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Sex before or after blood feeding: Mating activities of *Aedes aegypti* males under conditions of different densities and female blood feeding opportunities

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ABSTRACT

Blood feeding and mating are critical behaviors that regulate both mosquito population maintenance and disease transmission. However, our understanding of mosquito mating systems remains incomplete. One of the most critical issues is a lack of understanding regarding how and where males and females encounter one another. This study was performed to investigate changes in key mating behaviors of *Ae. aegypti* relative to female blood feeding opportunities, taking into account male density. We compared courtship latency and copulation activity between single and pooled males in a range of assays performed in the presence or absence of a blood source and after blood feeding. The time taken by grouped males to initiate courtship in the presence of a host was much shorter than that in single males. There was no significant difference in courtship latency between pooled and single males in the absence of a blood source or after blood feeding. At low male density, the presence of the host and blood meal ingestion provided better conditions for copulation. At high male density, however, copulation activity was decreased after blood feeding, but remained high regardless of the presence or absence of the host. In addition to providing insight into the mating ecology of *Aedes aegypti*, this study indicated that the presence of a blood source influences how males encounter and copulate with females. The observation that copulation activity decreases after blood feeding when males are numerous provides new avenues for improving mass release programs of sterile mosquitoes.

Introduction

Aedes aegypti is a known vector of several viruses that affect public health, including those that cause dengue, chikungunya (ECDC, 2016), and Zika virus disease (Hennessey et al., 2016; CDC, 2016). Together, these diseases pose significant and continuous public health threats (Fares et al., 2015) with great economic impacts worldwide (WHO, 2014). Alternative vector control strategies are needed due to the narrowing spectrum of effective insecticides (Dusfour et al., 2011) caused by the development of resistance (Dorta et al., 1993), along with the lack of effective vaccines and specific therapeutic agents to combat these diseases (Laughlin et al., 2012). Transmission of these viruses generally requires a female mosquito to bite an infected host (Cox et al., 2012) or lay viable infected eggs (Buckner et al., 2013). As blood feeding is the primary mechanism by which disease transmission occurs (Lehane, 2005), and mating, which can trigger vertical transmission (Martins et al., 2012), is closely related to blood feeding (Soghigian et al., 2014), focusing on these behaviors may facilitate the discovery of new strategies for vector control.

Due to the close interplay between blood feeding and mating in *Aedes* mosquitoes, and their roles in disease transmission and vector population maintenance, there have been a number of studies regarding their order of importance. Overall, these observational studies yielded controversial results or relied on unclear assumptions. Flight is an

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essential component of mating behavior in mosquitoes (Belton, 1994). In *Aedes*, males initiate mating in swarms—groups of flying males displaying stereotyped flight patterns—near blood hosts (Peyton, 1956; Klowden and Zwiebel, 2005) to increase the likelihood of finding a female mate (Hartberg, 1971; Cator and Harrington, 2011). Females fly into the clouds formed by these synchronized flights and depart paired with a male (Dao et al., 2008). Usually, non-blood-fed females show increased flight activities, whereas their blood-fed counterparts are inactive (Klowden and Briegel, 1994); immobile blood-fed females are commonly observed in caged *Aedes* populations in the laboratory environment. As reported by Lima-Camara et al. (2014), blood-fed females are much heavier than unfed females. As females must fly to enter the swarming arena, it is likely that such females have not recently fed on blood.

