $X^2 = 37.312$, df = 3, $p = 0.805$; Fig. 4C). Females in the old age groups suffered a significant decline in fecundity compared to control females (F-test ANOVA: $F_{2,6} = 55.352$, $p < 0.001$; Fig. 4B): 2-week and 3-week-old females were similarly fertile to each other, but females of both groups laid significantly fewer eggs than control females (Fig. 4B; Supplemental File 1). However, the fertility (shown as hatch percentage) of old females did not significantly differ from control females (Deviation test GLMM: $X^2 = 87.107$, df = 2, $p = 0.223$; Fig. 4D).

4. Discussion

In insects, the fertility of a mating pair can be affected by the age of the mating individuals (Grotewiel et al., 2005; Miller et al., 2014). Increased female age has been shown to impact fertility in a number of insect species (e.g., David et al., 1975; Fricke et al., 2013; Tabata and Teshiba, 2018; Tasnin et al., 2021), but little is known about the possible impacts of mating shortly after female emergence. In this study, we

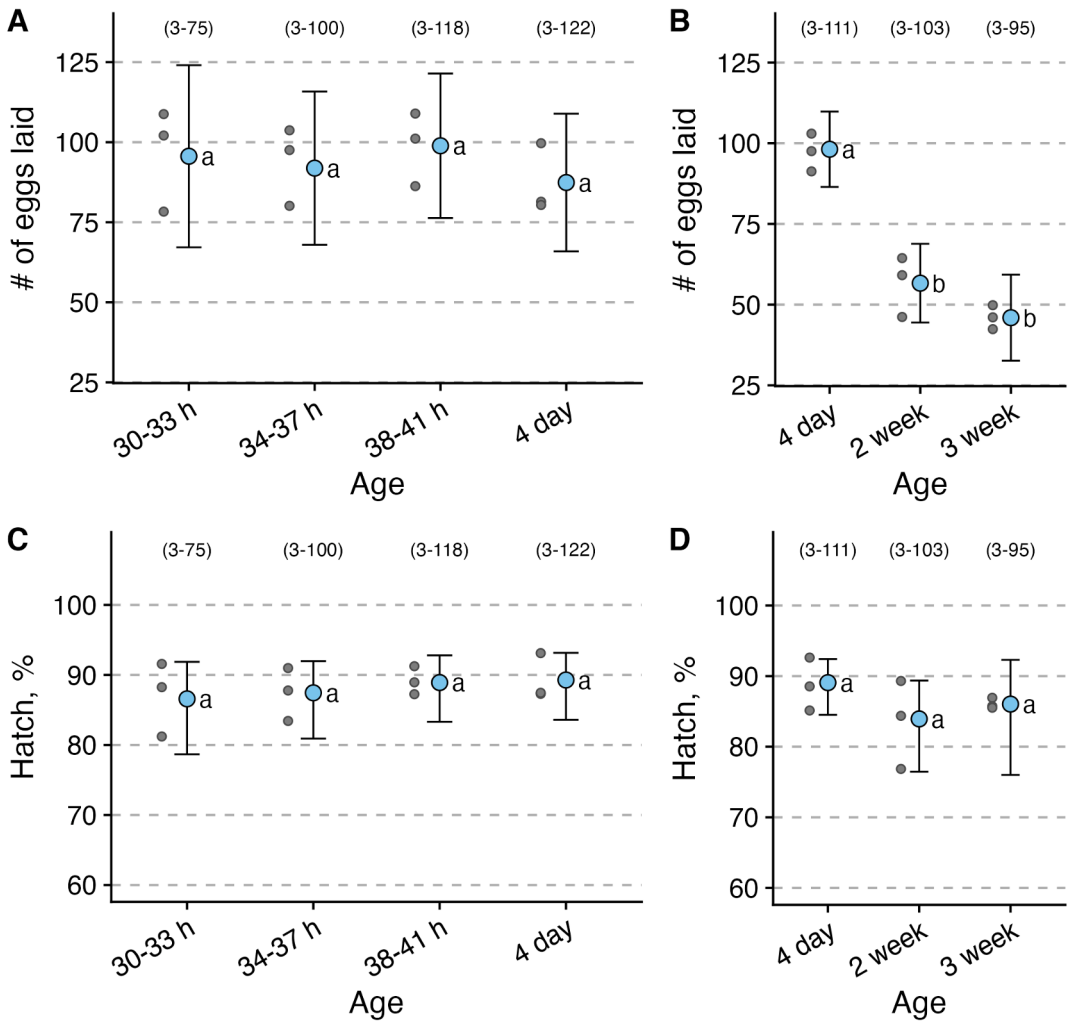

Fig. 4. Mean number of eggs laid (**A**, **B**) and the proportion of eggs that hatched (**C**, **D**) of females in the young (**A**, **C**) and old (**B**, **D**) age groups. Means for all replicates are shown in blue, with error bars representing the 95% confidence intervals. Means for individual replicates are shown as grey dots next to each bar. The number of replicates performed and the total sample size for each group are shown above the bars. Different letters next to the bars represent significant differences ($p < 0.05$) between female age groups according to multiple comparison tests with an FDR adjustment.

assessed reproductive parameters of female *Ae. aegypti* inseminated shortly after becoming sexually receptive, and asked whether they suffer age-related declines in fertility common in insects. As female *Ae. aegypti* typically mate only once in their lifetime (Degner and Harrington, 2016a), the possible effects of female age at an initial mating could potentially impact their lifetime fertility.

Given the large age range reported for the post-emergence sexual refractory period of female *Ae. aegypti* (Gunathilaka et al., 2023; Gwadz et al., 1971a; Gwadz and Craig, 1968; Spielman et al., 1969), and the timing of the first blood-meal (Christophers, 1960), we first determined the age when insemination and blood-feeding were likely to occur in our *Ae. aegypti* laboratory strain. Even as our assays placed high