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Evidence of Polyandry for *Aedes aegypti* in Semifield Enclosures

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Abstract. Female *Aedes aegypti* are assumed to be primarily monandrous (i.e., mate only once in their lifetime), but true estimates of mating frequency have not been determined outside the laboratory. To assess polyandry in *Ae. aegypti* with first-generation progeny from wild mosquitoes, stable isotope semen-labeled males (^{15}N or ^{13}C) were allowed to mate with unlabeled females in semifield enclosures (22.5 m^3) in a dengue-endemic area in southern Mexico. On average, 14% of females were positive for both labels, indicating that they received semen from more than one male. Our results provide evidence of a small but potentially significant rate of multiple mating within a 48-hour period and provide an approach for future open-field studies of polyandry in this species. Polyandry has implications for understanding mosquito ecology, evolution, and reproductive behavior as well as genetic strategies for mosquito control.

INTRODUCTION

Aedes aegypti is a principle vector of yellow fever, Chikungunya, and dengue viruses. Recently, there has been a resurgence of dengue worldwide, which has been attributed to urbanization, globalization, and lack of effective vector control.[1–3] With no vaccine or clinical treatment commercially available,[4,5] novel genetic control techniques provide promising tools to reduce vector populations and pathogen transmission.[6–11] These genetic approaches require a thorough knowledge of the mating behavior of field populations,[12] but little is known about the mating behavior of *Ae. aegypti* in nature. Contrary to anopheline mosquitoes that usually mate in large outdoor crepuscular swarms,[13,14] *Ae. aegypti* typically mates indoors close to its human host at low densities.[15,16] Mating is initiated in flight and lasts on average between 10 and 30 s.[17]

Polyandry (i.e., to mate with more than one male) is a common mating strategy for many female insects.[18] In the majority of medically important mosquitoes, however, it is generally assumed that females are monandrous (i.e., mate only one time in their lifetime).[16,17] This assumption has not been assessed in field populations of *Ae. aegypti*. A number of experiments conducted with phenotypically distinct inbred strains or using other characteristics of mating (i.e., extended bursa) indicated that female *Ae. aegypti* engaged in multiple mating in the laboratory. Polyandry was observed after mating with semen-depleted males[19] or over multiple egg-laying cycles.[20] However, laboratory conditions and densities used in these experiments might have resulted in an overestimation of polyandry.[21] Despite some evidence for polyandry, results from other laboratory-based experiments[22–25] strengthened the belief that monandry is the rule for *Ae. aegypti*. Consequently, for several decades, there was little incentive to study the ecological and evolutionary complexities of multiple mating by this species. Here, to determine if polyandry in *Ae. aegypti* can be observed outside the laboratory, we conducted experiments in semifield enclosures (22.5 m^3)[26] subject to ambient conditions such as temperature, humidity, and lighting.

Observations on polyandry in field populations of *Ae. aegypti* require the use of techniques to determine paternity. Genetic markers have been successfully used in some mosquito vectors of malaria parasites to define low rates of polyandry in wild swarming *Anopheles freeborni*[27] and *An. gambiae*.[28] Use of genetic markers for determining paternity in *Ae. aegypti* has been more challenging because of the low abundance of microsatellites[29] and the presence of multilocus microsatellite families linked with transposable elements,[30] although some microsatellite markers have been identified.[29,31] Genetic markers are currently being used in at least one study to examine polyandry in field-collected *Ae. aegypti* (Scott T, unpublished data). In this study, we apply a stable isotope semen-labeling technique to determine polyandry.[32–34] Mosquitoes used in the experiments were from an F_1 generation of field-collected insects to minimize inbreeding or laboratory adaptation effects. Small or large body-sized females, representing the range that we see in nature, were used to determine the role of body size on polyandry rate. Insects were introduced at two densities (i.e., 2.7 or 5.3 insects per m^3) to determine the role of density on multiple mating frequency.

METHODS

Mosquitoes. *Ae. aegypti* mosquitoes used in this study were an F_1 generation derived from approximately 600 fourth instar larvae and pupae collected from larval habitats close to Tapachula, Mexico (14°54′ N, 92°15′ W). Mosquitoes were reared and maintained in an insectary (27°C and 70% relative humidity [RH]) before introduction in the field enclosure. Adult mosquitoes were continuously supplied with a 10% sucrose solution.

