Factors Associated with Male Mating Success of the Dengue Vector Mosquito, *Aedes aegypti*

Alongkot Ponlawat and Laura C. Harrington*

Department of Entomology, Cornell University, Ithaca, New York

**Abstract.** We studied the effects of male *Aedes aegypti* age, body size, and density on mating success under laboratory and field conditions. Older males under field conditions transferred the greatest number of sperm to females (1,152 by 1-day-old males to 1,892 sperm by 10-day-old males). Larger males inseminated females with more sperm than smaller ones. Male age, female body size, and density also influenced male mating success. Larger females successfully mated with males more often than smaller females, especially with older males (> 25 days old). Female insemination rates in small high-density laboratory cages (0.009 m³) were artificially high (81.6–98.7%) compared with rates (65.4–84.6%) in large, low-density field cages (9 m³). This is the first study to systematically evaluate the effect of *Ae. aegypti* male body size and age on sperm transfer to females and the first one to evaluate the mating performance of males in a field setting.

**INTRODUCTION**

Dengue fever and dengue hemorrhagic fever are the most important arthropod-borne viral diseases of humans that infect millions of people in tropical and subtropical countries annually. Dengue is transmitted to humans by the *Aedes aegypti* mosquito, which is the principle vector of dengue viruses and one of the most medically important mosquitoes worldwide. Current, the only effective way to prevent dengue fever and dengue hemorrhagic fever is through vector control. The use of sterile or genetically modified male releases into dengue-endemic areas is a technique to control mosquitoes and reduce dengue, yet this approach relies on a sound knowledge of mosquito mating biology. Unfortunately, little information is available about the mating biology of mosquitoes especially in natural settings.

Instead of forming traditional station keeping swarms, male *Ae. aegypti* tend to aggregate around hosts who represent the primary female encounter site. Males grasp females in flight as they approach or leave hosts after blood feeding. Mating is initiated when the male grasps the female and orients himself so he is venter-to-venter with his mate. *Aedes aegypti* complete copulation in an average of 10 seconds; with a range of 9–31 seconds. The duration of copulation seems to be shorter in field populations than with laboratory-adapted strains (unpublished data). During copulation, sperm and seminal fluid are rapidly transferred from the male into the female’s bursa copulatrix. Seminal fluid of male *Ae. aegypti* contains a large number of proteins that are transferred to females during mating and may have important influences on female biology and behavior.

There are many physical traits that influence mating biology and reproductive success of insects and other animals. Body size is a well-documented factor influencing mating success for both males and females in many Dipterans including fruit flies, *Drosophila* spp., and the Mediterranean fruit fly, *Ceratitis capitata*. Larger male *Drosophila melanogaster* and *D. pseudoobscura* inseminated virgin females more often than smaller ones, and a negative relationship between male *D. melanogaster* body size and female egg production has been reported. In *C. capitata*, the number of sperm stored in females was positively related with female body size. The advantage of body size is also well known for female mosquitoes, where body size is proportional to gamete number. Previously, we showed a similar trend of body size and sperm number for male *Ae. aegypti*. Larger male *An. freeborni* collected from California mated more often than smaller males, based on the morphology of male accessory glands and testes. In other species, such as *Ae. cantans*, the success of reproduction and survival of larger females was higher than small ones. In addition, caged laboratory observations with male *An. gambiae* indicate that they prefer to mate with large females when given a choice. In contrast, studies of *An. gambiae* and *An. funestus* in the wild did not report a male size preference for larger females.

In addition to body size, male age is known to play an important role in insect reproduction. The mating success of the oblique-banded leafroller, *Choristoneura rosaceana*, increased for males up to 3 days old and then declined. Copulated male crickets (*Gryllus veletis* and *G. pennsylvanicus*) collected from the field were significantly older than solitary males. In the tsetse fly, sexual receptivity also is related to fly age. Older *Glossina pallidipes* males copulated more frequently than younger ones. In radiation-sterilized Mediterranean fruit flies, the number of sperm stored in mated females decreased with increasing age of their male mates, suggesting that older males transferred fewer sperm.

In addition to the effect of body size on sperm capacity, we recently showed that male age had a significant impact on the total number of sperm in *Ae. aegypti*. Sperm numbers in virgin males increased up to 10 days after eclosion and decreased at ages beyond 20 days at 25 ± 3°C. Beyond basic descriptions of mating biology, the factors influencing mosquito mating success in mosquitoes from dengue-endemic areas have rarely been studied.

