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Male contributions during mating increase female survival in the disease vector mosquito *Aedes aegypti*

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ABSTRACT

Aedes aegypti is a vector of medically important viruses including those causing Zika, dengue, and chikungunya. During mating, males transfer a number of proteins and other molecules to the female and these components of the male ejaculate are essential in shifting female post-mating behaviors in a number of insect species. Because these molecules are highly variable by species, and female post-mating behavior by species is also varied, behavioral assays testing the function of the ejaculate are necessary before we can develop control strategies targeting the mating system to reduce mosquito populations. Because increased survival in mosquitoes strongly increases vectorial capacity and can influence population sizes and potential risk we tested the effect of mating on female survival. The ejaculate can either promote or reduce female survival, as both have been shown in multiple insect species, yet this effect has not been directly assessed in mosquitoes. We compared survival of females in four treatment groups: mated females, virgin females, and virgin females injected with either an extract from the male reproductive glands or a saline control. Survival, blood feeding frequency, fecundity and cumulative net reproductive rate (R_0) were determined after multiple feedings from a human host. Our results confirm that male reproductive gland substances increase female fecundity and blood feeding frequency, resulting in dramatic increases in fitness (R_0). We also demonstrate, for the first time, an effect of male reproductive gland extracts alone on female survival, regardless of whether or not the female ingested a vertebrate blood meal. Thus, the effects of MAG extract on survival are not secondary effects from altered blood feeding. Collectively, we demonstrate a direct role for *Ae. aegypti* male-derived molecules on increasing female fitness, reproductive success and, ultimately, transmission potential for vector borne pathogens.

1. Introduction

Aedes aegypti is a global mosquito vector of arboviruses including dengue, chikungunya, and Zika (Fernández-Salas et al., 2016; Gubler, 2002; Levy-Blitchtein and del Valle-Mendoza, 2016; Meaney-Delman et al., 2016; Montero, 2015). *Ae. aegypti* is an effective vector due to its association with human hosts and its reliance on humans for shelter and habitat (Harrington et al., 2001b; Scott et al., 1993a). One means of controlling this insect vector is by manipulating its reproductive biology (Ferguson et al., 2010; Helinski and Harrington, 2013). However, to implement this control strategy, we need a deeper understanding of the mechanisms by which mating affects female reproductive fitness and survival. This includes filling the gaps in our knowledge surrounding the specific function of male contributions to female post-mating behavior and physiology.

Though sperm transfer is important to fertility, other materials transferred through the male ejaculate are often responsible for short- and long-term behavioral and physiological changes in the female. In most insect species, males possess glands accessory to their reproductive tract that produce proteins and other substances that are transferred to the female though their ejaculate. Experiments involving organ transplantation or injection of tissue extracts demonstrate that secretions from male accessory glands (MAG) cause a variety of female post-mating behaviors in mosquitoes (reviewed in Clements (1999)) and several other insects (reviewed in Avila et al. (2011) and Perry et al. (2013)). In *Aedes* spp. specifically, unidentified MAG substances

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were found to promote blood digestion rate (Downe, 1975), increase blood meal size (Adlakha and Pillai, 1976), reduce re-mating behavior (Fuchs et al., 1968; Fuchs and Hiss, 1970; Helinski et al., 2012; Ramalingam and Craig, 1976), stimulate egg development (Klowden, 1993; Klowden and Chambers, 1992, 1991), and increase fecundity (Hiss and Fuchs, 1972; Leahy and Craig, 1965; Ramalingam and Craig, 1976).

The male ejaculate as a whole has been found to influence female survival positively or negatively in a variety of insect species (Chapman et al., 1995; Arnqvist and Nilsson, 2000; Wagner et al., 2001; Lewis and South, 2012), yet no studies have explored the impact of mating and MAG substances on female survival in *Ae. aegypti* – a trait that profoundly affects capacity for disease transmission. Females that survive longer not only produce more offspring through multiple gonotrophic cycles, but also are more likely to survive through extrinsic incubation periods, allowing infected mosquitoes to transmit pathogens, contributing to their vectorial capacity (Garrett-Jones and Shidrawi, 1969; Kramer and Ebel, 2003). Early studies found that female *Ae. aegypti* live longer when associated with males than when isolated (Liles, 1965; Liles and Delong, 1960). Recent work suggests that transferred ejaculate may influence survival, as females mated to males whose ejaculates were depleted exhibited reduced survival compared to those females mated to non-depleted males (Helinski and Harrington, 2011). However, the males in that study were depleted for both sperm and seminal fluid; therefore, a direct cause to the increased survival could not be established.

Here, we assessed the impact of mating and MAG-injection on female survival and blood meal size. In an effort to address these effects comprehensively and under a natural context, we conducted multiple sets of life table experiments. While frequent sugar feeding in nature is considered rare for *Ae. aegypti* (Costero et al., 1998) especially in Thailand which is the origin of our mosquito strain (Edman et al., 1992; Spencer et al., 2005), we wanted to account for effects on survival both with and without sugar supplementation to cover the spectrum of potential sugar feeding. In addition, in nature, mosquitoes routinely blood feed multiply over their lifespan, though little is known about whether mating, either directly or indirectly, increases their propensity for blood feeding. Therefore, here we conducted experiments to address whether there were survival differences after no blood feeding, a single blood meal, or multiple blood meals. We also tested the effects on survival of sugar supplementation in combination with these various amounts of blood feeding and/or with MAG injection. Importantly, our experiments included survival measurements for females that were not allowed to ingest blood to determine whether there was a direct link between MAG substances and female survival.