Stable isotope labeling. Mosquitoes were labeled in the larval stage with ^{15}N-glycine (NLM-202-1; Cambridge Isotope Laboratories Inc., Andover, MA) or ^{13}C-glucose (CLM-1396-1) using procedures described in the work by Helinski and others.[32–34] Eggs were vacuum-hatched, and 200 first instar larvae were counted and placed in a tray with 1 L sterile water to obtain medium body-sized males. Larvae were fed a fixed diet, and amounts of 38, 75, 113, 150, and 113 mg diet, consisting of a 1:1 mixture of lactalbumin and yeast, were added to trays on days 1, 3, 4, 5, and 6, respectively. The stable isotope label was added to the larval water on the same day as the first instar larvae were introduced for the ^{15}N-labeled

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trays, and each tray received 41.1 mg label (i.e., 15% enrichment based on nitrogen content in diet). For the ^{13}C-labeled trays, the stable isotope label was added for several days to prevent excess bacterial growth. A solution containing 144.6 mg in 50 mL water was made (i.e., 25% enrichment based on carbon content in diet), and 3.9, 7.7, 11.5, and 26.8 mL were added on days 1, 3, 4, and 5, respectively. Two to four replicate rearing trays with larvae were established for each stable isotope label per experiment. In addition, one tray without label was used to obtain unlabeled males for the control treatment. Pupae were collected daily. Males were maintained in 30-cm^3 cages as virgins (i.e., sexing was done based on pupal size and checked within 12 hours after emergence) until the start of experiments. Larval survival was normal for all trays except one ^{15}N-labeled tray in replicate 4 for unknown reasons.

Females. To obtain small and large unlabeled females for replicate 1, larvae were reared at a density of 500 larvae in 3 L water and fed either a low (0.25 mg/larvae) or high diet (0.83 mg/larvae) of ground fish food (Microbites; Mascotas y Acuariofilia, Ecatepec, Mexico). In all other replicates, small and large females were obtained using the same lactalbumin: yeast diet and feeding schedule as above for males with varying density. Large females were reared as 75 larvae in 1 L water; small females were obtained by rearing 750 larvae in 1 L water. Large and small females in each replicate were significantly different in size (*t* test, rep 1: $t = 25.8$, degrees of freedom (df) = 97, $P < 0.01$; rep 2: $t = 31.1$, df = 72, $P < 0.01$; rep 3: $t = 25.2$, df = 36, $P < 0.01$; rep 4: $t = 29.0$, df = 52, $P < 0.01$). Females in rep 4 were significantly larger than females in the other replicates, but differences were small (large females: $\chi^2 = 28.42$, df = 2, $P < 0.01$, reps 1–3: 2.93 ± 0.10 (standard deviation) mm, rep 4: 3.03 ± 0.06 mm; small females: $\chi^2 = 49.51$, df = 2, $P < 0.01$, reps 1–3: 2.27 ± 0.11 mm, rep 4: 2.48 ± 0.08 mm). Females were maintained as virgins (i.e., sexing was done based on pupal size and checked within 12 hours after emergence) until the start of experiments. None of the virgin females dissected as controls (see below) were positive for sperm, indicating that this method was effective in assuring virginity.

