Spermathecal Filling in *Aedes aegypti* and *Aedes albopictus*: Effects of Female and Male Body Sizes and Species

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Subject Editor: Theodore Andreadis

Received 1 May 2018; Editorial decision 14 August 2018

Abstract

The mosquitoes *Aedes aegypti* (L.) (Diptera: Culicidae) and *Aedes albopictus* (Skuse) (Diptera: Culicidae) are ecologically similar species that have evolved independently in their native ranges, *Ae. aegypti* in Africa and *Ae. albopictus* in Asia. Where their invasive ranges overlap, interspecific mating is facilitated by both species swarming to mate around bloodmeal hosts during daylight. Here, we test hypotheses to account for variation in spermathecal filling in females of *Ae. aegypti* and *Ae. albopictus*, particularly, the effect of mosquito size on the number of spermathecae containing sperm after intraspecific and interspecific matings of these two species. Results show that significantly more spermathecae contained sperm in large than in small *Ae. albopictus* females, but there was no effect of *Ae. aegypti* female body size on the average number of spermathecae filled. Among inseminated females, there was no effect of male size or cross-type on number of spermathecae with sperm. Differences in the effect of female size on the number of spermathecae that store sperm in *Ae. aegypti* and *Ae. albopictus* imply differences in the mating biology of these two species.

Key words: spermathecae, sperm storage, mating, size

In many animal species that have internal fertilization, with the exception of most mammals, females may store sperm, transferred by males during mating, for prolonged periods of time (Pitnick et al. 1999). Stored sperm are held internally in the females' reproductive tract, where they are kept viable, and are released when fertilization of the ovum or egg takes place (Davey 1965, Birkhead and Moller 1992, Degner and Harrington 2016a). Different organs are used to store sperm in some or most of the species of the following groups: the uterus, the uterotubal junction, and the oviduct in bats (Racey 1979); the sperm storage tubules in birds and reptiles (Fox 1963, Birkhead and Moller 1992); the spermathecae in some arthropods (Gleson 1990) and amphibians (Sever et al. 2004); and the seminal receptacle in other arthropods (Waddy and Aiken 1990, Pitnick et al. 1999).

Females of most insects store and maintain sperm internally in the spermathecae (Gullan and Cranston 2010, Simmons 2001). Most insect species have a single spermatheca (Gullan and Cranston 2010, McAlpine et al. 1981), but the number of spermathecae varies from one to three (McAlpine et al. 1981). One exceptional insect

species, the earwig *Diplatys macrocephalus* (Palisot de Beauvois) (Dermaptera: Diplatidae), has 10 spermathecae (Popham 1965).

In different mosquito species, the number of spermathecae varies from one to three (Downes 1968, Clements, 1999). The genera *Anopheles*, *Uranotaenia*, *Aedeomyia*, some *Ficalbia*, and certain species of *Aedes* have a single spermatheca, while in the genus *Mansonia*, of the subgenus *Mansonioides*, one or two of the three spermathecae may be very small and difficult to detect. The genus *Hodgesia* has three, of which the third may be minute. The genera *Culex*, *Toxorhynchites*, and most aedine mosquitoes have three spermathecae (Edwards 1941, Neveu-Lemaire 1902). *Aedes aegypti* (L.) (Diptera: Culicidae) and *Aedes albopictus* (Skuse) (Diptera: Culicidae) have one large, medial and two smaller, lateral spermathecae (Clements 1999). Although mosquitoes are generally considered to be monandrous (Clements 1999), some mosquito species have spermathecal features associated with polyandry and others do not (Yuval 2006). Multiple spermathecae have been associated with cryptic female choice in polyandrous insects, allowing females to differentially utilize sperm of alternative mates (Eberhard 1996,

Hellriegel and Ward 1998, Yuval 2006). The influence of females on the process of sperm storage has been studied in yellow dung flies *Scathophaga stercoraria* (L.) (Diptera: Scathophagidae) that are polyandrous and have a singlet and a doublet spermatheca. In single matings, the number of spermathecae with sperm is influenced by male size and amount of stored sperm (Ward 1993). Double-mated females are able to differentially store sperm of their mates. Sperm storage may be influenced by female's body size, the size of the second male, and the relative sizes of the two male mates (Ward 1993), suggesting that multiple spermathecae allow females to have a better control of offspring paternity (Ward 1993, 1998; Otronen et al. 1997; Hellriegel and Bernasconi 2000). However, other authors argue that nonrandom paternity in yellow dung flies is explained solely by sperm competition (Simmons et al. 1996).