2. Methods

2.1. Mosquito rearing

A wild type Thai strain of *Aedes aegypti* was used in this study. The mosquitoes originated from collections in Bangkok, Thailand (15°72′N, 101°75′E) and have been maintained in colony since 2009. The colony was supplemented with eggs from field-caught mosquitoes annually. Mosquitoes were reared in an environmental chamber at 71.9% ± 9.5% RH and 29 °C ± 1.0 °C, and with a 24 h photoperiod consisting of 10 h light, 10 h dark, and 2 h simulated dawn/dusk. Mosquitoes were reared to produce uniform, medium-sized adults as in Helinski and Harrington (2011). Sexes were isolated prior to eclosion by transferring male and female pupae to individual test tubes. After eclosion, males and females were held separately in 8L bucket cages with access to 10% sucrose *ad libitum* for approximately three days prior to experimentation.

2.2. Experiment one: survival in the presence of blood meals and MAG extract, blood feeding frequency

2.2.1. Treatment preparation and injection

Male reproductive gland extract was prepared by dissecting pairs of male accessory glands, along with their attached seminal vesicles, from 60 virgin, 3–5 day old males into 60 µl of *Aedes* saline (Hayes, 1953). The dissected tissue solution was homogenized on ice for 10 s and sonicated for 15 s at 4 °C to release molecules from the tissue, and then centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant was placed into a fresh, sterile tube. For all replicates, a fresh protein extract was prepared. For the first two replicates, the supernatant was stored at −20 °C prior to injection. Previous injections of fresh or frozen MAG extract yielded no difference in activity, indicating that freezing did not destroy the survival-promoting activity (unpublished data). To further confirm that freezing extracts in replicates did not affect protein activity, the supernatant was injected on the day of collection for Replicate 3.

Four age-matched treatment groups were established to assess the effect of accessory gland products on female post-mating behavior: virgin females (V), virgin females injected with saline (VS), virgin females injected with MAG and seminal vesicle protein extract (VMAG), and mated females (M). All individuals were three to five days old prior to the experiment. The mated female treatment group was created by introducing virgin males into the female cage two days after eclosion (in a 2:1 male:female ratio) and allowing the mosquitoes to mate for two days with males. All females in the mated treatment group produced viable offspring, confirming they had mated successfully (data not shown).

For saline-injected and extract-injected treatment groups, virgin females were chilled on ice (for 20 min or less) and then injected into the thorax via a fine glass capillary needle. We used a Nanoject II injector, (Drummond Scientific, Broomall, PA, USA) with either 0.25 µl of extract (effective dose based on Helinski et al. (2012)) or 0.25 µl *Aedes* saline as a control (Hayes, 1953). To compensate for female death and for non-feeding females, yet still maintain a sample size of 40–60 females per treatment, 60–100 females were injected. Females (n = 20 per bucket) were transferred into damp paper towel covered and parafilm-wrapped recovery cages with access to 10% sucrose *ad libitum*. After one day of recovery, females were collectively held in 8L bucket cages based on their treatment prior to their first blood feeding and offered a blood meal from a human host (LCH). After the first blood feeding, females were maintained in individual cups for the duration of the experiment and offered 10% sucrose. Wings were collected from a subset of mosquitoes after each experiment and measured to estimate body size as described previously (Nasci, 1990).

2.2.2. Survival

To assess the impact of MAG proteins on survival, mortality was recorded daily for the duration of the three replicates. Replicate 1 and Replicate 2 were terminated 13 and 19 days after the first blood meal, respectively. The lengths of these experiments were chosen based on the period of transmission for infected *Ae. aegypti* to transmit diseases to human hosts (Rigau-Pérez et al., 1998) as well as the estimated survival time of wild *Ae. aegypti* mosquitoes (Maciel-de-freitas et al., 2007). Because male-derived molecules transferred to females may be depleted throughout a female's life, for Replicate 3, we monitored mortality up until the last female death (54 days after the first blood feeding).

2.2.3. Blood meal size and feeding frequency

To assess the effect of blood consumption on survival, females were offered a series of blood meals from a human host (LCH). The first blood meal was offered at 7–9 days post-emergence (3–4 days after injection). Mosquitoes were allowed to feed on the host for 20 min. For this first blood meal, females were weighed to determine blood meal size (fraction of female weight attributed to the blood meal). Blood meal

size was calculated by anesthetizing freshly-fed females on ice and then weighing each female using a microbalance (Model C-31, Cahn Inst. Inc., Cerritos, CA). After weighing, each female was transferred individually to a 0.5 L cardboard cage. Within each treatment cohort additional unfed females were weighed. The average non-blood fed weight was subtracted from each individual blood fed female's weight to determine blood meal size. After the first feeding, females were maintained in individual cups and fed through the mesh of the cup lid. All females were required to take the primary blood meal to continue in the experiment. Supplemental blood meals were offered every three days for a total of four (Replicate 1), five (Replicate 2), or six (Replicate 3) supplemental blood meals (ending on experimental day 12, 15, and 18 respectively). Whether or not a female blood-fed was tracked for the duration of the experiment. The day before a blood meal, sucrose was removed to promote subsequent feeding. Once blood feeding had stopped, females were given access to 10% sucrose *ad libitum* for the remainder of the experiment. Feed number was incorporated as a time-varying covariate in the survival analysis described in Section 2.5. Because few females fed more than four times (n = 3), females that took four, five, or six total blood meals were analyzed together.

2.2.4. Fecundity

All females ingested at least one blood meal and therefore were stimulated to lay eggs (Judson, 1968; Klowden and Chambers, 1991). To assess the effect of treatment on egg laying, females were provided with an oviposition substrate (90 ml distilled water-filled cup, lined with moist oviposition paper) after the primary blood meal and for the duration of the experiment. Fresh water was added to the cups as needed. When a female died, her eggs were collected and counted. For mated females, fertility was verified by the presence of larvae once eggs were hatched. Because blood-feeding greatly impacts fecundity (Clements, 1992; Colless and Chellapah, 1960), female fecundity was standardized by dividing total eggs laid by the number of blood meals consumed (Helinski and Harrington, 2011; Helinski et al., 2012). Because daily egg laying was not monitored, egg data was excluded from the survival analysis (see Section 2.4).