Experimental setup. The experimental protocol was reviewed and approved by the Instituto Nacional de Salud Pública (INSP) committees of ethics, biosecurity, and research. In addition, representatives of the community situated in close proximity to the field enclosures were informed about the study and gave collective consent. Experiments were performed in field enclosures ($2.5 \times 5 \times 1.8$ m)[26] located close to the village of Rio Florido, approximately 15 km from Tapachula, Mexico (14°54′ N, 92°15′ W). Hobo data loggers (Onset, Bourne MA) were used to record hourly temperature and RH inside the enclosures. The two enclosures were almost identical in ambient conditions during the experiments (*t* test, temperature: $t = -0.37$, df = 1,074, $P > 0.05$; RH: $t = -0.40$, df = 1,074, $P > 0.05$). Four identical resting sites consisting of black plastic buckets (23 L) with wet black cloths inside and one black cloth covering the majority of the bucket entrance were set up in each enclosure. Four replicates of the experiment were performed. Replicates 1 and 2 (September of 2009) were performed with male mosquitoes from the same cohort during the rainy season (average = 2.1 m rainfall/year). Replicates 3 (December of 2009) and 4 (January of 2010) were performed during the dry season (average = 0.2 m rainfall/year). Replicate 3 was performed under cooler conditions (22.4 ± 4.5°C [SD]) than replicates 1, 2 (28.0 ± 4.4°C), and 4 (25.6 ± 5.4°C). RH was 82.3 ± 15.8 (SD) for replicates 1 and 2, 85.7 ± 14.6 for replicate 3, and 76.6 ± 17.2 for replicate 4. In each replicate, two field enclosures, A and B, were used (Table 1). Insects were introduced at high- (120 insects [5.3 insects/m3]: 30 ^{15}N-labeled males, 30 ^{13}C-labeled males, 30 small females, and 30 large females) or low-density treatments (60 insects [2.7 insects/m3]: 15 ^{15}N-labeled males, 15 ^{13}C-labeled males, 15 small females, and 15 large females). Each density treatment was replicated three to five times

TABLE 1
Percentage of females from field enclosures positive for ^{13}C, ^{15}N, both labels, or no label

Enclosure	Density	♀ Size	^{13}C	^{15}N	G-test* (*P* value)	Both labels	No label†	*N*
Rep 1‡								
A	60 males × 60 females	Small	60.0	36.7	3.73 (0.05)	3.3	0.0	30
		Large	57.7	26.9		15.4	0.0	26
B	60 males × 60 females	Small	34.8	60.9	1.00 (0.32)	0.0	4.3	23
		Large	41.7	45.8		12.5	0.0	24
Rep 2‡								
A	30 males × 30 females	Small	26.7	60.0	0.93 (0.33)	0.0	13.3	15
		Large	46.2	46.2		7.7	0.0	13
B	60 males × 60 females	Small	55.6	29.6	3.03 (0.08)	7.4	7.4	27
		Large	57.9	31.6		10.5	0.0	19
Rep 3								
A	30 males × 30 females	Small	75.0	25.0	0.07 (0.80)	0.0	0.0	4
		Large	36.4	45.5		9.1	9.1	11
B	30 males × 30 females	Small	27.3	36.4	0.20 (0.65)	9.1	27.3	11
		Large	53.8	30.8		0.0	15.4	13
Rep 4								
A	30 males × 30 females	Small	30.8	53.8	0.03 (0.86)	7.7	7.7	13
		Large	50.0	21.4		28.6	0.0	14
B	30 males × 30 females	Small	23.1	76.9	0.04 (0.85)	0.0	0.0	13
		Large	66.7	26.7		0.0	6.7	15

*G test results compare the number of females inseminated by ^{13}C- and ^{15}N-labeled males (i.e., the number of females with both were added to the overall count for each male type), and data from small and large females were combined for each replicate and enclosure.
†Of these results, five samples were false negatives; all other samples were negative for sperm as determined by microscopy before sample analysis (Table 4).
‡Males used were from the same cohort.
N is the number of females analyzed.

TABLE 2
Recovery and insemination data of mosquitoes from field enclosures

	Percent recovery						Percent insemination (N)	
	Labeled males			Females			Females	
Enclosure	^{13}C	^{15}N	Age	Small	Large	Age	Small	Large
Rep 1								
A	87	100*	3–4	100*	90*	3–7	100 (30)	100 (26)
B	93	93		74*	81*		96 (23)	100 (24)
Rep 2								
A	80	73	7–8	94*	81*	3–6	100 (15)	100 (13)
B	77	77		87*	65*		96 (27)	100 (19)
Rep 3								
A	87	100	2–3	33	80	2	100 (4)	91 (11)
B	100	100		73	87		73 (11)	85 (13)
Rep 4								
A	100*	87	3–6	87	93	4–6	92 (13)	100 (14)
B	67	87		87	100		100 (13)	100 (15)

*One too many introduced.
Percent of live mosquitoes recovered and females inseminated. Insemination was determined by microscopy observing sperm in spermathecae. *N* is the number of mosquitoes dissected. Not all females recovered were dissected because of accidental escapees or dissection error. Age in days of males and females used at start of experiment is indicated.

