Age and Body Size Influence Sperm Quantity in Male Aedes albopictus (Diptera: Culicidae) Mosquitoes

A. J. Hatala, L. C. Harrington, and E. C. Degner

Department of Entomology, Comstock Hall, Cornell University, Ithaca, NY 14853, and 1Corresponding author, e-mail: ecd77@cornell.edu

Subject Editor: Patricia Scaraffia

Received 20 December 2017; Editorial decision 17 February 2018

Abstract

Aedes albopictus (Skuse) (Diptera: Culicidae) is a vector of several arboviruses impacting human health, including dengue, chikungunya, and potentially Zika. Vector control strategies that deploy modified males into the field are in use or under development and require a solid understanding of male biology; unfortunately, there has been limited effort to understand male Aedes albopictus reproductive biology, including sperm production and capacity. We tested whether body size and age affect spermatogenesis in Aedes albopictus. In general, older and larger males produced more sperm than their younger or smaller counterparts. Large males continued spermatogenesis well after 10-d post-eclosion (dpe), augmenting their reserves by 39%. By contrast, small males stopped producing sperm at 10 dpe. These results contribute to a deeper understanding of Aedes albopictus reproductive physiology. We discuss the usefulness of these findings in the context of Aedes albopictus life history and their utility in optimizing male mosquito release strategies.

Key words: larval diet, spermatogenesis, modified male release, mating, male reproductive success

Aedes albopictus (Skuse) (Diptera: Culicidae) is a vector of more than 20 arboviruses (reviewed in Grant 2004, Paupy et al. 2009), most notably dengue, chikungunya (Delatte et al. 2008), and Zika (Wong et al. 2013, Grard et al. 2014). While Aedes aegypti (Linnaeus) (Diptera: Culicidae) is responsible for a majority of arbovirus transmission in the tropics, Aedes albopictus also contributes to epidemics (Grant 2004, Borgherini et al. 2007), has been solely responsible for several arbovirus outbreaks (Effler et al. 2005, Reiter et al. 2006, Tsatsarkin et al. 2007), and may act as a bridge vector that sparks new epidemics (Mondet et al. 1996, Grant 2004). Being more cold tolerant than Aedes aegypti, Aedes albopictus has expanded its range considerably in recent years, threatening temperate regions that once had no risk of transmission (reviewed in Shragai et al. 2017). Improved methods of controlling this mosquito are imperative.

Some tools for mosquito population suppression involve the release of modified male mosquitoes to interfere with reproduction (reviewed in Macias et al. 2017). The oldest of these approaches, sterile insect technique (SIT), deploys males sterilized by radiation (Klassen and Curtis 2005). Because most females mate only once (Boyer et al. 2012, Helinski et al. 2012a), those females that mate to an SIT male experience reproductive failure (Lees et al. 2015). A variation on traditional SIT uses males that harbor Wolbachia endosymbionts (Walker et al. 2011); wild females that mate with released males are unable to fertilize eggs due to cytoplasmic incompatibility (O’Connor et al. 2012; Zhang et al. 2015a,b; Mains et al. 2016). Finally, released males may be genetically modified so that they pass lethal genes to their offspring, preventing them from reaching adulthood (Alphey et al. 2010). This technique has the advantage of reducing adult mosquito populations. While also producing larvae that compete for resources in containers with wild larvae, regardless of the strategy, the success of each approach hinges on laboratory reared, altered males competing for and successfully inseminating wild females.

Results of modified male mosquito releases have been mixed (Reisen 2003, Harris et al. 2011), and failures could be due to factors of male biology that should not be overlooked (Helinski and Harrington 2013). To assist in designing effective releases of mosquitoes, other authors have examined male vigor, insemination capacity, mating compatibility, and survival (Oliva et al. 2012; reviewed in Helinski and Harrington 2013; Oliva et al. 2013a,b). We propose that sperm quantity is another parameter that may be optimized in future releases. While females are normally monogamous, up to 26% of Aedes albopictus females in the wild mate with and produce progeny from multiple males (Boyer et al. 2012). Thus, released males that transfer more sperm to a polyandrous female may more effectively reduce offspring production by a wild mate. In addition, the capacity to produce excess sperm likely represents a surplus of nutrition that will allow males in a release scenario to be reproductively competitive. Therefore, in this study, we quantify the effect of body size and age on sperm quantity in male Aedes albopictus.
Materials and Methods

Mosquito Rearing
We established a laboratory colony of *Ae. albopictus* with field-collected eggs from Mercer County, NJ. All mosquitoes in this study originated from this colony within one year of its inception. At all times, we maintained mosquitoes in environmental conditions described in Degner and Harrington (2016). We submerged eggs in deionized water for 30 min with a pinch of pulverized fish food (crushed Cichlid Gold fish food pellets; Hikari, Himeji, Japan) and subsequently placed them under vacuum pressure for 30 min to induce hatching. Larvae grew for one day before transfer to mass rearing trays with different feeding regimes according to treatment. We isolated pupae to ensure virginity; after eclosion, we transferred males to 8.4-liter bucket cages at densities of 200 males per cage with a separate cage for each size class. Adults were offered 10% sucrose ad libitum.