2.3. Experiment two: survival based on extract injection, in the absence of blood meals

We carried out a second set of experiments to test if changes in female survival post-mating were due to the activity of molecules in the male extracts, or to a nutritive effect of those molecules after injection into females. To remove any impact of blood feeding or sugar feeding on survival, this experiment was performed without supplying females with any blood meals or sucrose.

For this experiment, we tested five treatments of virgin females (n = 30 each injected with 0.25 µl liquid in the same manner as described in Section 2.2.1). The five treatments were: saline (VS), MAG and seminal vesicle protein extract (prepared as described in Section 2.2.1; VMAG), an aliquot of VMAG heated for 5 min at 95 °C to denature proteins (VHmag), proteins extracted from male mosquito heads in a manner similar to VMAG (VHead), and bovine serum albumin (VBSA) protein prepared by suspending BSA powder in saline. All solutions for injections were adjusted to a protein concentration of ~1 µg/µl; protein concentration was verified via BCA protein assay (Pierce BCA Protein Assay Kit, Thermo Scientific, Rockford, IL). Daily monitoring of female survival was performed for up to ten days, by which time only a few females remained alive. This experiment was replicated four times (n = 30 per treatment), with the exception of the VHead treatment group, which was included in only two of the four replicates. We analyzed all replicates together and then, because VHead was not included in all replicates, we performed a second analysis in which we separated these replicates into two Cox Regressions based on inclusion (or not) of the VHead treatment. We found similar results whether or not Vhead was included (see Supplementary Table 4).

2.4. Experiment three: survival and fecundity based on MAG injection, in the presence of sugar with one blood meal

To test for the possibility of confounding effects of sugar supplementation, multiple blood meals and/or any retained eggs across the treatments in Experiment One, we conducted an additional experiment with three replicates. For this experiment, we reared mosquitoes to ensure uniform body size across all three treatments, we injected fresh MAG extracts, provided females with only a single blood meal and gave them continuous sugar access. We also reduced the mating interval to 2 h, continued the life table until all mosquitoes had died, and we dissected every female to check them for retained eggs. Mosquitoes were reared as described above in Section 2.1. Eight age-matched female treatment groups were established that included MAG-injected non-blood fed (MAGNBF); MAG injected blood fed (MAGBF); saline injected blood fed (SBF); saline injected non-blood fed (SNBF); virgin blood fed (VBF); virgin non-blood fed (VNBF); mated blood fed (MBF): and mated non-blood fed (MNBF). MAG was prepared and mosquitoes were injected as described in Section 2.2.1. To reduce potential for remating, mosquitoes in this experiment were held with males for a 2 h interval 3 days after injection. Females in the blood-fed groups were offered a blood meal 2 days after injection and then transferred to individual cartons. No additional blood meals were offered. Assessments of survival and fecundity were performed as described above with one exception: when a female died, she was dissected to check for any potential retained eggs. Those eggs were counted and scored as mature (Christophers' stage V) or immature (Christophers' stage IIIa-IVb; (Clements, 1984). Retained eggs were reported separately from deposited eggs. No immature stage retained eggs were found; therefore, they were added to the total deposited mature eggs to obtain a value for total eggs produced.

2.5. Statistical analyses

All survival curves were generated using Kaplan-Meier estimates. Females that were alive at the conclusion of all survival experiments were considered right censored for the analysis. The few females that escaped or were accidentally killed were removed from the analysis (n = 20). Survival analysis for the experiment described in Section 2.2 was performed via a Cox model, with a treatment and a time-varying feed number effect added as covariates (Cox, 1972; Kalbfleisch and Prentice, 2002). To assess the overall effect of survival across all three replicates, replicate was added as a strata variable. The model was also repeated independently for each replicate to assess any inter-replicate variability in survival. Survival analysis for this experiment was performed using SAS (Version 9.4, SAS Institute Inc., Cary, NC).

Blood meal size was analyzed using a two-way ANOVA, with treatment, replicate, and their interaction as factors. Comparisons between these groups was performed post hoc using a Tukey's HSD. The pattern of blood feeding frequency was separately analyzed via a longitudinal data analysis, with replicate, treatment, and their interaction as factors (Generalized Estimating Equation, Logit link function, with blood meal number as the within-subject effect, correlation matrix: unstructured). The primary blood meal was excluded in this analysis. In addition, supplemental blood meal six of Replicate 3 was excluded from this analysis due to a low sample size.

To confirm the previously reported effect of MAG proteins on egg laying, two separate analyses were performed on the data collected in Experiments One and Three. For Experiment One, total laid eggs were recorded and analyzed using a two-way ANOVA, with treatment, replicate, and their interaction as factors. For Experiment Three, total laid eggs and total eggs (laid and retained) were analyzed using two, two-way ANOVAs, with treatment, replicate, and their interaction as factors. All pairwise comparisons between groups was performed post hoc using a Tukey's HSD.

Overall life table fitness measures of cumulative net reproductive

rate (R_0), intrinsic rate of increase (r), and generation time (T_c) for females were calculated (Southwood, 1978) by considering all eggs laid as potential progeny, regardless of reproductive state. We first estimated r from the natural log of R_0 divided by the generation time T_c. The actual value of r was calculated using successive approximation (Begon et al., 1996; Harrington et al., 2001a). Generation time is traditionally overestimated using this method for continuously reproducing animals and therefore is not reported.