In the mosquitoes *Ae. aegypti* and *Ae. albopictus*, there is scanty information about spermathecal filling. Dissections of mated females of both species reveal variation in numbers of capsules with sperm, usually two or three. Studies on spermathecal filling by Jones and Wheeler (1965) with *Ae. aegypti* of the Bangkok strain reared in an insectary and allowed to copulate freely in cages, reported that 8% of the females stored sperm in two spermathecae and 92% in all three spermathecae. For an *Ae. albopictus* colony from Rimini, Italy, reared under laboratory conditions, it has been reported that females stored sperm mainly in two spermathecae (Oliva et al. 2013). Among *Ae. aegypti* and *Ae. albopictus* colonized from the southern United States, most mated females contained sperm in two spermathecae, except for a few *Ae. albopictus* that had sperm in three (De Jesus & Reiskind, 2016). Among wild-caught specimens from Florida, Tripet et al. (2011) found that most *Ae. aegypti* and *Ae. albopictus* females had sperm in two spermathecae. These authors reported that some interspecific matings, which produced no progeny, between these two species in Florida showed evidence of multiple inseminations. Molecular analysis of sperm in spermathecae of these wild-caught females showed either mixed sperm of both species in one spermatheca or segregation of conspecific and heterospecific sperm in different spermathecae (Tripet et al. 2011).

Aedes aegypti and *Ae. albopictus* are ecologically similar species (Murrell et al. 2011), that have evolved independently (Leahy and Craig 1967). They belong to the subgenus *Stegomyia*, and were originally confined to different tropical and subtropical regions of the Old World. *Aedes aegypti* belongs to the *Aegypti* Group native to the Afrotropical Region (Huang 2004), while *Ae. albopictus* is part of the *Scutellaris* Group, originally from Southeast Asia and Pacific Islands (Huang 1972). Differences in the mating biology of *Ae. aegypti* and *Ae. albopictus* were detected by De Jesus and Reiskind (2016). In intraspecific matings, more sperm were stored in spermathecae of *Ae. albopictus* compared with *Ae. aegypti*, and fecundity increases were observed in *Ae. albopictus* females, but not in *Ae. aegypti*, that mated with larger males. Differences were not found between *Ae. aegypti* and *Ae. albopictus* females that mated with large or small males in the number of sperm stored in spermathecae. However, in *Ae. aegypti*, the number of sperm in spermathecae declined in gonotrophic cycles three and four. Using females reared in a moderate density larval environment, it was shown that female size and number of sperm stored in *Ae. aegypti* or *Ae. albopictus* spermathecae were not correlated (De Jesus and Reiskind 2016).

Postcopulatory female control of sperm storage has been implied in mosquitoes, as sperm may arrive by their own motility from the female bursa to the spermathecal vestibule, and then it is likely that they are transported through the spermathecal ducts by actions of the female (Linley 1981, Linley and Simmons 1981, Clements 1999,

Degner and Harrington 2016a). On the other hand, male influence on female behavior and physiology can be pronounced, especially through the postcopulatory actions of male accessory gland substances, which differ between these species (Boes et al. 2014) and have different effects after interspecific matings (Tripet et al. 2011). We examined the influence of mosquito body size on spermathecal usage in *Ae. aegypti* and *Ae. albopictus*, after intraspecific and interspecific matings in light of the evidence of different numbers of spermathecae used by these species and differential effects of their male accessory gland products in cross-matings. As heterologous male accessory gland proteins stimulate oviposition (Leahy and Craig 1967), and refractoriness to further mating (Tripet et al. 2011) in *Ae. aegypti* females, but not in *Ae. albopictus* females, and larger *Ae. albopictus* males may transfer more seminal fluid substances during mating and induce in females more pronounced postmating responses (De Jesus and Reiskind 2016), we hypothesized that *Ae. aegypti* and *Ae. albopictus* females inseminated by large *Ae. albopictus* males would have more spermathecae containing sperm than crosses involving *Ae. aegypti* males. Our experimental design allowed us to quantify how body size affects insemination rates, as well as the variations observed in filling the second and third spermathecae, in these two mosquito species.