We manipulated larval density to produce two different adult size classes. For large body sizes, we placed 75 first-instar larvae in plastic trays with 1 liter of DI water; for small males, we transferred 750 larvae per tray. Each tray received four fish food pellets (as above). As a proxy for body size, we measured wings of all 240 males in this study, as in Ponlawat and Harrington (2007). Both sizes had normally distributed wing lengths (Shapiro–Wilk normality test, *W* = 0.99, *P* > 0.05), with large males’ wings (2.48 ± 0.08 mm; mean ± SD) significantly longer than small males’ wings (2.11 ± 0.09 mm; *t* = 34.6; *df* = 237, *P* < 0.001).

Sperm Quantification
We obtained total sperm counts using the methods of Ponlawat and Harrington (2007) with slight modification. Briefly, we dissected the testes, vas deferentia, and seminal vesicle, transferred them to 200-µl deionized water, and used minutien pins to release all sperm and homogeneously mix the solution. In a pilot study, we verified that no sperm remained on the dissecting tools with this method by washing with 1% Triton and viewing the wash solution under a compound microscope with darkfield illumination. After transferring ten 5-µl aliquots of sperm to a glass slide, we fixed and stained sperm with Giemsa dye. We counted nuclei using darkfield illumination at 100× magnification of sperm to a glass slide, we fixed and stained sperm with Giemsa dye.

We manipulated larval density to produce two different adult size classes. For large body sizes, we placed 75 first-instar larvae in plastic trays with 1 liter of DI water; for small males, we transferred 750 larvae per tray. Each tray received four fish food pellets (as above). As a proxy for body size, we measured wings of all 240 males in this study, as in Ponlawat and Harrington (2007). Both sizes had normally distributed wing lengths (Shapiro–Wilk normality test, *W* = 0.99, *P* > 0.05), with large males’ wings (2.48 ± 0.08 mm; mean ± SD) significantly longer than small males’ wings (2.11 ± 0.09 mm; *t* = 34.6; *df* = 237, *P* < 0.001).

Data Analysis
After verifying that residuals of sperm counts from both cohorts were normally distributed and had homoscedastic variance, we tested the effects of age and body size on sperm quantity using a univariate general linear model (UGLM) with body size, age, and cohort as fixed factors. We constructed our model iteratively, beginning with a full factorial design and an intercept and removing the least significant term in each iteration until all remaining terms were significant. Post-hoc pairwise comparisons were based on estimated marginal means and Bonferroni corrected. All statistics were conducted using SPSS (IBM SPSS Statistics for Windows, v24). Raw data can be accessed at https://doi.org/10.7298/X4P26W7B.

Results
Our final corrected model included four terms (excluding the intercept) that significantly predicted sperm quantity in *Ae. albopictus* (UGLM; *df* = 8,231; *F* = 388.8; *P* < 0.001; *r*² = 0.931; Supp Table 2 [Online only]). Both body size and age influenced sperm quantity (UGLM; *df* = 3,231; *F* ~ body size ~ 1,231; *F* ~ age ~ 665.3, *F* ~ body size × age ~ 769.8, *P* ~ age, body size ~ < 0.001; Fig. 1). At each age up to 10 dpe, large males had significantly more sperm (30–35%) than small males (*P* < 0.001). Likewise, males produced more sperm as they aged; in all but one comparison, older males had significantly more sperm than their younger counterparts of the same body size (*P* < 0.001). The only exception was the comparison of 10 and 20 dpe small males, which had similar sperm quantity (*P* = 1); this was accounted for in our model by a significant interaction between body size and age (UGLM; *df* = 3,231; *F* = 112.5; *P* < 0.001).

![Fig. 1. Sperm quantity of large and small male Ae. albopictus at different ages. Cross-hashed and dotted boxes indicate small and large males, respectively. Age and body size both significantly predicted sperm quantity (univariate general linear model; *df* ~ age ~ 3,231; *df* ~ body size ~ 1,231; *F* ~ age ~ 665.3, *F* ~ body size ~ 769.8, *P* ~ age, body size ~ < 0.001). In addition, there was a significant interaction between these terms (univariate general linear model; *df* = 3,231; *F* = 112.5; *P* < 0.001). All pairwise comparisons between groups of the same age or the same body size are significantly different except for small males at 10 and 20 dpe (α = 0.05). Figure includes data from two independent cohorts. Boxes indicate inner quartiles, and whiskers and outliers are drawn using the Tukey method. n.s. = not significant.](image-url)
This experiment was replicated twice with separate cohorts of mosquitoes. We tested 'cohort' as a factor in our model to verify the repeatability of our experiment, and our final model included it as a significant predictor (UGLM; df = 1,231; F = 7.105; P = 0.008). Further analysis revealed that males in the first cohort had slightly more sperm than those in the second cohort (average of 4%, or 162 more sperm). However, wing lengths did not differ between cohorts for either size class (large males: t = 1.221; df = 116.9, P = 0.22; small males: t = 0.747, df = 118, P = 0.46). Despite this difference, our statistical and biological conclusions remain the same whether we analyze the first, second, or both cohorts together.