Our goal in this study was to address this gap in our knowledge by examining two key components of mating success. Although mating success could involve many different factors, for the purposes of this study, we define male mating success as the number of gametes transferred to females during mating and the number of females inseminated. We tested the hypothesis that the number of sperm transferred to females and the number of females inseminated is influenced by male age, female body size, and density (i.e., degree of crowding). We began examining these effects in the laboratory and studied them in the field with wild captured mosquitoes to determine whether artificial laboratory conditions may influence our results. In addition to building on our knowledge base of mating biology for the dengue vector mosquito, information on optimal physical traits and environmental conditions may
improve the efficacy of vector control programs that rely on release of genetically modified or sterile males.

MATERIALS AND METHODS

Mosquito strains. A laboratory colony of *Ae. aegypti* (Thai wild strain) was established from mosquitoes originally collected from Nakhon Ratchasima Province (15°05′N, 101°54′E), Thailand from May to August 2006 and had undergone approximately four generations from field collections. This mosquito strain was maintained in an environmental chamber set with a temperature range of 22–30°C (8 degree-days) and 80% relative humidity (RH) with a photoperiod of 14-hour light: 10-hour dark. The light phase began and ended with a 2-hour period of dawn and dusk.

Wild mosquitoes collected as pupae directly from the field were used for experiments conducted from May 2006 to May 2007. Pupae were collected from dengue endemic villages in Ban Nong Suang Pattana (15°05′N, 101°54′E), Nong Suang subdistrict, Kham Thale So District, Nakhon Ratchasima Province, a region located ~260 km northeast of Bangkok, Thailand. Permission to conduct the research in selected villages was obtained, and local meetings with villagers and health clinic officers were conducted before beginning the study. *Ae. aegypti* pupae were collected from water storage jars, cement basins, and other breeding sites. Pupae were separately distributed into vials (15 mL) containing 3 mL of water from the breeding sites and plugged with cotton wool to prevent escape of emerged adults. After eclosion, adults were removed from vials using a mouth aspirator, anesthetized on wet ice, and identified to species using morphological characteristics according to Rattanarithikul and Panthusiri. Effect of crowding and female body size on male mating success. In this experiment, we examined the effect of male age, female body size, and crowding on male mating success in the field. We used different size cages (small bucket cages [0.009 m³] and large screened untreated bed net cages [9 m³]) to determine whether crowding under artificial laboratory holding conditions was one factor influencing our previous results.

Effect of crowding and female body size on male mating success in small (0.009 m³) bucket cages. Medium-sized virgin *Ae. aegypti* males 1, 5, and 10 days after emergence and different body size *Ae. aegypti* females (large and small) at 3 days after emergence (F₁ generation) were used in this experiment. During each replicate, five virgin males of the same age group were released into a small cage (0.009 m³) covered with a mesh lid with 20 virgin females (10 females per body size group) and held together 24 hours. Sugar solution was provided inside the cage during the experiment. After the 24-hour period, all mosquitoes were removed from the cage using a mouth aspirator and anesthetized on wet ice. Wing measurements were used to determine mosquito body size. Right wings of each specimen were removed using dissecting forceps, placed on adhesive over glass slides, and microscopically measured. Spermathecae from each female were dissected using small insect pins and fine forceps on a glass slide. The spermathecae were covered and cracked with a gentle tap on the cover-slip. Each specimen was examined carefully for the presence of sperm under a phase-contrast microscope. More than 20 replicate cages were used.

Effect of crowding on mating success in large screened cages (9 m³). During each replicate, five medium-sized virgin males of the same age group (1, 5, and 10 days old) were released into a large screened bed net cage (225 × 200 × 200 cm: 9 m³) inside a vacant house in the field site with 20 virgin females (10 females per body size group) 3 days of age and cohabited for 24 hours. Sugar solution was provided inside the cage during the experiment. After 24 hours, all mosquitoes were removed from the cage using a small modified vacuum aspirator (Black & Decker, Towson, MD) and anesthetized on ice. The right wing was removed as described above as a measure...
of body size. Female spermathecae and bursa were dissected and examined for the presence of sperm as described above.