Materials and Methods

Aedes aegypti were F₁₀–F₁₁ progeny from collections in Key West, Florida. *Aedes albopictus* were F₁₁–F₁₂ progeny from Vero Beach, Florida. Mosquitoes were maintained in two insectaries at the Florida Medical Entomology Laboratory one at 26.78 ± 0.87°C, 89.18 ± 9.57% RH, and the other at 26.99 ± 0.28°C, 80.72 ± 4.67% RH, and both illuminated with a 14L:10D day length.

Eggs were hatched under vacuum and first instar larvae were placed in a tray with 1 l of water (Barbosa and Peters 1969). To produce differences in adult body size, larvae were reared in groups of different numbers (75 per tray for large mosquitoes and 500 for small mosquitoes). Food (1:1 yeast: lactalbumin) was added to each tray in the following schedule: 75, 38, 75, 113, 150 mg on day: 1, 3, 4, 5, and 6, respectively (Ponlawat and Harrington 2007). Pupae were sexed based on morphological differences of terminalia and put in emergence containers with no more than 20 individuals, in order to confirm the sex of adults before setting up the cage crosses. If a mistake in sexing was detected, the container was discarded. Intraspecific and interspecific crosses were set up, exposing large or small *Ae. aegypti* or *Ae. albopictus* females to large or small conspecific or heterospecific males. The following crosses were performed: large females × large males, large females × small males, small females × large males, small females × small males. As two intraspecific crosses (*Ae. aegypti* females × *Ae. aegypti* males, and *Ae. albopictus* females × *Ae. albopictus* males) and two interspecific crosses (*Ae. aegypti* females × *Ae. albopictus* males, and *Ae. albopictus* females × *Ae. aegypti* males) were set up, in total 16 different combinations were performed. Each cross combination was replicated in three sequential repetitions. In each cross, 150 three-d-old females were caged with 150 three-d-old males for 3 wk in Bug Dorm cages (30 cm³). Sugar solution (10% sucrose) was constantly provided.

Females were dissected 3 wk after exposure to males. Females were anesthetized with chloroform and spermathecal dissections were performed in water immediately thereafter under a dissecting microscope. Spermathecae were observed under a phase contrast microscope. To see sperm moving inside the spermathecae, no more than three females were anesthetized and dissected at the same time,

as sperm stop moving a few minutes after dissection. Observation of sperm movement helped to assess if a spermatheca contained sperm, particularly when few were present. The insemination status of each female was recorded. A female was considered inseminated if she had sperm in any of the three spermathecae. For inseminated females, the number of spermathecae that were positive for sperm (1, 2, or 3), and their location: central (large), and/or one or two of the lateral (smaller) spermathecae were recorded.

Female and male mosquitoes from the same developmental cohorts, that were not used for the cage crosses, were dried in aluminum cups for 24 h at 55°C in an oven (Fisher Isotemp, Asheville, NC) and weighed individually on an Orion Cahn C-33 microbalance (Thermo Scientific, Waltham, MA).

Statistical Analysis

A logistic linear model using Proc GLIMMIX (SAS 2011) was used to analyze the effects of cross type (*Ae. aegypti* ♀ × *Ae. albopictus* ♂, *Ae. albopictus* ♀ × *Ae. aegypti* ♂), female size (large, small), and male size (large, small) on the proportions of inseminated females. When significant effects were found, follow-up analyses were conducted by post hoc Tukey–Kramer comparisons method.

The average number of spermathecae with sperm in inseminated females was analyzed as a full factorial linear fixed-effects model with three fixed categorical effects, cross-type, female size, and male size using Proc GLIMMIX (SAS 2011). Separate analyses were run for the *Ae. aegypti* female crosses (*Ae. aegypti* ♀ × *Ae. aegypti* ♂, *Ae. aegypti* ♀ × *Ae. albopictus* ♂) and for *Ae. albopictus* female crosses (*Ae. albopictus* ♀ × *Ae. albopictus* ♂, *Ae. albopictus* ♀ × *Ae. aegypti* ♂) to test for species-specific responses. When significant effects were found, follow-up analyses were conducted by post hoc Tukey comparisons. Variations among treatments in mosquito dried body mass were analyzed with analysis of variance (ANOVA) using SPSS Statistics (SPSS 2007).