This inactivity after a blood meal taken together with the assumption that females must enter the swarms and be grasped by males during flight for copulation to proceed (Ponlawat and Harrington, 2009) would suggest that blood-fed females have lower chances of successful copulation and/or take longer to copulate than their unfed counterparts. The suggestion that aedine females must actively fly to enter the swarms and be grasped by males during flight for copulation (Cabrera and Jaffe, 2007; Ponlawat and Harrington, 2009) is inconsistent with reports that the rate of mating after blood feeding is high in *Aedes* mosquitoes. To address this issue, O'Meara (1985) suggested that mating can occur before or after blood feeding (O'Meara, 1985). Blood-fed female *Aedes triseriatus* mosquitoes—a species that is capable of transovarial transmission of RNA viruses (Hughes et al., 2006) and is closely related to *Ae. aegypti*—were reported to show a high incidence of copulation (Wright and Venard, 1967). Ponlawat and Harrington (2009) suggested that males of this species copulate with females as they approach or leave hosts after blood intake. In nature, blood feeding has been reported to precede mating (Teesdale, 1955). Many groups have reported that mating can occur without swarming (Downes, 1955; Sullivan, 1981; Yuval and Bouskila, 1993; Cabrera and Jaffe, 2007). This seems true for *Ae. aegypti*, for which swarming behavior is non-essential (Cabrera and Jaffe, 2007) and swarms being composed of only a few individuals (Goeldi, 1905; McClelland, 1959; Hartberg, 1971; Gubler and Bhattacharya, 1972). In fact, swarming is not required in *Ae. aegypti*, and compatible partners can form a mating pair (Oliva et al., 2013). A similar observation was reported by Gubler and Bhattacharya (1972), who noted that mating occurred freely between single males and females in nature. *Ae. aegypti* males have also been observed mating during biting catches (McClelland, 1959). These reports are consistent with our observation of single pairs seen on approaching encaged populations of *Ae. aegypti* in the laboratory or minutes after entering natural habitats of dengue mosquitoes, suggesting that the presence of a blood source stimulates mating activity, as indicated elsewhere (Dieng et al., 2007).

During mating, male mosquitoes transfer sperm and seminal fluids (Helinski and Harrington, 2011), leading to broad transient changes in gene expression in the female (Alfonso-Parra et al., 2016), as does blood feeding in *Ae. aegypti* (Akbari et al., 2013; Roy et al., 2015). The levels of transcription of many genes, including some that are involved in metabolism, are modulated by seminal proteins (McGraw et al., 2004). In *Ae. aegypti*, copulation initiates a wide range of transcriptional changes relative to blood feeding and blood use for egg production (Alfonso-Parra et al., 2016). As mating and blood feeding are interconnected, gene expression processes during blood assimilation may be influenced by the gene expression cascade triggered by copulation and mating, or vice versa. These male-induced changes in the regulation of gene expression in the female have attracted a great deal of research interest due to their potential for the discovery of molecules critical for female fertility (Alfonso-Parra et al., 2016). The identification of such molecules may be useful in the development of new mosquito management strategies, such as insecticides that render fertilizing molecules inaccessible for females or inhibitors that prevent the production of fertile eggs. Therefore, accurate knowledge regarding when mating readily occurs is relevant, especially in dengue vectors in which female re-mating is rare and polyandry droopy after mating (Degner and Harrington, 2016). The present study was performed to examine whether and to what extent *Ae. aegypti* males can copulate with females in the absence or presence of a host and after blood meal uptake. The impacts of such sexual environments on courtship latency were also examined.

Materials and methods

Rearing of mosquitoes

A colony of *Aedes aegypti* established in 2015 at the Entomology Unit of the Faculty of Resource Science and Technology (Universiti Malaysia Sarawak, Kota Samarahan) was used in this study. The mosquito populations were maintained at a temperature of 27 °C – 30 °C and relative humidity of 60%–86% with a photoperiod of 14:10 h (L:D). Adults were kept in plastic cages (30 cm × 30 cm × 30 cm, BugDorm; MegaView Science Co., Ltd., Taichung, Taiwan) with free access to sugared water (10%). Once a week, females were given blood meals from a restrained hamster placed inside the cage for 35 min. Three days after blood feeding, egg collection devices (2 cm in diameter, 8 cm in depth, equipped with a section of filter paper as an oviposition substrate) were placed inside the cage. Eggs were allowed to dry under insectary conditions for 4 days before being processed and stored as a stock colony, as described elsewhere (Dieng et al., 2013a). Samples of eggs were immersed in dechlorinated water and newly hatched larvae were reared in replicates at densities of 200–300 in white plastic trays (As One Corporation, Osaka, Japan) holding 1 L of tap water as described elsewhere (Gary and Foster, 2004). Larvae were provided 0.25–3 g of cat food pellets (ProDiet Cat Food, Malaysia) every two days and the rearing media once changed per generation. The resulting pupae were placed in plastic vials containing 10–15 mL of water and transferred into cages for emergence.