We employed a Cox Regression analysis for the survival experiments described in Sections 2.3 and 2.4. For these experiments, we compared the effect of different protein injections and blood feeding on female survival. We added replicate as a strata term and the hazards for each treatment were compared to saline. To assess all pairwise comparisons beyond the comparisons to saline, multiple Log-rank (Mantel-Cox) tests were used and a Bonferroni correction was applied to account for the multiple comparisons. All analyses, excluding the Cox model survival analysis performed in SAS (above), were conducted using SPSS (Version 22.0, IBM Corp., Armonk, NY).

3. Results

3.1. Survival in the presence of multiple blood meals and MAG extract

3.1.1. Mated and MAG-extract injected females live longer than virgin or saline-injected virgins

Survival was significantly affected both by mating status (Cox model; Fig. 1; $X_3^2 = 69.9$, $P < 0.0001$) and by number of blood meals ($X_4^2 = 10.7$; $P = 0.03$). There was no time by treatment effect ($P = 0.45$), indicating that the effect of treatment was consistent across time. In addition, there was no interaction between treatment and number of blood meals ingested on female survival ($P = 0.25$), indicating that the effect of mating and MAG proteins on survival is independent of the number of blood meals consumed.

Because the replicates of Experiment One varied in their end-points, this survival analysis was run twice, first with all replicates analyzed together, and second with each replicate as its own separate survival analysis. For the first analysis, pairwise comparisons between treatments demonstrated that virgin females die sooner than mated females (Supplementary Table 1; $P < 0.0001$) and females injected with MAG extracts exhibited a similar survival benefit as mated females ($P = 0.09$). When analyzed separately, the effect of survival based on treatment was maintained across replicates, except Replicate 1 and Replicate 2 lost the significant difference between saline and VMAG survival (Supplementary Table 1; $P = 0.08$, 0.23 respectively). Replicate 3, which was assessed for longer, had a larger sample size and exhibits a more pronounced effect of treatment, similar to the results when all three replicates were analyzed together.

3.1.2. Mated and MAG-extract injected females generally feed more than virgins or saline-injected females

Overall treatment affected how often a female fed (longitudinal data analysis; Supplementary Table 2), with mated and MAG-extract injected females feeding in a pattern significantly different from that of virgin and mated females (Fig. 2). Overall M and VMAG females exhibited increased blood feeding for the second and fourth meal, however both replicate and the interaction between treatment and replicate are also significant (Supplementary Table 2). The interaction was due to a specific trend in Replicate 2 where mated and MAG-injected females did not show increased feeding for the second and fourth blood meal (Bonferroni-corrected pairwise comparisons, $P < 0.05$).

There were inconsistent effects of treatment on blood meal size. Treatment, replicate, and their interaction all had a significant effect (Supplementary Table 3; Supplementary Fig. 1). Replicate variation makes it difficult to observe the effect of mating on female blood meal size, though mated females tended to take a larger blood meal in two out of the three replicates.

3.1.3. Mated and MAG-extract injected females lay more eggs than virgins or saline-injected females

As predicted, mated and MAG-extract injected females laid significantly greater numbers of eggs than saline-injected and virgin females (Fig. 3; $F_{3,330} = 102.20$, $P < 0.01$). However, there was a

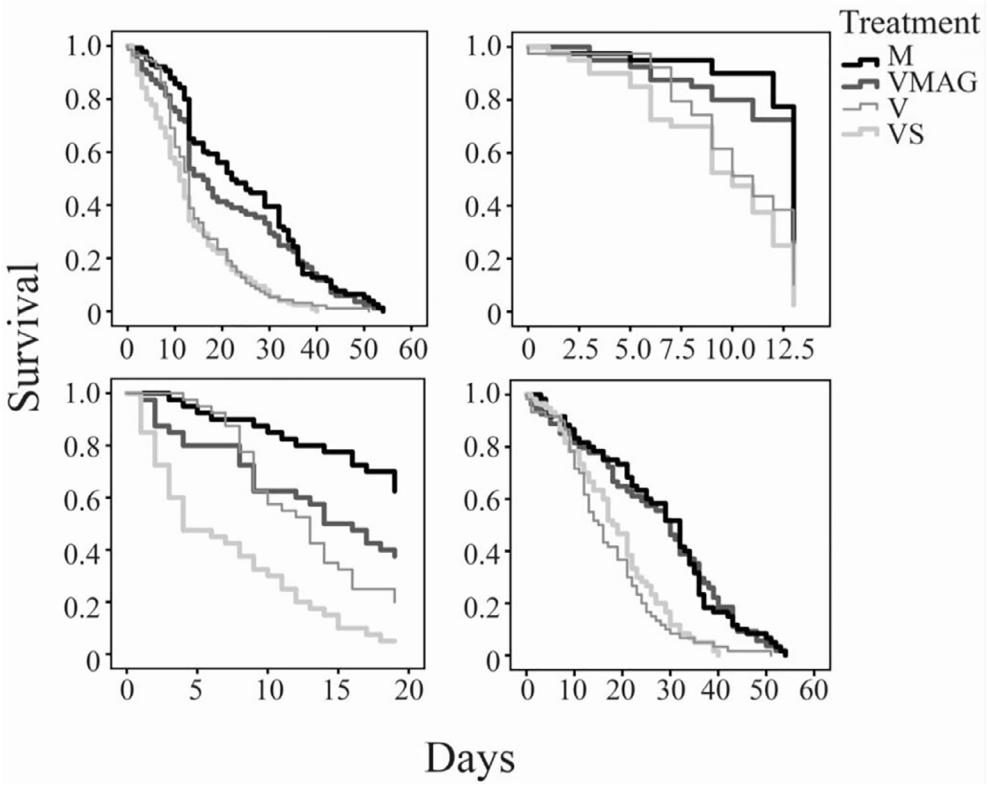

Fig. 1. Female *Ae. aegypti* survival (Top Left = All three replicates, combined; Top Right = Replicate 1 (n = 39–40 per treatment); Bottom Left = Replicate 2 (n = 40 per treatment); Bottom Right = Replicate 3 (n = 54–60 per treatment) by MAG/mating status (VMAG = virgin, injected with MAG extract; M = mated; VS = virgin injected with saline; V = virgin).