**Effect of male age on female insemination rate. Laboratory conditions.** In each replicate, 20 medium-sized virgin females (3 days old) were placed in a cage (0.009 m³) with 5 virgin males (1 and 5 days old) from the large body size group. They were held together for 24 hours. Eighteen replicates using the same number of females and males at the same ages were conducted inside large screened cages as described above. Sucrose solution-soaked cotton balls were provided. All mosquitoes in the experimental cage were removed by a mouth aspirator and knocked down on wet ice. Spermathecae were dissected as described above. Spermathecae were put into a drop of PBS, covered with glass coverslips, and examined under a phase-contrast microscope for the presence of sperm.

**Field conditions.** Twenty medium-sized virgin females (3 days old) from the field colony (F₁ generation) were put into a cage as described above using a mouth aspirator. Five large-sized males from the same age group (1, 5, and 10 days old) were released into the cage. Sucrose solution was provided during conduction of the experiments. After 24 hours, all mosquitoes in the cage were removed by a mouth aspirator and immediately anesthetized on wet ice. Wings were measured, and spermathecae were examined as described above.

**Statistical analysis.** Data for each experiment were tested for conformation to the assumptions of normality and homoscedasticity (Shapiro-Wilk test). The difference in total sperm transferred to females among male age and group size was analyzed using general linear models (GLMs) and Tukey honestly significant difference (HSD) with MINITAB software (Minitab, State College, PA). A generalized estimating equation (GEE)²⁴ (SAS/STAT; SAS Institute, Cary, NC) was used to test the null hypothesis that there was no difference in the number of large and small females inseminated among males of different ages and crowding conditions. Relationships between percent insemination and physical traits, male age and female body size, were examined using GLMs (Minitab).

**RESULTS**

**Number of sperm transferred to females.** For the Thai laboratory colony, the average number of sperm transferred to females ranged from 1,262 by 5-day-old males to 1,655 by 10-day-old males (Table 1). A significant difference in the total number of sperm transferred to females by different age (1, 5, and 10 days) and body size (large versus small) males was detected (F = 2.42, P = 0.04). Large 10-day-old males inseminated and transferred the greatest number of sperm to females (Table 1). In contrast, no differences in age after controlling for body size were observed. The number of transferred sperm per millimeter male wing length among 1-, 5-, and 10-day-old males was not significantly different (F = 1.66, P = 0.151). In addition, no significant differences in sperm transferred were found by male body size (large versus small) within the same age group (P > 0.05).

Our field experiments confirmed results from the laboratory. The number of sperm transferred by 10-day-old males was significantly higher than 1- and 5-day-old males (F = 11.01, P < 0.001). Average number of sperm transferred to females ranged from 1,154 by 1-day-old males to 1,892 by 10-day-old males. In addition, the number of sperm transferred per millimeter male wing length by 10-day-old males was higher than 1- and 5-day-old males (F = 6.49, P = 0.002; Table 1).

Compared with laboratory experiments, sperm transferred per millimeter male wing length by wild 10-day-old males was higher than those transferred by 10-day-old males under laboratory conditions (Table 1). No significant differences were detected in number of transferred sperm per millimeter male wing length between field and laboratory colony males at 1 and 5 days old (P > 0.05).

**Effect of male age, female body size, and crowding on mating success.** We measured mating success by comparing the number of females inseminated per male in our study. Overall, 1,295 females were dissected. We detected a significant effect of male age (P = 0.0039), female body size (P < 0.0001), and adult crowding (P < 0.0001) on mating success for the Thai wild strain. In addition, a significant interaction effect of male age and female body size on insemination rate also was observed (P = 0.0035). Larger females tended to mate with older males (> 25 days old).

**Effect of crowding on mating success in small (0.009 m³) cages.** Insemination in small cages occurred more frequently than in the large ones (P < 0.0001) when overall number of mating pairs was kept the same. We observed that the very young male *Ae. aegypti* (1 day old) mated with small females (90.0%) more often than with large females (81.6%; F = 6.72, P = 0.013; Figure 1). In contrast, both 5- and 10-day-old males mated with large females more often than small females (98.7% versus 87.8%, F = 20.96, P < 0.001 for 5-day-old males; 91.5% versus 83.0%, F = 6.14, P = 0.018 for 10-day-old males, respectively; Figure 1).

**Effect of crowding on mating success in large screened cages (9 m³).** Figure 2 presents the significant effect of female body size on male mating success in large cages. In all male age groups (1, 5, and 10 days old), large females were inseminated more often than small females (90.0% vs. 81.6%; P = 0.013).