Obtaining of virgin experimental adults

To produce virgin experimental adults, we followed a slight modification of the procedure described previously (Dieng et al., 2013b; Satho et al., 2015). Briefly, samples from the egg store were hatched as described above for the colony. Groups of 300 newly eclosed larvae were bred in quintuplet in plastic trays filled with 1 L of water. Larvae were provided 0.15 g of cat food every 48 h and the rearing water in each tray was substituted with fresh water before the second food supply. Pupated individuals were individually placed in 1.5-mL Eppendorf tubes containing 0.15 mL of water. Upon emergence, the sex of all adults was determined under a stereomicroscope (SZ-LED; Kenis, Osaka, Japan). Males were pooled in a cage labeled “CM” (Cage for Males) and females were divided into two groups placed in distinct cages each equipped with a 10% sucrose solution supplier. One cage was designated as “CSF” containing sugar-fed females; the other cage labeled “CBF” (cage holding blood-fed females). To obtain blood-fed females, individuals from the second CBF cage were allowed to take blood meals from a retrained hamster. Fully blood-engorged females that had just finished feeding were used as experimental blood-fed females. A fully blood-fed female was considered as defined elsewhere (Dieng et al., 2015). Identical rearing procedures described above were repeated to produce experimental adults (males, sugar-fed females, and blood-fed females) when necessary.

Experimental features

All bioassays were conducted in a mating device that consisted of a 250-mL transparent plastic container equipped with mosquito net-screened windows (1.5 cm × 1.5 cm). The middle of the lid of the

container had an opening with a 1.5-mL Eppendorf tube the bottom of which had been removed. This modified tube was used to release experimental mosquitoes; during the experiment, the upper opening of the tube was blocked with a cotton pad soaked with 10% sucrose solution. In all bioassays, experimental adult cohorts were allowed to cohabit for a period of 30 min.

Bioassays

We examined whether the mating behavior of *Ae. aegypti* males is influenced by female blood feeding opportunities. Briefly, a single sugar-fed virgin male (3–4-day old) was released into a mating container. After 10 min of acclimatization to the new environment, five sugar-fed virgin females (3–5 days old) were released into the container, adopting others (Patil et al., 2014). Immediately after release, the opening was sealed with saturated cotton pad and the interactions between the male and females were monitored. The first copulation attempt was recorded and copulations were enumerated for 30 min. Nine additional replicates of each of the two treatments (one mating container with one male + five females and another mating container with five males + five sugar-fed females) were set up on the same or different days and observed as described above for the two first containers. To examine the mating rituals of *Ae. aegypti* males in cages with the presence of a host, 10 other mating containers processed and treated as described above were also prepared. These 10 containers were divided into two groups of five containers each. Each replicate for each group was ascribed to one of the following treatments: i) one male + five females + a restrained hamster, or ii) five males + five females + a restrained hamster. Ten additional containers processed as outlined above were also set up to examine whether the mating behavior of males is impacted by blood feeding. These containers were divided into two lots of five containers each. Each replicate in each lot was assigned to one of the treatments: i) one male + five freshly blood-fed females, and ii) five male + five freshly blood-fed females. For both the latter bioassays (presence of a host, i.e., a restrained hamster and after blood feeding, blood-fed females), the interactions between sexes were monitored as described for the first bioassay.