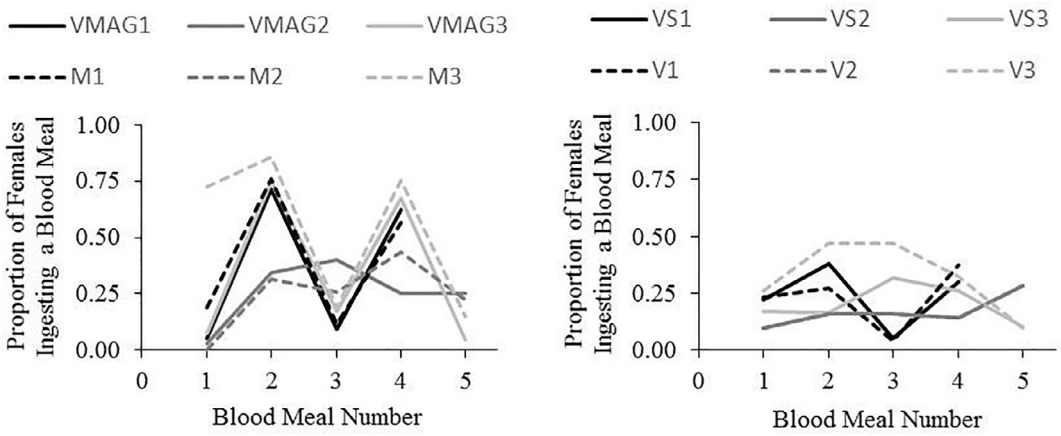

Fig. 2. Proportion of female *Ae. aegypti* blood feeding at each blood feeding event (after the initial blood meal) by MAG/mating status (VMAG = virgin, injected with MAG extract; M = mated; VS = virgin injected with saline; V = virgin). Numbers next to treatment name indicate replicate.

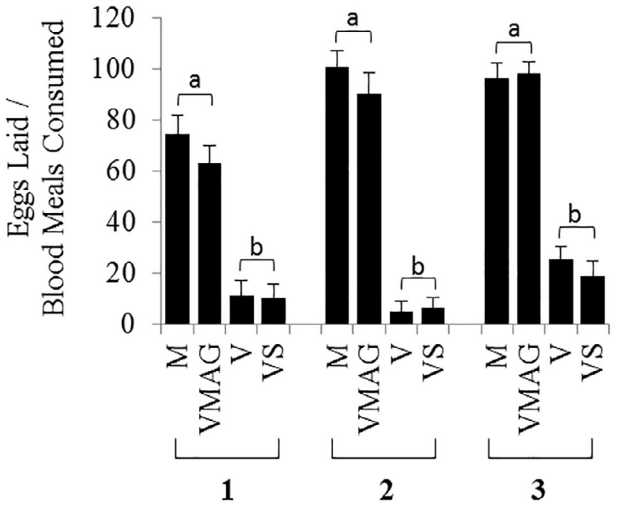

Fig. 3. Female *Ae. aegypti* fecundity (divided by the number of blood meals consumed) by MAG/mating status [VMAG = virgin, injected with MAG extract (n = 22–45); M = mated (n = 26–53); VS = virgin injected with saline (n = 10–35); V = virgin (n = 13–38)]. Data are μ ± SE. Letters indicate Tukey HSD comparisons within each replicate.

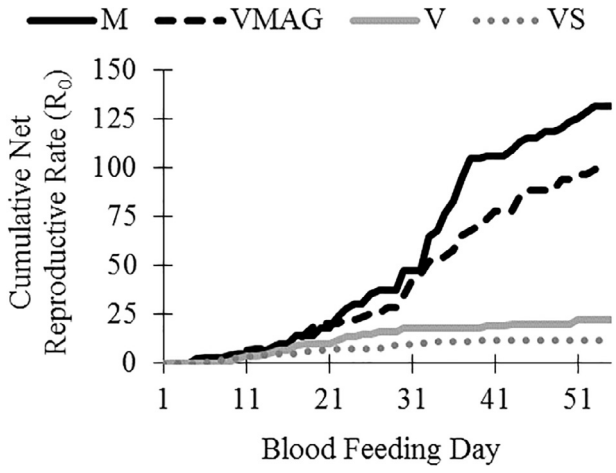

Fig. 4. Cumulative net reproductive rate in *Ae. aegypti* by MAG/mating status (VMAG = virgin, injected with MAG extract; M = mated; VS = virgin injected with saline; V = virgin).

significant effect of replicate ($F_{2,330} = 9.59$, $P < 0.01$), with both control and experimental females in Replicate 1 laying fewer eggs overall. Females reared for Replicate 1 were smaller than those used for Replicates 2 and 3, likely contributing to this difference ($\mu \pm SD$: Replicate 1 = 1.70 ± 0.41 mg, Replicate 2 = 2.16 ± 0.45 mg, Replicate 3 = 2.31 ± 0.58 mg; Univariate ANOVA: $F_{2,212} = 30.60$, $P < 0.001$). There was no significant interaction between treatment and replicate ($F_{6,330} = 1.64$, $P = 0.14$).

Our analysis demonstrated dramatic fitness advantages for mated or MAG-extract injected females when compared to virgin and saline-injected females (Fig. 4). Mated and MAG-extract injected females had higher reproductive rates and experienced a larger intrinsic rate of increase (Table 1).