**Table 1**

<table>
<thead>
<tr>
<th>Male age</th>
<th>Male wing length ± SE (mm)</th>
<th>N</th>
<th>Range of sperm transferred (mean)</th>
<th>Range of sperm/mm male wing length (mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (field/small)</td>
<td>1.72 ± 0.02</td>
<td>35</td>
<td>467–1,237 (1,154)</td>
<td>267–1,381 (673)</td>
</tr>
<tr>
<td>1 (laboratory/large)</td>
<td>2.15 ± 0.01</td>
<td>20</td>
<td>525–2,185 (1,349)</td>
<td>250–1,040 (628)</td>
</tr>
<tr>
<td>1 (laboratory/small)</td>
<td>1.77 ± 0.01</td>
<td>18</td>
<td>459–1,899 (1,373)</td>
<td>255–1,117 (799)</td>
</tr>
<tr>
<td>5 (field/small)</td>
<td>2.04 ± 0.01</td>
<td>35</td>
<td>350–2,264 (1,289)</td>
<td>167–1,132 (635)</td>
</tr>
<tr>
<td>5 (laboratory/large)</td>
<td>2.27 ± 0.01</td>
<td>20</td>
<td>1,045–2,250 (1,489)</td>
<td>454–1,978 (657)</td>
</tr>
<tr>
<td>5 (laboratory/small)</td>
<td>2.00 ± 0.02</td>
<td>21</td>
<td>821–2,011 (1,262)</td>
<td>391–970 (633)</td>
</tr>
<tr>
<td>10 (field/small)</td>
<td>2.06 ± 0.01</td>
<td>23</td>
<td>390–4,267 (1,892)</td>
<td>186–2,032 (917)</td>
</tr>
<tr>
<td>10 (laboratory/large)</td>
<td>2.31 ± 0.01</td>
<td>20</td>
<td>1,083–2,234 (1,655)</td>
<td>492–931 (712)</td>
</tr>
<tr>
<td>10 (laboratory/small)</td>
<td>2.07 ± 0.01</td>
<td>21</td>
<td>989–4,356 (1,357)</td>
<td>470–1,126 (655)</td>
</tr>
</tbody>
</table>

Numbers followed by the same letter are not significantly different from each other (P > 0.05; ANOVA. LS separation of means).
more often than small ones \( (P < 0.001) \). Percentages of insemination of 1-, 5-, and 10-day-old male insemination with large females were 82.0\%, 81.5\%, and 84.6\%, respectively. Small females were inseminated less by 1-, 5-, and 10-day-old males in the large screened cages with 65.4\%, 71.0\%, and 73.6\%, respectively (Figure 2).

**Effect of male age on female insemination rate.** \textit{Laboratory conditions}. Male age (between 1 and 5 days old) had an effect on insemination rate under laboratory conditions. From 15 replicates, 74.2 \( \pm \) 5.9\% and 89.0 \( \pm \) 2.08\% of cohabited medium size females (2.2 \( \pm \) 0.01-mm wing length) mated with 1- and 5-day-old males (2.33 \( \pm \) 0.01-mm wing length), respectively (Table 2).

**Field conditions.** From 25 replicates, there was no significant difference in insemination rates by large 1-, 5-, and 10-day-old males (2.32 \( \pm \) 0.01-mm wing length; \( P > 0.05 \)). Percentage insemination rate of medium-sized females (2.2 \( \pm \) 0.01-mm wing length) mated by large 1-, 5-, and 10-day-old males were 87.5 \( \pm \) 4.01, 89.5 \( \pm \) 2.83, and 96.7 \( \pm \) 1.67, respectively (Table 2). In addition, an effect of rearing conditions (laboratory versus field) on insemination rate was detected. Insemination rate of wild 1-day-old males was greater than their laboratory-reared counterparts \( (P < 0.05) \).