Data collection and analysis

In all bioassays, the time to first copulation attempt made by the single male or any one of the five grouped males on any female was monitored immediately after the collective release of the five females into the mating cup using a stopwatch. A copulation attempt was defined as a male approaching and attempting to attach itself to a female, in accordance with Roth (1948). The time between release and a copulation attempt was recorded for each mating cup replicate of each male density and female treatment group. In each case, the mean value of these times expressed in seconds was considered as a measure of courtship latency defined as the time taken by a male to initiate courtship of a female (Eastwood and Burnet, 1977). *Ae. aegypti* completes copulation within an average of 10 s (Roth, 1948; *Jones and Wheeler,* 1965). Therefore, we considered copulation as any effective genital contact of a mating pair, which lasted for at least 10 s, adopting the definition of Roth (1948). The mean numbers of such contacts were computed for each male density and female treatment, and used as mean numbers of copulations.

The differences in courtship latency and number of copulations were compared by analysis of variance (ANOVA) using Systat v.11 statistical software (Systat Software Inc., 2004). Where necessary, the means (± SE) were examined by Tukey's honestly significant difference (HSD) test. In all analyses, $P < .05$ was taken to indicate statistical significance.

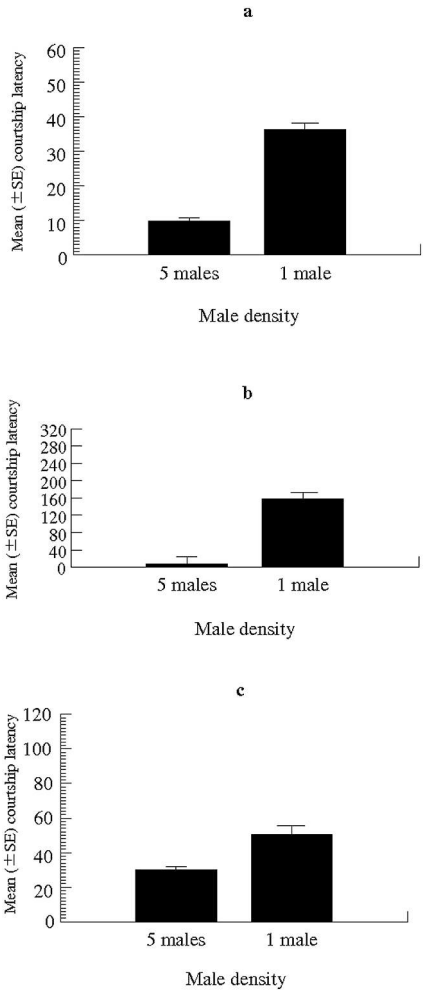

Fig. 1. Time taken by *Ae. aegypti* males at low and high densities to initiate courtship of sugar-fed females in the presence of a blood meal source (a), absence of a blood meal source (b), and after blood meal uptake (c).

Results

Courtship activities of males in environments with various mating opportunities

In the presence of a host, both single and crowded *Ae. aegypti* males successfully courted females, but the time taken to initiate courtship exhibited different patterns according to male density. For individual males, the mean courtship latency was 36.20 ± 7.45 s (range: 15–89 s). When males were in crowd, however, the time to initiation of courtship was 9.90 ± 3.11 s (range: 1–30 s). Thus, the courtship latency for single males was significantly longer than that for males in a group ($F = 10.59$, $df = 1$, $P = .004$) (Fig. 1a). In the absence of a host, the courtship latency of *Ae. aegypti* males was related to their density. The mean time taken to initiate courtship of females by males under crowded conditions (8.50 ± 2.22 s, range: 1–20 s) was significantly shorter than that of lone males (157.00 ± 64.93 s, range: 19–680 s) (Fig. 1b). After blood meal uptake, courtship occurred with latencies of 50.70 ± 19.49 s (range: 4–195 s) and 30.30 ± 7.42 s (range 4–69 s) for single males and those in groups, respectively. However, the difference between these two mean values was not significant ($F = 0.956$, $df = 1$, $P = .341$) (Fig. 1c).