(Table 1). Ages of the mosquitoes introduced were between 2 and 8 days for males and 2 and 7 days for females (Table 2).

All mosquitoes were dusted with different colors of fluorescent dusts (Day-Glo Color Corp., Cleveland, OH) to determine survival. Colors were alternated between labeled males and small and large females for the various replicates to exclude labeling color bias. Mosquitoes were transported to the field site and introduced either in the morning (8:00–10:00 AM in reps 1, 2, and 4) or late afternoon (5:00–6:00 PM in rep 3). Before release, sucrose (10%) and water on cotton were added to both enclosures. The next day, one or two people stood close to the enclosure for 45 minutes to provide mating stimuli (i.e., *Ae. aegypti* typically mates close to its human host),[15,16] and they observed ample mating activity in the enclosures. The following morning (after 40–48 hours of confinement), mosquitoes were removed from the enclosures with backpack aspirators. Mosquitoes were transported to the insectary on ice to prevent any mating in the collection cup. On arrival, males were checked for color and counted, and a wing was removed to estimate size; females were dissected. No significant differences were observed in body size of males labeled with either stable isotope in the majority of replicates (*t* test, reps 1 and 2: $t = 0.75$, df = 171, $P > 0.05$; rep 4: $t = -0.83$, df = 48, $P > 0.05$). In rep 3, ^{15}N-labeled males were significantly larger than ^{13}C-labeled males ($t = 3.67$, df = 53, $P < 0.01$), although differences were small (i.e., 2.22 ± 0.07 [SD] mm ^{13}C-labeled males and 2.28 ± 0.05 for ^{15}N-labeled males).

For each replicate, control treatments were set up in the insectary consisting of 20 males of each stable isotope label with 10 large and 10 small females in 5-L cages. In addition, 20 unlabeled males were mated with 10 large and 10 small females.

Sample preparation. After collection, spermathecae (sperm storage organs) were prepared for sample analysis in the mass spectrometer. Briefly, females were dissected in 1 × PBS (phosphate buffered saline), and their spermathecae were checked for sperm under 100 × magnification by compound microscopy. A wing was taken from each female to determine size. Spermathecae from an individual female were transferred to a small piece of quartz paper using a fine brush and placed in a tin cup. Tools were cleaned with ethanol after every dissection to prevent contamination. A spike solution (i.e., consisting of sucrose [for C] and ammonium sulphate [for N]) was added to each sample to attain sufficient nitrogen and carbon to be above the detection limit of the isotope ratio mass spectrometer.[32,33] In replicates 1 and 2, an error with the spike solution led to an elevated spiking of the samples with carbon, and each sample received 30 μg N and 50 μg C. In subsequent experiments, samples were spiked with the correct amount of 30 μg N and 37.5 μg C. Samples were dried at 55°C, and cups were closed. Blank samples consisting of a tin cup with quartz paper and the spike solution were added after every five samples as internal controls. Control samples from females mated to males in the insectary were also dissected and prepared as above. In addition, spermathecae from virgin females from the same batch of females used in field enclosure experiments were included. Samples were analyzed at the stable isotope facility at the University of California at Davis.