pressure on recently emerged females to mate, insemination was not observed for ~24 h, and only 11% of females were inseminated by 30 h post-emergence. This result aligns with a previous study that reported females were not receptive for 1–2 days post-emergence (Gwadz et al., 1971a) and suggests that recently emerged female *Ae. aegypti* exert control over their ability to be inseminated or are physically unable to be inseminated. Juvenile hormone (JH) appears to control sexual receptivity of female *Ae. aegypti*; topical application of a synthetic JH analog significantly reduces the post-emergence refractory period of female *Ae. aegypti* (Gwadz et al., 1971b), initiating sexual receptivity by acting on the terminal ganglion of the female nervous system (Gwadz, 1972). JH synthesis in the female corpora allata drastically increases by 12 h post-emergence (Rivera-Perez et al., 2014) and JH titers steadily increase in the hemolymph, peaking at ~48 h (Zhao et al., 2016). Thus, differences in post-emergence receptivity observed among the various studies may be due to strain-dependent timing of JH production of recently emerged females.

The consumption of blood by virgin females occurred much sooner than insemination, and the proportion of young females that blood-fed was greater than the proportion that mated at all timepoints. Although several studies have reported that female *Ae. aegypti* mate prior to blood-feeding (Facchinelli et al., 2023), a recent report showed that blood-meal size, blood-digestion rates, and host feeding avidity are similar between mated and virgin females that have been deprived of a sugar meal prior to analysis (League et al., 2021). Further, mated and virgin *Ae. aegypti* collected from the field are equally likely to have consumed a blood-meal (League et al., 2021). Given that >99% of virgin control females blood-fed in our assays, and that virgins of most age groups were more likely to blood-feed than mate, our results further suggest that mating is not a prerequisite to blood-feeding in this species. As sugar feeding can impede blood intake in virgin and mated *Ae. aegypti* (League et al., 2021), the differences reported for the post-emergence age at the first blood-meal in previous studies may have resulted from sugar feeding prior to analysis. However, although females in our assays

were deprived of sugar prior to our assays, young females were never given a sugar meal, which may have had subtle effects on the results reported here.