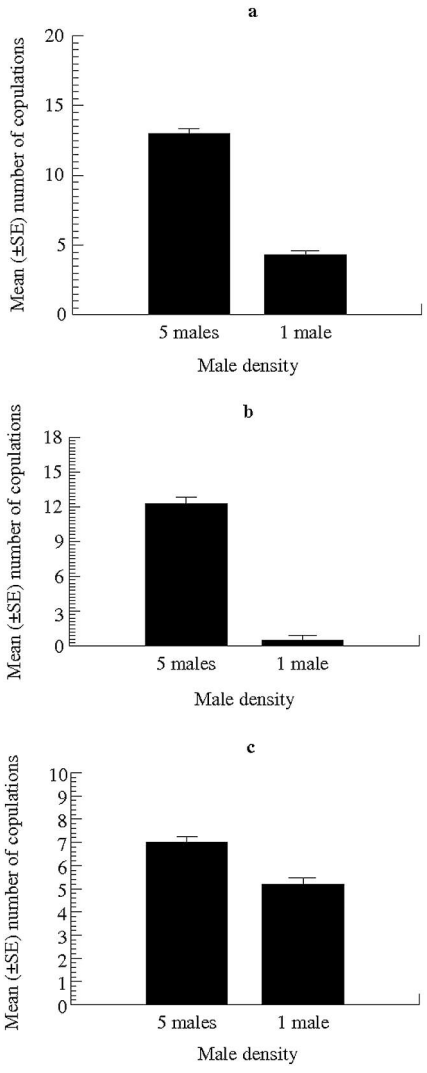

Fig. 2. Mean (± SE) number of copulations of *Ae. aegypti* males at low and high densities with conspecific females in the presence of a blood meal source (a), absence of a blood meal source (b), and after blood meal uptake (c).

Copulation activities of males in relation to female blood feeding opportunity

In the presence of a host, the mean number of copulations of *Ae. aegypti* males presented singly was 4.30 ± 1.08 (range: 0–9), while that for their counterparts presented in groups was 13.00 ± 1.48 (range: 7–20). This difference between the two means was significant ($F = 22.40$, $df = 1$, $P < .001$), with copulation activity occurring at a higher level for males under crowded conditions (Fig. 2a). Single and grouped males of *Ae. aegypti* exhibited different copulation activity levels in the absence of a blood source. For single males, the mean number of copulations ranged between 0 and 2, with an average of 0.50 ± 0.26. The mean number of copulations among their counterparts presented in groups was 12.30 ± 2.20, ranging from 4 to 27. Crowded males showed significantly greater copulation success than their individual counterparts ($F = 28.18$, $df = 1$, $P < .001$) (Fig. 2b). *Ae. aegypti* males cohabiting with conspecific females after blood feeding showed courtship behavior, but the incidence differed according to male density. Crowded males showed more copulations (mean ± SE = 7.00 ± 1.09, range: 3–13) than their single counterparts (mean ± SE = 5.20 ± 1.06, range: 0–10), but the difference between these two mean values was not significant ($F = 1.391$, $df = 1$, $P = .254$) (Fig. 2c).