Table 1
Comparison of life-table attributes.

Parameter	Saline (VS)	Virgin (V)	VMAG	Mated (M)
R_0 = cumulative net reproductive rate	11.79	22.26	101.16	131.58
r = intrinsic rate of increase	0.1955	0.2000	0.2821	0.3229

3.2. Increased female survival results from injection of MAG extracts

In a separate set of survival experiments, survival was investigated in females lacking access to blood meals to determine whether MAG substances alone causes increased female survival. In addition to VS (negative control) and VMAG (positive control), we injected females with VHmag (expected to have killed any survival-enhancing activity while still containing the same amino acid composition as MAG), an unheated extract from an unrelated tissue, the head, and pure bovine serum albumin (BSA). The protein treatment that a female was subjected to significantly affected her survival (Fig. 5; Cox Regression: $X_4^2 = 78.12$, $P < 0.001$). Females injected with head extract or with BSA protein showed similar levels of survival as females that had been injected with saline ($P = 0.180$ and 0.328 respectively), indicating that protein *per se* was not the cause of increased survival after MAG-extract injection. Among the treatments where females were injected with proteins derived from males, MAG-extract injected females maintained a survival benefit ($P = 0.001$), whereas females injected with head extract did not, indicating that the survival-extending activity is not a general tissue product – it is found in the MAG but not the head. Pairwise comparisons between all treatments indicated that heating the MAG extract significantly reduced the beneficial effect of MAG proteins on female survival, but was still significantly different from saline (Log Rank test, $P < 0.01$, Bonferroni corrected). These data suggest that a heat-labile MAG component, such as protein, is likely partially

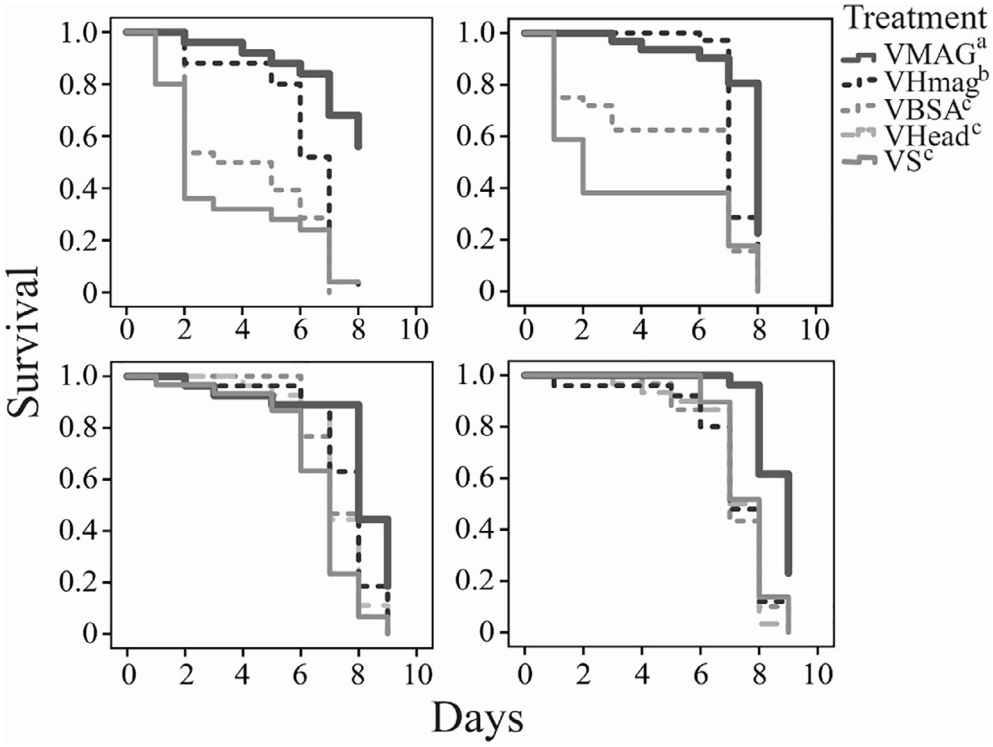

Fig. 5. The effect of injected molecules on female *Ae. aegypti* survival over four replicates. Virgin females were injected with male accessory gland and seminal vesicle extract (VMAG), heated VMAG (VHmag), head protein extract (VHead), BSA protein (VBSA) or a saline control (VS). Lower case letters indicate significantly different treatments/pairs of treatments based on the Log Rank test (Bonferroni corrected).

responsible for the increase in female survival seen in mated females.

3.3. Increased survival is not a purely nutritive effect from injected MAG extracts

Because variability between replicates in Experiment One potentially obscured effects of MAG-extract injection on female survival, a separate analysis was run in which we controlled for effects of blood feeding, as well as for whether the number of eggs retained by females varied across treatments.

Treatment had an overall significant effect on survival (Cox Regression: $X^2_7 = 184.446$, $P < 0.001$; hazard ratios are reported in Table 2). Any pairwise relationship between variables can be revealed by breaking apart treatment into three binomial (yes/no) factors: Blood-fed, injected, and "mated" (MAG-injected & mated versus saline-injected & virgin). The differences seen between the treatments in the previous analyses is largely associated with "mated" status ($X^2_1 = 45.476$, $P < 0.001$) and injection status ($X^2_1 = 6.002$, P = 0.014). Whether or not a female was blood fed did not influence survival ($X^2_1 = 0.219$, $P = 0.640$). We saw no significant two-way or three-way interactions between treatments.

Table 2
Hazard ratios (95% CI) derived from the survival analysis of treatment by blood feeding in Experiment Three (Section 2.4).