Results

Mosquito Mass

ANOVA detected significant variation in dried body mass between small and large females ($F_{3, 185} = 162.57$, $P < 0.0001$) and between small and large males ($F_{3, 195} = 43.21$, $P < 0.0001$) (Tables 1 and 2).

Table 1. Average dried body mass of large and small *Ae. aegypti* and *Ae. albopictus* females and males

	Females (mg ± SD)	n	Males (mg ± SD)	n
Large <i>Ae. aegypti</i>	1.505 ± 0.304	45	0.587 ± 0.102	45
Large <i>Ae. albopictus</i>	1.451 ± 0.246	45	0.578 ± 0.138	45
Small <i>Ae. aegypti</i>	0.742 ± 0.168	50	0.391 ± 0.088	54
Small <i>Ae. albopictus</i>	0.745 ± 0.178	49	0.431 ± 0.099	55

Table 2. P values after Tukey multiple comparisons of dried body mass

Females		Males	
Pair comparisons	P	Pair comparisons	P
Large vs. small <i>Ae. aegypti</i> ♀	<0.0001	Large vs. small <i>Ae. aegypti</i> ♂	<0.0001
Large vs. small <i>Ae. albopictus</i> ♀	<0.0001	Large vs. small <i>Ae. albopictus</i> ♂	<0.0001
Large <i>Ae. aegypti</i> ♀ vs. small <i>Ae. albopictus</i> ♀	<0.0001	Large <i>Ae. aegypti</i> ♂ vs. small <i>Ae. albopictus</i> ♂	<0.0001
Small <i>Ae. aegypti</i> ♀ vs. large <i>Ae. albopictus</i> ♀	<0.0001	Small <i>Ae. aegypti</i> ♂ vs. large <i>Ae. albopictus</i> ♂	<0.0001
Large <i>Ae. aegypti</i> ♀ vs. large <i>Ae. albopictus</i> ♀	0.678	Large <i>Ae. aegypti</i> ♂ vs. large <i>Ae. albopictus</i> ♂	0.981
Small <i>Ae. aegypti</i> ♀ vs. small <i>Ae. albopictus</i> ♀	1.000	Small <i>Ae. aegypti</i> ♂ vs. small <i>Ae. albopictus</i> ♂	0.205

Tukey post hoc comparisons showed that interspecific comparisons of mean body masses between individuals of the same size class and sex were not significant (Table 2).

Insemination Rate

For the analysis of insemination rates, we only included the interspecific crosses (*Ae. aegypti* ♀ × *Ae. albopictus* ♂, and *Ae. albopictus* ♀ × *Ae. aegypti* ♂), as in the intraspecific crosses (*Ae. aegypti* ♀ × *Ae. aegypti* ♂, and *Ae. albopictus* ♀ × *Ae. albopictus* ♂) proportions of inseminated females were above 0.99. A significant three-way interaction was found between type of mating (*Ae. aegypti* ♀ × *Ae. albopictus* ♂ and *Ae. albopictus* ♀ × *Ae. aegypti* ♂), female size, and male size (Table 3). Owing to the much higher insemination rates in interspecific crosses of *Ae. aegypti* females, there was a much broader range in mean proportions inseminated compared to the converse crosses involving *Ae. albopictus* females (Fig. 1). The highest insemination rate of *Ae. aegypti* females was observed in the cross of large individuals of both sexes, but this pattern did not apply to *Ae. albopictus* females, where the large × large cross did not produce a significantly different mean proportion inseminated from other crosses. Most, but not all, interspecific insemination rates were significantly higher in crosses between *Ae. aegypti* females and *Ae. albopictus* males. There were no significant differences in insemination rates between the cross *Ae. aegypti* small female × *Ae. albopictus* large male and the cross *Ae. albopictus* small female × *Ae. aegypti* large male (Fig. 1).

Spermathecal Use

Sperm location

Overall, 1,801 females had sperm in three spermathecae, 1,865 had sperm in two spermathecae, and 53 had sperm in one spermatheca. In females containing sperm in two spermathecae, the sperm were detected in the central and in one of the lateral spermathecae in 99.95%, and in the two lateral spermathecae in 0.05% of the females. In the mosquitoes that had sperm in one spermatheca, sperm were found in the central spermatheca in 94.33% of the females, and in one of the lateral spermathecae in 5.66% of the individuals.