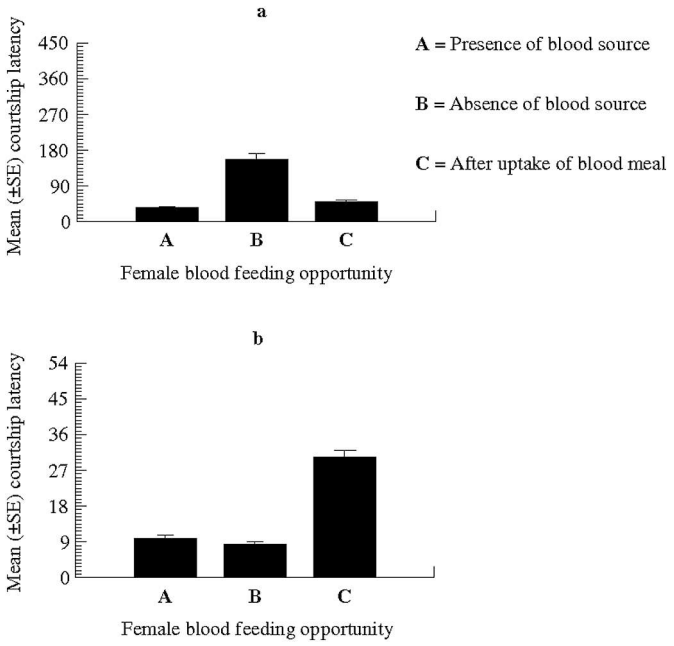

Fig. 3. Mean (± SE) time taken by *Ae. aegypti* males at low (a) and high (b) densities to initiate courtship of conspecific females under different blood feeding opportunities (A = Presence of a blood source; B = Absence of a blood source; and C = After uptake of a blood meal).

Courtship latency patterns in relation to female blood feeding opportunity

The courtship latency of single males was greater in the absence than in the presence of a host (157.00 ± 64.93 s and 36.20 ± 7.45 s, respectively), which in turn tended to be lower than that obtained after blood meal uptake (50.70 ± 19.49 s). However, the mean time taken by males to initiate courtship did not differ significantly between the three groups according to female blood feeding opportunities ($F = 2.80$, $df = 2$, $P = .078$) (Fig. 3a). Female blood feeding opportunity significantly affected courtship latency length for crowded males ($F = 6.40$, $df = 2$, $P = .005$). The time taken by males to initiate courtship was similar in the presence and in the absence of a host (9.90 ± 3.11 s and 8.50 ± 2.22 s, respectively; *Matrix of pairwise mean differences* (*MPMD*) = −1.400, $P = .977$), which in turn was shorter than that after blood feeding (30.30 ± 7.42 s; *MPMD* = 21.800, $P = .010$) (Fig. 3b).

Copulation success level in relation to female blood feeding opportunity

The single males showed significant differences in the occurrence of copulation between the different female blood feeding environments ($F = 7.845$, $df = 2$, $P = .002$). The mean number of copulations was significantly lower in the absence than in the presence of a host (0.50 ± 0.26 and 4.30 ± 1.08, respectively; *MPMD* = −3.800, $P = .015$), which in turn did not differ from that after blood meal uptake (5.20 ± 1.06; *MPMD* = 0.900, $P = .757$) (Fig. 4a). For crowded males, copulation activity varied significantly with female blood feeding opportunity ($F = 3.906$, $df = 2$, $P = .032$). The mean copulation number did not vary significantly between the absence or presence of a host (12.30 ± 2.20 and 13.00 ± 1.48, respectively; *MPMD* = −0.700, $P = .952$). This latter mean number of copulations was significantly higher than that after blood feeding (7.00 ± 1.09; *MPMD* = −6.000, $P = .042$) (Fig. 4b).