Sperm storage was suppressed in recently emerged and in 3-week-old females. Sperm storage in the female reproductive tract is a common process in insects, and one that requires both male and female contributions (Avila et al., 2011; Zhao et al., 2021). Given that 4–6-day-old males are likely to transfer optimal quantities of seminal components that contribute to the female PMR, including sperm (Agudelo et al., 2022) and seminal fluid proteins (Alfonso-Parra et al., 2014)—the latter required for efficient sperm storage in female insects (Avila et al., 2011; Rogers et al., 2009)—the reduced sperm storage levels observed is likely a female-specific effect. Proteins produced in secretory cells of the spermathecae are required for female sperm storage in *D. melanogaster* (Schnakenberg et al., 2011) and mating up-regulates spermathecal genes hypothesized to protect stored sperm from oxidative stress in *Anopheles gambiae* (Shaw et al., 2014). In *Ae. aegypti*, both mating and blood-ingestion regulate the expression of spermathecal genes (Camargo et al., 2020). Thus, the production of spermathecal proteins required for *Ae. aegypti* sperm storage, or the action of pathways that modulate protein secretion from the spermathecae, may not be optimal in recently emerged females, and may decrease in efficiency as females age. More investigation is required to determine the timing of spermathecae gene expression post-emergence, and if the production of proteins produced there is altered as females age.

Fecundity of recently emerged females did not differ from control females, showing that the slight reduction in the quantity of sperm stored and of blood consumed did not affect fecundity in the first gonotrophic cycle. That fertility of young females was similar to controls suggests that female *Ae. aegypti* are optimally fertile once they are sexually receptive, unlike in *Drosophila* (Fricke et al., 2013). Fecundity in old females was significantly impacted by 2-weeks of age, which aligns with other insect studies reporting declines in egg-laying that accompany increased female age (David et al., 1975; Fricke et al., 2013; Gunathilaka et al., 2023; Tabata and Teshiba, 2018; Tasnin et al., 2021), although we did not observe decreases in the fertility of older females previously reported for this species (Gunathilaka et al., 2023). Females typically have consumed blood and undergone one or more gonotrophic cycle by 2- and 3-weeks of age, with decreases in total eggs laid observed after the first gonotrophic cycles (De Jesus and Reiskind, 2016). Thus, our assays may not completely reflect physiological changes associated with increased age, as 3-week-old females that undergone one or more gonotrophic cycles are more similar to 5-day old females than those that have never consumed blood (Klowden and Lea, 1980). The decline in fecundity may be due to a decrease in stem cell proliferation (Zhao et al., 2008), a decline in protein production and/or secretion from the spermathecae, whose products are required for egg-laying (Pascini et al., 2020; Schnakenberg et al., 2011), a combination of both, or other factors associated with our experimental design. Further studies are needed to assess how increased female age alters key processes necessary for egg production and oviposition.

Aedes aegypti is an accomplished vector of numerous arboviruses that impact human health. Domestic strains of this vector prefer a human blood-meal (McBride et al., 2014) and females typically lay their eggs in natural and artificial containers in close proximity to human settlements (Metz et al., 2023), factors that facilitate disease transmission by this species. Thus, it is imperative that we expand our understanding of the reproductive processes of this species. In this report, we show that although *Ae. aegypti* females require 1–2 days to mate and are fully fertile once they can be inseminated, they can blood-feed within hours after their emergence. This suggests that females can potentially ingest an arbovirus infected blood-meal shortly after emergence. As young females consumed smaller blood quantities, they may take additional blood-meals, which can shorten the extrinsic incubation period of ingested viruses (Armstrong et al., 2020). As genetic background influences a multitude of phenotypes, more work is required to determine the variation in reproductive processes for *Ae. aegypti* populations to assess the risk of disease transmission and determine how reproductive output can vary with female age.

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CRediT authorship contribution statement

Luis Felipe Ramírez-Sánchez: Investigation, Methodology, Writing – review & editing. **Brenda Juliana Hernández:** Investigation, Writing – review & editing. **Pablo Andrés Guzmán:** Formal analysis, Validation, Visualization, Writing – review & editing. **Catalina Alfonso-Parra:** Conceptualization, Investigation, Supervision, Writing – review & editing. **Frank W. Avila:** Conceptualization, Investigation, Funding acquisition, Methodology, Supervision, Writing – original draft.

Declaration of Competing Interest

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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