Data analysis and interpretation. The raw δ-values of the spiked samples were used for data analysis. These values are referenced to the international standards for nitrogen (i.e., AIR) and carbon (i.e., Vienna Pee Dee Belemite). To determine if a spermatheca was inseminated by a ^{15}N- or ^{13}C-labeled male, conservative threshold values consisting of three standard deviations above a mean control value were determined.[33,35] Because samples from each experiment were run in separate batches over time and absolute values are not constant, for each replicate, separate threshold values were determined and applied to samples in that run. In replicates 3 and 4, the threshold value for ^{15}N was based on three standard deviations above the mean $\delta^{15}N$ value obtained for spermathecae inseminated by ^{13}C-labeled control males. Vice versa, the threshold for ^{13}C was based on the mean $\delta^{13}C$ values obtained for spermathecae inseminated by ^{15}N-labeled control males. In replicates 1 and 2, a large number of control samples were lost during sample analysis because of initial erroneous spiking; therefore, the threshold value was determined based on three standard deviations above the mean values observed for spermathecae inseminated by unlabeled males. Overall, the threshold values applied resulted in 98% (170/173) of control samples classified correctly (Table 3). There were some spermathecae from the field enclosure experiments that could not be classified by mass spectrometry, although they were positive for sperm as determined by microscopy (false negatives) (Table 4), but their number was low. Likewise, one false positive sample (i.e., spermathecae identified as not containing sperm by microscopy but after sample analysis, positive for one label) was identified. This finding was likely the result of a microscopy error.

TABLE 3
Overview of correctly classified samples from control experiments

	Percent correctly classified control samples (*N*)				
	Spermathecae inseminated by				
Rep	^{13}C ♂	^{15}N ♂	Unlabeled ♂	Virgin ♀	Blanks
1, 2	100 (5)	100 (4)	100 (7)	100 (10)	96 (46)
3	100 (10)	89 (9)	100 (10)	100 (5)	100 (14)
4	100 (10)	100 (10)	100 (10)	100 (5)	100 (18)

Percentage of correctly classified control samples of spermathecae from females inseminated by ^{13}C, ^{15}N, or unlabeled control males in the laboratory, virgin females, and blank samples (i.e., consisting of the tin cup with quartz paper and spike only). The number of samples analyzed is in parentheses.

TABLE 4
Overview of correctly and incorrectly classified samples from field enclosure experiments

	Correctly classified		Incorrectly classified		
Rep	Positive	Negative	False positive	False negative*	*N*
1	98.1 (101)	0.0 (0)	1.0 (1)	1.0 (1)	103
2	94.6 (70)	1.4 (1)	0.0 (0)	4.1 (3)	74
3	84.6 (33)	15.4 (6)	0.0 (0)	0.0 (0)	39
4	96.4 (53)	1.8 (1)	0.0 (0)	1.8 (1)	55

*Four of five samples classified as false negatives were from small females.

Percentage of correctly and incorrectly classified spermathecae from females from the field enclosure experiments. Correctly classified positive samples were from females observed to be inseminated by microscopy and classified as containing label by threshold values. Correctly classified negative samples were from females identified by microscopy as uninseminated and classified by threshold values as having no label. False positive samples were spermathecae identified as not containing sperm by microscopy, but after sample analysis, they were positive for one label. False negative samples were spermathecae positive for sperm from which we did not detect a label. The number of samples analyzed is in parentheses.

Nonetheless, > 95% of experimental samples were classified correctly according to their insemination status (Table 4). The stable isotope technique used here permits only for the identification of polyandry if a female mates with both types of labeled males. It is assumed that multiple mating also occurs in undetected cases (i.e., C and C, N and N), and thus, the remating rate estimate is approximately twofold higher.

Data were analyzed with SPSS (version 18; SPSS Inc., Chicago, IL), and a P value less than 0.05 was considered significant. Transformation of recovery and insemination data did not result in normality, and data were analyzed using non-parametric tests (Kruskal–Wallis or Mann–Whitney U). Multiple mating frequencies were normally distributed and analyzed with general linear models with replicate, density, and size as fixed factors. The number of females (small and large combined) inseminated by either ^{15}N- or ^{13}C-labeled males in each replicate and enclosure were analyzed with G-goodness of fit tests (G tests).[36] Spermathecae positive for both labels were added to the number of females inseminated by either male. A pooled G test was performed, because the replicates were not significantly different as determined by the heterogeneity G value ($P > 0.05$).[36]