Blood Fed	Treatment			
	Mated	MAG-Injected	Virgin	Saline-Injected
Yes	0.467^{AB} (0.352, 0.620)	0.630^{C} (0.474, 0.837)	1.068^{D} (0.811, 1.407)	1.103^{DE} (0.834, 1.460)
No	0.371^{A} (0.278, 0.494)	0.496^{BC} (0.372, 0.661)	Ref *	1.425^{E} (1.073, 1.892)

Letters denote significance ($P < 0.05$) based on Bonferroni-corrected pairwise comparisons.

* Virgin, non-blood fed was not included in one of the three replicates and therefore was not included in the Log-rank test for pairwise comparisons across strata.

Table 3
Hazard ratios (95% CI) derived from the survival analysis of treatment, ignoring blood feeding, in Experiment Three (Section 2.4).

Mated[A]	Treatment		
	MAG-Injected[B]	Virgin[C]	Saline-Injected[D]
0.564 (0.505, 0.631)	0.755 (0.675, 0.631)	Ref	1.670 (1.495, 1.864)

Letters denote significance ($P < 0.05$) based on Bonferroni-corrected pairwise comparisons.

Re-running the survival analysis of the data from Experiment Three without distinguishing blood-fed vs. non-blood-fed, yielded similar results to what we observed in Experiment One (Table 3). Specifically, mated females survived the longest, followed by MAG-injected females. Females from both of these treatments survived longer than both virgin and saline-injected; saline-injected females had the poorest survival (Fig. 6 and Table 3; $X^2_3 = 173.722$, $P < 0.001$).

Few non-blood-fed females laid eggs or retained eggs, regardless of treatment (Mated, n = 7; MAG-injected, n = 2; Virgin, n = 2; Saline-injected, n = 2). When we plotted the number of eggs laid by each female condition (discounting blood feeding) the results where consistent with those shown in Fig. 3: there was some variability between replicates but MAG-injected and mated females laid significantly more eggs than virgin and saline-injected females (two-way ANOVA: $F_{3,401} = 73.82$, $P < 0.001$; "mated" versus non-mated comparisons via Tukey HSD, $P < 0.05$).

Most mated or MAG-injected blood-fed females produced eggs (96% and 93%, respectively). All retained eggs in females across all treatments were mature (Christophers' stage V). However, treatment played a significant role in whether or not females had retained eggs (Fig. 7; $F_{3,241} = 4.08$, $P = 0.008$). Mated females retained the fewest eggs (11%; and laid the most eggs). Of the unmated females that produced any eggs, virgin (76%) and saline-injected females (78%) retained the most. Few MAG-injected females retained eggs (11%), and those that did retained an intermediate number of eggs. There was no effect of replicate ($F_{2,241} = 2.89$, $P = 0.057$) or interaction ($F_{6,241} = 1.11$,

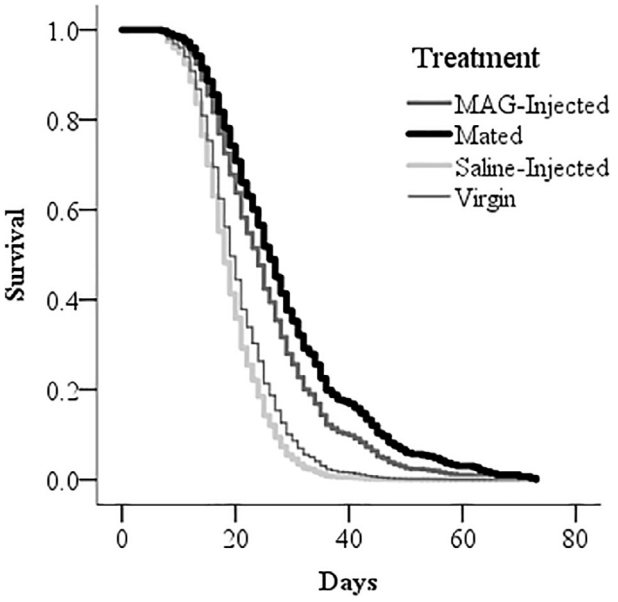

Fig. 6. Female *Ae. aegypti* survival by treatment in Experiment Three (Section 2.4).

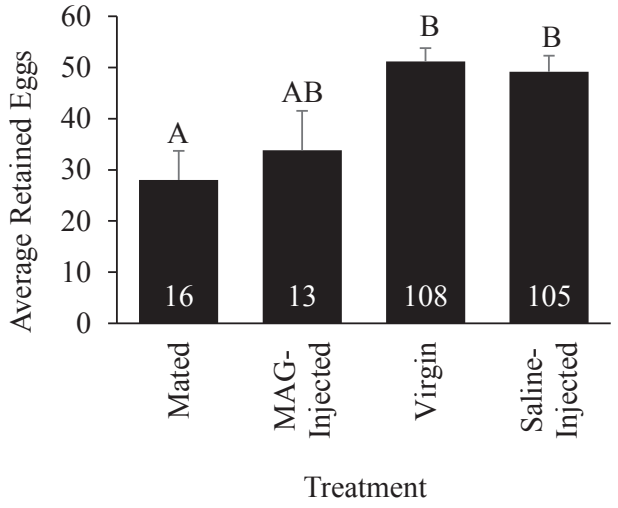

Fig. 7. Eggs retained by female *Ae. aegypti*. Data are μ ± SE. Letters indicate Tukey HSD comparisons. Numbers at base indicate the number of females with retained eggs. Proportion of females that retained eggs across replicates: Mated = 0.12, MAG-Injected = 0.11; Virgin = 0.76; Saline-injected = 0.78.

$P = 0.357$).