Discussion

The results of the present study indicated that *Ae. aegypti* males

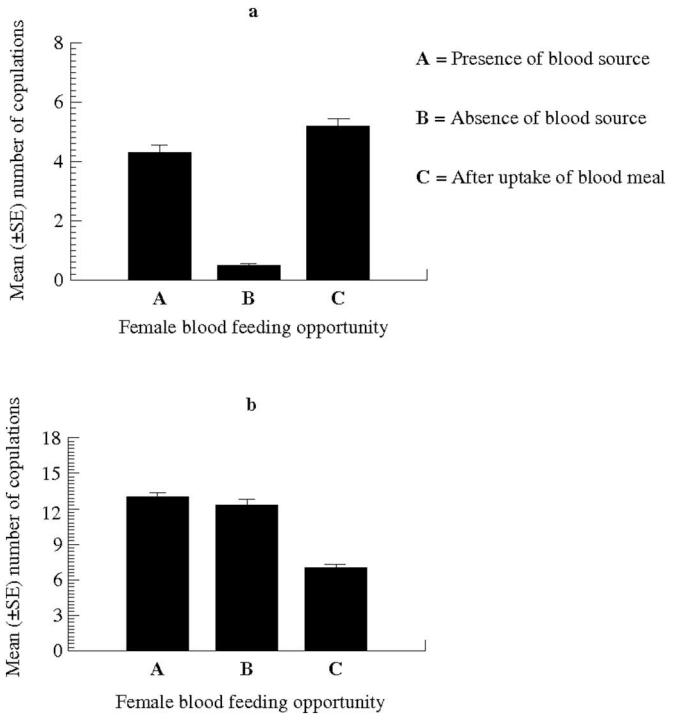

Fig. 4. Mean (± SE) number of copulations of *Ae. aegypti* males at low (**a**) and high (b) densities with conspecific females under different blood feeding opportunities (A = Presence of a blood source; B = Absence of a blood source; and C = After uptake of a blood meal).

courted and copulated females whenever they were present and regardless of their density or female opportunity to take a blood meal. However, courtship latency and copulation success showed different patterns according to male abundance and female blood feeding status.

Pooled males initiated courtship much earlier than their lone counterparts in the presence or absence of a blood source, as well as after blood uptake, indicating that the presence of increased numbers of males stimulated courtship initiation behavior. Similar to many mosquito species, *Ae. aegypti* males form swarms consisting of several males exhibiting stereotyped flight patterns near a physical object or a living organism (Klowden and Zwiebel, 2005; Peyton, 1956) that provide increased chances of copulating with females (Oliva et al., 2013).

Aedes aegypti can enhance mating opportunities in a number of other ways in addition to swarm formation, including aggregating around hosts (Clements, 1999) and single pairs (Oliva et al., 2013; Marcela et al., 2015). Male density is closely related to the scale of swarming. Indeed, the presence of increased numbers of males will produce larger swarms than lower male densities. Marcela et al. (2015) reported a correlation between male density and swarming level in *Ae. aegypti*. They reported that a reduction in male abundance triggered a decrease in swarming. The intensity of swarming is also closely related to the attractiveness of males to females. Cabrera and Jaffe (2007) reported that the attraction to females and copulation success are positively correlated with the number of males swarming in *Ae. aegypti*. In the present study, bioassays were performed with two male densities, i.e., one male and five males, in 250-mL plastic containers with the same numbers of females. *Ae. aegypti* males are sexually aggressive (Black et al., 1989). Indeed, containers with five males and females will tend to have greater amounts of flight activity than containers with one male and females. The increased flight activity will also lead to greater numbers of male–female interactions, which is also likely to be associated with increased occurrence of copulation. Although male aggregation was not assessed in the present study, such assemblages are more likely to occur at a greater rate in containers with five males simply because of their number. There may also be more encounters with females at a high male density, which would decrease chance that females would avoid copulation. In *Ae. aegypti*, both sexes are believed to produce volatile pheromones, also called aggregation pheromones, that modulate swarming behavior, stimulate female flight activity and facilitate male–female interactions (Cabrera and Jaffe, 2007; Fawaz et al., 2014). Although we did not measure such volatile agents in the present study, containers with ten adults (five males + five females) would likely have higher levels of aggregation pheromones, and therefore their effects were likely to be greater in these containers.

Single males courted and copulated females both in the absence of the blood source and after blood feeding. Males of *Aedes* mosquitoes do not require the formation of swarms to attract females and mate (Oliva et al., 2013). In *Ae. aegypti*, such mating behavior is not obligatory (Cabrera and Jaffe, 2007) and is rare presumably due to the possibility of copulation through other strategies (Oliva et al., 2013).