RESULTS

Mass spectrometry analysis of female spermathecae confirmed that males labeled in the larval stage with the stable isotopes ^{13}C or ^{15}N carried the label through in their semen in sufficient quantities to allow detection in spermathecae of individual mated females (Table 3). When insects mated freely in the field enclosure for 40–48 hours, on average, 7 ± 5% (SD) of females were positive for both ^{13}C and ^{15}N isotopes, indicative of multiple mating activity(Table 1). Assuming that multiple mating also occurs in undetected cases, our remating rate is a conservative estimate of approximately 14%. Similar levels of polyandry were observed at high (i.e., 5.3 insects/m^3) or low insect density (2.7 insects/m^3; $F_{1,6} = 0.30$, $P > 0.05$) (Table 1). There were no significant differences in female polyandry rates between replicates ($F_{3,6} = 0.21$, $P > 0.05$) or between large and small body-sized females ($F_{1,6} = 1.51$, $P > 0.05$). In addition, there were no significant differences in the mating ability of ^{13}C- or ^{15}N-labeled males to inseminate females (pooled G test = 1.16, df = 1, $P > 0.05$) (Table 1 has individual results).

The percentages of males and females recovered alive after 40–48 hours were similar for both sexes ($\chi^2 = 1.12$, df = 3, $P > 0.05$), and the average was 85 ± 14% (SD). Only small females in replicate 3 in one enclosure (A) had a reduced survival (i.e., 33%) (Table 2). The large majority of females (73–100%) recovered from the field enclosures were positive for sperm (Table 2). No significant differences were observed in insemination rates between small and large females ($U = 26.0$, df = 1, $P > 0.05$) or between females of different ages ($\chi^2 = 7.60$, df = 3, $P > 0.05$).

DISCUSSION

We have shown that a significant proportion (i.e., 14%) of female *Ae. aegypti* will engage in multiple matings when tested in large outdoor enclosures under ambient conditions within a 48-hour period. We examined rates of polyandry by mosquito density (i.e., 2.7 or 5.3 insects per m^3) and female body size. We found no differences in multiple mating frequency among the two mosquito densities tested, and we did not find differences for females based on large or small body size.

In this study, a combination of semifield enclosures and stable isotope semen labeling to examine female multiple mating frequency was used. Performing research in semifield enclosures offers a unique opportunity to study insects under ambient conditions. Conditions in the enclosures were suitable for mosquito survival and mating in this experiment, and continuous populations have been maintained in these enclosures for weeks.[26] The colonization and rearing of mosquitoes in the laboratory potentially modifies insect behavior, and for this reason, we used an F_1 generation of mosquitoes derived from local field-collected larvae and pupae. Given the brief exposure of our experimental mosquitoes to laboratory conditions, it seems unlikely that major behavioral differences occurred. However, our findings may not be representative of free-ranging mosquitoes, and confirmation of our multiple mating findings with field-collected females that were unrestricted in movement and behavior are still warranted. The frequency of polyandry in *Ae. aegypti* was higher than observations for some field-collected anopheline mosquitoes (i.e., around 3%),[27,28] but members of this genus have distinctly different mating biology than *Ae. aegypti*.[16]

Stable isotope labeling of *Ae. aegypti* larvae resulted in a semen marker that could be reliably detected in spermathecae using mass spectrometry, which was previously shown in *An. arabiensis*.[32–34] Both types of labeled males obtained similar numbers of mates, suggesting that there was no effect of either label on mating performance. Similarly, ^{13}C labeling of *An. arabiensis* mosquitoes did not impact on their mating ability.[33] Thus, stable isotopes can provide an easy and safe method to test for paternity in insects where genetic markers are difficult to obtain or apply. Natural levels of stable isotopes also can be used to study mating and oviposition processes.[37] In our study, stable isotope labeling of semen (i.e., sperm and seminal fluid proteins) was used to infer polyandry. Although we have no evidence that males of any Dipteran species, including mosquitoes, would transfer seminal fluid without sperm, future studies should confirm the presence of sperm from both males in dual matings. This approach can only be accomplished with genetic markers.

However, microsatellites commonly used for this purpose are challenging to use in *Ae. aegypti*.[29,31]

The mosquito densities used in this study represent the high range of natural densities detected in endemic areas (Harrington L, et al., unpublished data from Thailand, Mexico, and Puerto Rico)[38,39]; therefore, it is possible that density-related effects may occur at lower densities. Although our enclosures represented ambient light, humidity, and temperature, mosquitoes were still contained within them, and this enclosure may have led to higher multiple mating frequencies than in a field setting where mosquitoes are not physically confined, despite the short flight range of this species.[40] Females have been observed, however, to successfully resist male mating attempts in the laboratory during flight (Harrington L, et al., unpublished data).[41] Different-sized females were reared by varying larval density or food, and although there may be other physiological differences between the size cohorts, we found no differences in the proportion of females positive for both labels in our study.