Average number of spermathecae used

***Aedes aegypti* female crosses:** In intraspecific matings, the average number of spermathecae used was 2.43, and in matings between *Ae. aegypti* females and *Ae. albopictus* males the average number of spermathecae used was 2.36. No significant differences were found between cross type (*Ae. aegypti* ♀ × *Ae. aegypti* ♂ and *Ae. aegypti* ♀ × *Ae. albopictus* ♂), female size, or male size (Fig. 2). Interactions were not significant (Table 4).

***Aedes albopictus* female crosses:** In intraspecific matings, the average number of spermathecae used was 2.57 and in matings between *Ae. albopictus* females and *Ae. aegypti* males the average number of spermathecae used was 2.56. There was a significant effect of female size, as larger females had a higher mean number of spermathecae filled (2.67) than smaller (2.46) counterparts. No significant differences were found between cross type (*Ae. albopictus* ♀ × *Ae. albopictus* ♂ and *Ae. albopictus* ♀ × *Ae. aegypti* ♂), or male size (Fig. 2). Interactions were not significant (Table 5).

Discussion

Analysis of the proportion of inseminated females revealed an interaction between type of mating (*Ae. aegypti* females × *Ae. albopictus* males, *Ae. albopictus* females × *Ae. aegypti* males), female size, and male size. Interspecific insemination was higher in most of the crosses between *Ae. aegypti* females and *Ae. albopictus* males compared with crosses between *Ae. albopictus* females and *Ae. aegypti* males, as has been shown in previous studies that did not sort mosquitoes by size (Bargielowski et al. 2013, Nasci et al. 1989). In our study, the cross *Ae. aegypti* large female × *Ae. albopictus* large male had the highest insemination rate among interspecific pairings, and three of the *Ae. albopictus* female × *Ae. aegypti* male crosses (large female × large male, large female × small male, small female × small male) had the lowest insemination rates. However, not all interspecific insemination rates were significantly higher between *Ae. aegypti*

females and *Ae. albopictus* males. Significant differences were not found between the cross *Ae. aegypti* small female × *Ae. albopictus* large male and the cross *Ae. albopictus* small female × *Ae. aegypti* large male.

The analysis of the average number of spermathecae used in crosses with *Ae. albopictus* females showed that larger females had more spermathecae containing sperm compared with smaller ones, and that in crosses with *Ae. aegypti* females, there was no effect of female body size on the average number of spermathecae positive for sperm. De Jesus and Reiskind (2016) found no correlation between female size and number of stored sperm for either *Ae. aegypti* and *Ae. albopictus*. However, they did not discriminate between large and small females, and produced various sized females by rearing larvae in intermediate crowding conditions. In our study, female size significantly affected the number of spermathecae with sperm in *Ae. albopictus*, but not in *Ae. aegypti*, suggesting that female body size plays a different role in the two species, determining whether two or three spermathecae are filled in mated females. This implies differences in the mating biology of these two species.

We did not see an effect of male size on the number of spermathecae containing sperm. In *Ae. aegypti*, the independence of the size of the male and its effects on postmating behaviors of the female has been shown by Dickinson and Klowden (1997). In their experiments, small males reared on a low-quality diet had less protein in their male accessory glands and transferred less protein during mating compared to large males reared on a high-quality diet. However, both large and small males induced female refractoriness to further mating. In a different experiment, it was shown that in *Ae. aegypti*, the effect on female postmating behavior was modulated by the nutritional state of the adult male. Starved males had less total protein in their male accessory glands, transferred fewer proteins during mating, and were less efficient than sugar-fed males at inhibiting the host-seeking behavior of the gravid females that they mated (Fernández and Klowden 1995). De Jesus and Reiskind (2016), found an effect of male body size on fecundity of *Ae. albopictus*, but not of *Ae. aegypti*. These authors suggested that larger *Ae. albopictus* males transferred more seminal fluid products to females. In our experiment, we generated large and small mosquitoes, rearing the larvae under different crowding conditions, using the same type of food. In future experiments, it would be interesting to generate

Table 3. Effect of cross-type (*Ae. aegypti* ♀ × *Ae. albopictus* ♂, and *Ae. albopictus* ♀ × *Ae. aegypti* ♂), male and female size on the proportion of inseminated females

Effect	Num DF	Den DF	F value	P
Cross	1	16	247.18	<0.0001
Female size	1	16	29.66	<0.0001
Cross* female size	1	16	19.69	0.0004
Male size	1	16	19.65	0.0004
Cross* male size	1	16	4.38	0.0525
Female size * male size	1	16	0.43	0.5191
Cross* female size * male size	1	16	29.11	<0.0001

Fig. 1. Mean proportion of inseminated females in eight interspecific crosses. Same letter indicates no significant difference (Tukey-Kramer for Cross*Female size * Male size. Alpha = 0.05).