Discussion

We found that older and larger *Ae. albopictus* males produced more sperm than their younger and smaller counterparts. Old males in our small body treatment were an exception to this trend, with sperm counts that plateaued at 10 dpe. In contrast, large males continued to produce sperm beyond this age. Although we did not quantify sperm at ages between 10 and 20 dpe, based on the rate of spermatogenesis between 5 and 10 dpe, it is likely that males produced sperm at least until 17 dpe ([Supp Analysis [Online only]])—the oldest recorded sperm production in a mosquito to date ([Hausermann and Nijhout 1975, Clements 1992]). The fact that sperm production plateaus at different ages depending on size suggests a divergence in resource availability or allocation; large *Ae. albopictus* males are able to invest more in sex than small males. A similar age- and size-dependent pattern of spermatogenesis exists in *Ae. aegypti* ([Ponlawat and Harrington 2007]), with one key difference: *Ae. aegypti* males' sperm count peaked at 10 dpe, regardless of size. This interspecific difference in gamete production may reflect nuances in the biology of *Ae. aegypti* and *Ae. albopictus*, or it may be an artifact of slightly different larval diets in the two experiments.

Sperm counts in this study should be interpreted with two caveats in mind. First, males were not allowed to mate or cohabit with females. This allowed precise and accurate enumeration of total sperm production, but under natural conditions, it is possible that mating or a natural sex ratio would accelerate sperm production. While this has not been documented in mosquitoes, plasticity in the rate of spermatogenesis has been demonstrated in *Drosophila bifurca* ([Björk et al. 2007]) and *Drosophila melanogaster* ([Moat et al. 2014]). Second, our methods do not identify sperm that are dead or incomplete. Sperm are easily and precisely quantified. Furthermore, because spermatogenesis continues into old age, even in the absence of mating, analysis of a single old male may reveal how well equipped he is to allocate nutritional reserves to reproductive efforts. Thus, sperm capacity is a straightforward means of quantifying male reproductive investment and may be used to fine-tune rearing protocols for male releases.

Supplementary Data

Supplementary data are available at *Journal of Medical Entomology* online.

Acknowledgments

We thank Sylve Pitcher for technical expertise and Dr. Alongkot Ponlawat for experimental guidance. This work was funded in part by a Cornell Graduate School fellowship (to E.C.D.) and NIH/NIAID grant R01AI095491.

References Cited

Alfonso-Parra, C., F. W. Avila, P. Decewathanawong, L. K. Sirot, M. F. Wolfner, and L. C. Harrington. 2010. Synthesis, depletion and cell-type expression of a protein from the male accessory glands of the dengue vector mosquito *Aedes aegypti*. J. Insect Physiol. 70: 117–124.

Alphey, L., M. Benedict, R. Bellini, G. G. Clark, D. A. Dame, M. W. Service, and S. L. Dobson. 2010. Sterile-insect methods for control of mosquito-borne diseases: an analysis. Vector Borne Zoonotic Dis. 10: 295–311.

Björk, A., R. Dallai, and S. Pitnick. 2007. Adaptive modulation of sperm production rate in *Drosophila bifurca*, a species with giant sperm. Biol. Lett. 3: 517–519.

Borgherini, G., P. Poubeau, F. Staikowsky, M. Lory, N. Le Moullec, J. P. Becquart, C. Wengling, A. Michault, and F. Paganin. 2007. Outbreak of chikungunya on Reunion Island: early clinical and laboratory features in 157 adult patients. Clin. Infect. Dis. 44: 1401–1407.

Alfonso-Parra, C., F. W. Avila, P. Decewathanawong, L. K. Sirot, M. F. Wolfner, and L. C. Harrington. 2010. Synthesis, depletion and cell-type expression of a protein from the male accessory glands of the dengue vector mosquito *Aedes aegypti*. J. Insect Physiol. 70: 117–124.

Alphey, L., M. Benedict, R. Bellini, G. G. Clark, D. A. Dame, M. W. Service, and S. L. Dobson. 2010. Sterile-insect methods for control of mosquito-borne diseases: an analysis. Vector Borne Zoonotic Dis. 10: 295–311.

Björk, A., R. Dallai, and S. Pitnick. 2007. Adaptive modulation of sperm production rate in *Drosophila bifurca*, a species with giant sperm. Biol. Lett. 3: 517–519.

Borgherini, G., P. Poubeau, F. Staikowsky, M. Lory, N. Le Moullec, J. P. Becquart, C. Wengling, A. Michault, and F. Paganin. 2007. Outbreak of chikungunya on Reunion Island: early clinical and laboratory features in 157 adult patients. Clin. Infect. Dis. 44: 1401–1407.