Previous studies reporting monandrous behavior in *Ae. aegypti* showed that it is induced after injection of whole or partially purified male accessory gland homogenates in the female, suggesting that molecules transferred in the ejaculate cause monandry.[22,25,42] The time from injection to induction of monandrous behavior was not clear from these studies, because females were allowed to recover for 24 hours post-injection. In *Drosophila*, a small peptide known as the sex peptide has been implicated to temporarily reduce female sexual receptivity after mating.[43,44] It is not known which specific seminal fluid peptide or protein (Acps) is associated with monandry in *Ae. aegypti*. Recent studies have been undertaken to identify the suite of Acps transferred by *Ae. aegypti* males to females during mating,[45,46] which will enable the functional characterization of individual proteins. Despite the observed refractory state of *Ae. aegypti* females injected with male accessory gland extracts described above, polyandry has been shown for *Ae. aegypti* in laboratory studies in small cages, although rarely. Gwadz and Craig[19] observed multiple mating at a low frequency (i.e., 7.5%) when *Ae. aegypti* females were exposed simultaneously to males of several genotypes. This finding suggests that the effect of Acps in inducing monandry might not be immediate, which could have accounted for our findings. Williams and Berger[20] detected multiple mating after more than four gonotrophic cycles; however, females in our experiment could not engage in blood feeding or oviposition behavior. Gwadz and Craig[19] showed that *Ae. aegypti* females mated with semen-depleted males or females engaging in an interrupted mating remated nearly 100% of the time. It is unlikely that semen depletion led to the observed rates of polyandry observed here. Our previous work has shown that three to four consecutive matings are required before sperm depletion occurs in a single male.[47]

In many other female insects, polyandry is a common mating strategy, where females can increase lifetime offspring production with moderate multiple mating rates.[18] Whether *Ae. aegypti* females that remate benefit similarly remains to be determined. If benefits are present, sexual conflict over female mating rate is expected to occur.[18,48,49] Interestingly, *Ae. aegypti* females have greater capacity for sperm storage and manipulation than *Anopheles* mosquitoes, because they have three spermathecae compared with one in anophelines. In polyandrous insects, multiple sperm storage organs can be used to exercise cryptic female choice if a female mates with more than one male.[16,50] Future studies that record the number of spermathecae positive for sperm or analyze individual spermatheca to determine how sperm of two males is allocated would provide more information on whether *Ae. aegypti* females use cryptic choice mechanisms. In addition, sperm usage patterns of polyandrous females can be studied to determine to what extend the second male's sperm is used to fertilize eggs. Data from previous studies that determined polyandry using genetically marked males show that sperm of the second male is used in insemination, because mixed offspring are observed,[19,51] but the exact mechanisms are not clear.

Ae. aegypti is the main vector of three important human arboviruses. With few alternatives other than traditional vector control, to reduce disease burden, genetic control strategies are being devised and evaluated for *Ae. aegypti*.[6–11,52,53] The data gathered here will inform genetic control programs to optimize the spread of desired genotypes. Contrary to earlier interpretations,[54] monogamy of females is not a prerequisite for effective genetic control strategies.[55] Released males, however, do need to be able to successfully compete with wild males for mates for the technology to have the desired outcome, and if polyandry is common, it potentially could drive up costs for sterile insect technique (SIT) programs, because more males need to be released.

Our results from semifield enclosures confirm the few laboratory findings of polyandry and challenge the long-standing belief that all *Ae. aegypti* females mate only once in their lifetime. Our results add to the growing body of research indicating that mating biology of mosquitoes is considerably more complex than previously thought.[56,57] Understanding female mating behavior will improve our understanding of the spread of epidemiologically important phenotypes (i.e., vector competence and insecticide resistance) and highlights the needs and opportunities for research on the ecology and evolution of mosquito reproductive behavior.

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