Fig. 2. Average number of spermathecae containing sperm. Numbers of females with spermathecae positive for sperm in each cross are shown above the error bars.

Table 4. Effect of cross-type (*Ae. aegypti* ♀ × *Ae. aegypti* ♂, and *Ae. aegypti* ♀ × *Ae. albopictus* ♂), male and female size on the average number of spermathecae containing sperm

Effect	Num DF	Den DF	F value	P
Cross	1	16	1.22	0.2855
Female size	1	16	0.17	0.6861
Cross * female size	1	16	0.02	0.9018
Male size	1	16	0.07	0.7977
Cross * male size	1	16	0.17	0.6848
Female size * male size	1	16	0.00	0.9589
Cross * female size * male size	1	16	0.71	0.4124

Table 5. Effect of cross-type (*Ae. albopictus* ♀ × *Ae. albopictus* ♂, and *Ae. albopictus* ♀ × *Ae. aegypti* ♂), male and female size on the average number of spermathecae containing sperm

Effect	Num DF	Den DF	F value	P
Cross	1	16	0.02	0.8856
Female size	1	16	6.02	0.0260
Cross * female size	1	16	1.83	0.1953
Male size	1	16	0.25	0.6242
Cross * male size	1	16	0.35	0.5646
Female size * male size	1	16	0.01	0.9173
Cross * female size * male size	1	16	0.97	0.3405

large and small individuals, also altering the quality of the larval food (Dickinson and Klowden 1997), and to vary the nutritional state of the adult male (Fernández and Klowden 1995) in order to better resolve the effect of male quality on spermathecal filling in *Ae. aegypti* and *Ae. albopictus*.

In our experiments, there was no evidence of an effect of cross-type on the number of spermathecae containing sperm. It has been shown that injections of heterologous male accessory gland substances influence the switch to postmating behavior in *Ae. aegypti* females, but not in *Ae. albopictus*, in the same way that conspecific

male accessory gland substances trigger oviposition (Leahy and Craig 1967) and refractoriness to mating (Tripet et al. 2011), so other postmating processes that are influenced by male accessory gland proteins could have a similar response in interspecific crosses between *Ae. aegypti* females and *Ae. albopictus* males. However, in some *Ae. aegypti* females exposed to *Ae. albopictus* males, sperm storage does not occur as male accessory gland products and sperm may be transferred to the female bursa, but not to the spermathecae (Carrasquilla and Lounibos 2015), and some observers of interspecific matings have detected dead or immobilized sperm in spermathecae (Leahy and Craig 1967, Nasci et al. 1989). Our results for heterospecific crosses between *Ae. albopictus* females and *Ae. aegypti* males show that in the relatively few females that are inseminated (10.52%), patterns of sperm storage are similar to those found in intraspecific matings. In crosses between *Ae. albopictus* females and *Ae. aegypti* males, the presence of dead sperm in spermathecae has also been reported, but not as frequently as in interspecific crosses between *Ae. aegypti* females and *Ae. albopictus* males (Leahy and Craig 1967). Even though we were not studying sperm viability when we were assessing if spermathecae had sperm, we could see sperm moving. Thus in both interspecific crosses, females that are inseminated have similar patterns of sperm storage and maintenance compared to conspecific matings. However, further studies will be necessary to understand the specific mechanisms behind this process. In intraspecific matings, it is broadly known that sperm storage is influenced by sperm locomotion and active transport in the female reproductive tract (Clements 1999, Degner and Harrington 2016a). Male accessory gland substances transferred during mating may play a role in sperm localization in the spermathecae and sperm storage, as it has been shown in anopheline mosquitoes (Rogers et al. 2009) and *Drosophila* (Avila and Wolfner 2009, Ram and Wolfner 2009, Avila et al. 2011).