**DISCUSSION**

In this study, we examined the effect of male age, body size, and rearing conditions on two measures of male mating success: the number of sperm transferred to females (as potential mating capacity) and the total number of females inseminated. Based on laboratory results, a significant difference in the total number of sperm transferred to females by different age and body size males was detected. Large 10-day-old males inseminated and transferred the greatest number of sperm to females. These results support those from our earlier study, showing that older and larger virgin \textit{Ae. aegypti} males \( (> 10 \text{ days old}) \) produce and store more sperm in their reproductive organs than younger males. We also detected an effect of male age on number of sperm transferred to females during mating in wild \textit{Ae. aegypti}. These results indicate that older and larger \textit{Ae. aegypti} males have a greater competitive advantage over other males. They produce and store more sperm in their reproductive tracts, can transfer the greatest number of sperm to females, and can mate with the largest number of females per unit time. Some 1-day-old males may not have fully completed terminus rotation when they were initially placed in cartons with females, potentially explaining the trend toward low insemination rates for these males. Further studies to determine whether mating with large males translates to greater lifetime reproductive success for females will be important and are currently underway. In addition to the effects of male age and body size, crowding conditions and ambient environmental factors play important roles in insemination success. In previous studies, we found that environmental conditions in the field affected male sperm capacity resulting in greater numbers of sperm stored in the reproductive organs of older males. It is not clear what conditions in the field provide optimal male fitness, but better nutrients and more ideal temperature or humidity may influence reproductive success.

Based on our observations, the number of sperm transferred to females potentially depends on males completing copulation. Successful mating without interruption probably increases the quantity of seminal complements transferred as well as sperm. Artificially high crowding, such as conditions experienced in some laboratory cages, probably reduce the number of transferred sperm through interruption of copulation by jostling of competing or close-flying males. Furthermore, incomplete transferring of sperm and seminal fluid may lead to multiple inseminations. In its natural range in Thailand, the density of adult \textit{Ae. aegypti} is low, which may increase mating time and the frequency of complete copulations. A high frequency of successful and complete matings may lead to greater monandry in \textit{Ae. aegypti}. One potential problem with genetically modified or sterile males is re-mating of females with wild-type males if a full complement of sperm is not transferred. This suggests that re-mating with wild-type males is a serious problem with genetically modified or sterile males. Artificial insemination, the technique of transferring sperm to females artificially, may be an alternative method to prevent this problem.
or seminal fluid is not transferred from genetically modified or sterile males. Based on theoretical models, the impact of female re-matings with wild males may not be a detriment unless the frequency is high.26 Our study provides a baseline for comparing and screening viability of modified Ae. aegypti. However, the fitness traits we examined are not an exclusive list. Other factors that may influence male mating success are male effects on female fecundity and survival, levels of nutrient reserves, adult nutrition, predator avoidance, and mate finding in the field. In addition, future studies should address the role of male physical and behavioral traits on the number of viable offspring produced.

Female body size is deemed to be a major physical factor affecting female mating success. Our results showed that larger virgin females were inseminated by all age group males more frequently than smaller ones. The mechanism for this apparent preference of male mosquitoes for larger females is not known. It is also not clear if smaller females suffer fitness costs or if the cost is reduced if they are eventually mated in nature. Mature males may use visual cues, female flight tones, and volatile pheromones to find larger females for copulation and insemination.27 Alternatively females may be the selecting sex. Although we observed males grasping females in flight, females may ultimately decide to accept or reject copulation with that male during the pre- or postcopulatory stage. To date, there is no evidence for the presence of female choice in the Ae. aegypti mating system, but we cannot rule out this possibility.

In our study, we compared our results under laboratory and field conditions with a low-generation laboratory colony and wild mosquitoes collected as pupae directly from the field. Our results show that laboratory conditions can affect mating behaviors of Ae. aegypti. Although it is not always practical to run replicates, controlled studies of mosquito mating biology in the field, we encourage researchers to validate results from laboratory studies in the field whenever possible. Laboratory colony mosquitoes may behaviorally adapt to mating under artificially high densities within the confines of small cages.

In conclusion, these results suggest that older and larger males as well as larger females of the dengue vector, Ae. aegypti, have the greatest mating success. Density and ambient environmental conditions are influential factors of mating biology of dengue vector mosquitoes. Further studies of wild populations in dengue-endemic areas under field conditions are clearly needed to understand the natural mating behavior of Ae. aegypti. Knowledge of mosquito mating behavior and optimal physical traits is highly relevant to dengue vector control, especially to improve efficiency of genetically modified or sterile male programs.

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Authors’ addresses: Alongkot Ponlawat, Department of Entomology, Cornell University, 3132 Comstock Hall, Ithaca, NY 14853, Tel: 607-255-7040, Fax: 607-255-0939, E-mail: ap285@cornell.edu. Laura C. Harrington, Department of Entomology, Cornell University, 3138 Comstock Hall, Ithaca, NY 14853, Tel: 607-255-4475, Fax: 607-255-0939, E-mail: lch27@cornell.edu.

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