The number of copulations at high male density was low when females had fed on blood, compared to when females were fed sugar and presented with or without a host. Flight and flight tone synchronization have been reported to play a key role in copulation success in mosquitoes (Belton, 1994; Cator et al., 2009). Ponlawat and Harrington (2009) reported that aggregated *Ae. aegypti* males often grapple females in flight as they approach or leave hosts after a blood meal. In general, female mosquitoes draw more than their body weight in blood, thus rendering them heavy and slower moving after feeding (Paskewitz, 2009). This effect of blood feeding has been well documented in dengue vectors. Jones (1981) examined flight activity partly in relation to blood ingestion in *Ae. aegypti*, and observed almost total inactivity for about 2 days and spontaneous or frenzies activity. In a related study, Lima-Camara et al. (2014) investigated the effects of blood feeding on the locomotor activity of female *Ae. aegypti* mosquitoes. They observed significantly higher levels of locomotor activity among non-blood-fed females compared to those that had taken a blood meal. They suggested that a fully engorged female is much heavier than an unfed counterpart, and that the abdominal distension due to the presence of the blood meal stalled flight activity. In support of this suggestion, dengue mosquitoes were reported to search for a resting place after a blood meal to digest the blood and complete egg development (Kaufmann et al., 2013). The copulation activity study was performed with three types of female, i.e., sugar-fed females presented with a host, sugar-fed females, and blood-fed females. Based on these reports and our experimental design, it is likely that females did not exhibit sufficient flight activities in the containers with five males and five blood-fed females, probably because they were heavy. It is also clear that such effects of blood meal weight on flight activity did not occur in containers with five males and sugar-fed females.

This study was performed to examine the knowledge gaps in mating biology of *Aedes aegypti*, especially mate-finding. We examined courtship behavior and copulation performance in the dengue vector, *Ae. aegypti*, taking into consideration male density and female blood feeding opportunity with respect to the potential implications for mass release programs of sterile mosquitoes. Our results indicated significant effects of male density on early mating behavior in this mosquito species: the time taken to initiate courtship was much shorter when males were numerous. In addition, copulation success was much higher in the presence of increased numbers of males; pooled males that cohabited with blood-fed females showed less copulation success than their counterparts with sugar-fed females. The sterile insect technique (SIT) and incompatible insect technique (IIT) techniques are based on the use of laboratory colonized individuals, which are sterilized and released into the wild (Oliva et al., 2012), an environment that contains many different animals. In nature, mosquitoes, including dengue vectors, feed on many types of animals, including cows, pigs, rats, cats, birds, horses, dogs, and brushtail possums (Ponlawat et al., 2005; Carvalho et al., 2014). Evidently, in operational SIT programs, the presence of such hosts will tend to constantly generate blood-fed females. With the increased prevalence of blood-fed females, released males have reduced

chances of acquiring mates due to inactivity, blood digestion, or completion of egg development. The increased presence of blood-fed females will therefore lead to decreased chances for successful copulation, as sterilization reduces the competitiveness of males (El Gazzar and Dame, 1983) that must compete for mates with their wild counterparts (Oliva et al., 2012). In addition, this study provided information regarding the sexual ecology of *Ae. aegypti* to gain insight into the order of precedence between blood feeding and mating in *Aedes* mosquitoes. More importantly, the results suggest that failure of any program involving the mass release of sterile blood-feeding insects would also be related to the permanent presence of blood feeding opportunities in the area of the target insect species. It is important for any SIT program targeting a blood-feeding insect to carefully remove or reduce the prevalence of potential animal hosts, as these can favor blood meal uptake, thereby reducing the number of mate-seeking females and increasing mating competition between released males and their wild counterparts.

Conflict of interest

We (author) declare no conflict of interest.

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