In *Ae. aegypti*, after copulation, following deposition of sperm into the female's bursa, sperm reach the spermathecal vestibule. Sperm motility may be important to lead cells to the vestibule. However, sperm are carried through the spermathecal ducts into the spermathecal capsules by action of the female (Linley and Simmons

1981, Clements 1999). It has been postulated that in *Ae. aegypti*, following deposition of semen in the female bursa, sperm may reach the spermathecal vestibule by their own motility. However, it is likely that they are carried through the spermathecal ducts into the spermathecal capsules by action of the female (Linley and Simmons 1981, Clements 1999). Sperm are delivered to the bursa and are not transferred to the spermathecae until after the female and the male have separated (Spielman 1964). After mating, sperm are rapidly stored in the spermathecae, where they remain viable (Christophers 1960). Spermathecal filling in *Ae. aegypti* and *Ae. albopictus* is completed 5 or 6 min after copulation (Spielman 1964, Jones and Wheeler 1965, Oliva et al. 2013). Sperm that stay in the bursa and are not transferred to the spermathecae lose their motility by the second day after copulation (Spielman 1964). In the bursa, coagulation of the semen occurs, and 48 h after mating, only small remnant materials are observed (Oliva et al. 2013).

In order to better understand the influence of the female on spermathecal usage in *Ae. aegypti* and *Ae. albopictus*, it would be interesting to observe patterns of spermathecal filling, right after mating, and some time after mating in females that have been kept awake and in mosquitoes that have been anesthetized. In yellow dung flies *S. stercoraria*, which are polyandrous, Ward (1998) found that in individuals that were dissected immediately after first copulation, larger females had more sperm in their spermathecae than smaller ones. In females that were anaesthetized for 30 min, larger females had even more sperm in the spermathecae, showing that sperm were able to move on their own in the female reproductive tract. However, if females were kept awake for 30 min before dissection, the relationship was inverse, and larger females had less sperm in their spermathecae than smaller ones (Ward 1998). This author proposed that larger females may be more selective than smaller ones, about the particular sperm that they store.

Mosquitoes are generally monandrous (Craig 1967, Clements 1999). However, under some circumstances, polyandry may occur, particularly if a second mating takes place immediately following the first insemination (Craig 1967). Helinski et al. (2012) conducted a semifield study labeling males with stable isotopes and reported that around 14% of *Ae. aegypti* females re-mate. Richardson et al. (2015) using microsatellite loci detected in a natural population of *Ae. aegypti* that out of 48 families, three showed evidence of multiple paternity. Degner and Harrington (2016b) reported that for *Ae. aegypti* the frequency of polyandry in cages ranged from 24% immediately after mating to 3% at 20 h after mating. For *Ae. albopictus*, through observations of the bursa, it has been reported that 11% of the females mated twice in an interval of less than 40 min (Oliva et al. 2013). The highest frequency of polyandry, 26% was reported in a wild population of *Ae. albopictus* on the island of La Reunion using microsatellite markers (Boyer et al. 2012). In our experiments, it is likely that polyandry did occur but we cannot assess how female re-mating may affect sperm storage. However, the similarities in frequencies of polyandry in previous studies of these two species (see above) lead us to speculate that our conclusions about the influences of body size are not compromised by any polyandry that occurred in our experiments.

Our results show that larger *Ae. albopictus* females have more spermathecae containing sperm compared with smaller females, and that there is no effect of *Ae. aegypti* female body size on the average number of spermathecae used. We did not find an effect of male size or cross-type. The different effect that female size has on spermathecal usage in *Ae. aegypti* and *Ae. albopictus* provides more evidence that there are differences in the mating biology of these two species. Our experiments are limited by the fact that they were performed under laboratory conditions, therefore comparison with mosquitoes from the field remains desirable.

Acknowledgments

We are very grateful to Jorge Rey, Dan Hahn, and Jane Brockman for advice during the development of this research. We acknowledge James Colee for his valuable help with statistical analyses, and Steve Juliano for statistical advice. We thank Naoya Nishimura for technical support and Tom Swan for his assistance in the lab. This research was supported by National Institutes of Health Grant R21 AI095780 (to L.P.L.) and a Colciencias (Colombia) graduate fellowship (to M.C.C.).

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