4. Discussion

Here we present evidence that MAG substances not only influence female blood feeding and fecundity, but also have a profound effect on components of female fitness including survival and egg production. In measurements made in the absence of blood or sugar feeding, our results parallel the differences in survival observed when mosquitoes were allowed to feed at their own discretion. *Aedes aegypti* females, either mated or injected with seminal proteins extracted from the MAG, exhibited increased survival compared to virgin controls. Although previous studies suggested that mating could improve female *Ae. aegypti* survival (Helinski and Harrington, 2011; Liles, 1965; Liles and Delong, 1960), our study is the first to experimentally test the cause of the increased female post-mating survival. When females were allowed to feed multiply, the number of blood meals they consumed did not significantly affect survival ($P = 0.25$). Additionally, we found that the enhanced survival can be attributed to mating/MAG-injection when females are not allowed to blood-feed or were only blood-fed once. These data indicate that the survival effect is not purely nutritive, and does not occur when females are injected with non-MAG-derived protein. Our data also indicate that MAG-derived effects do not diminish with time post-treatment, at least under laboratory conditions. The survival benefit is seen when experiments end early, to simulate wild mosquito lifespans (Harrington et al., 2001a,b; Maciel-de-freitas et al., 2007), as well as when they extend to the lab-reared mosquito's entire lifespan.

The protein composition of the seminal fluid varies between species of mosquitoes (*Anopheles gambiae:* Baldini et al., 2012; Dottorini et al., 2007; *Aedes albopictus:* Boes et al., 2014; *Aedes aegypti:* Sirot et al., 2011, 2008) as well as across *Drosophila* and other insect species (Avila et al., 2011; Perry et al., 2013). Even though much is known about the role of various seminal molecules in affecting female insect post-mating behavior, the exact agent in *Ae. aegypti* mosquitoes promoting survival is unknown. Because *Ae. aegypti* is an important vector of diseases, this work highlights the importance of species-specific identification of the proteins responsible for promoting female survival post-mating. Experiments are currently underway in our lab to identify the key *Ae. aegypti* proteins responsible for promoting female post-mating fecundity and survival.

The reproductive gland proteins we injected into females could be considered nuptial gifts, defined as "materials … provided by a donor to a recipient during courtship or copulation in order to improve donor fitness (Lewis et al., 2014)", assuming that the male gains a fitness advantage by promoting female survival. Though the term "gift" might imply a beneficial effect on the female recipient, few studies have found a survival benefit across various insect and spider groups. When a survival benefit is found it tends to be associated with nuptial feeding and/or polyandry (Arnqvist and Nilsson, 2000; Wagner et al., 2001; Vahed, 2007; Gwynne, 2008), which is not the case for *Ae. aegypti*.

Though nuptial gifts often contain components that provide a nutritive boost to the female, the survival enhancement seen in the present study does not appear to derive from a nutritive effect of MAG extracts. Females that were mated/MAG-extract injected in Experiment Two did not increase their blood feeding frequency, yet these females still exhibit increased survival. In addition, the effect of MAG extracts could not be recapitulated from an alternative source in the present study: females that received either a single protein (VBSA) or an extract from another region of the male's body (VHead) did not survive as long as MAG-injected females. The increased survival is therefore specific to MAG components received during mating.

There is some controversy regarding the effect of MAG secretions on female blood meal size ingested initially and subsequent feeding frequency. Female *Ae. aegypti* have been found to increase their blood meal size after mating or receipt of MAG components (Adlakha and Pillai, 1976). Houseman and Downe (1986) only found an effect when blood was offered 6 days after emergence and Klowden (Klowden, 1979) argued that there is no effect of mating on blood meal size. Our analysis indicates that initial blood meal size may increase in mated females, though this requires further study.

Studies on feeding frequency after mating or receipt of MAG extracts have argued that feeding frequency initially decreases then increases within the first 5 h after mating (Lavoipierre, 1958) or decreases after longer intervals for mated females (Judson, 1968). In our study, mating or MAG-injection was associated with increased blood feeding, likely linked to gonotrophic cycles. Feeding increased on day six and twelve when females had likely just finished ovipositing (unpublished data), though daily egg laying was not measured in this study. Mating may induce oviposition and therefore secondarily cause females to take multiple blood meals. Virgin females retained more, and oviposited fewer, developed eggs and therefore this could explain why they did not feed. During Replicate 2 of Experiment One, females did not

demonstrate increased blood feeding activity and the cause of this lack in consistency is unknown. The discrepancies between replicates of the effect of MAG on blood feeding activity and blood meal size highlights the importance of fully characterizing the factors that go into female host-seeking decisions, as they appear to be highly variable even within the controlled lab setting.

The effect of MAG components on increasing female fecundity is well documented for *Ae. aegypti* (Helinski and Harrington, 2011; Hiss and Fuchs, 1972; Leahy and Craig, 1965; Ramalingam and Craig, 1976; Yeh and Klowden, 1990) as well as for other mosquito species (*Ae. albopictus:* Klowden and Chambers, 1992; Klowden, 1993; *Anopheles gambiae:* (Baldini et al., 2013; Gabrieli et al., 2014). In *Ae. aegypti*, the amount of blood consumed correlates with fecundity (Colless and Chellapah, 1960; Lea et al., 1978). In the present study, we found that MAG extract and mating, either directly or indirectly promoted egg laying, even with only a single blood meal. Virgin females retained more eggs than MAG-injected or mated females, indicating that although egg production was initiated by blood feeding, a component of the male ejaculate (MAG secretions) is required for laying behavior. The transfer of juvenile hormone III has been implicated in activating egg development in *Ae. aegypti* (Clifton and Noriega, 2012; Kelly et al., 1981) and is transferred from the male to the female during mating (Clifton et al., 2014). Clifton & Noriega (2012) found that juvenile hormone mediates the relationship of nutrition with egg development, as females that receive juvenile hormone exhibit reduced follicle reabsorption and increased fecundity, even if the female took a smaller blood meal. The transfer of juvenile hormone in the MAG extract could explain why females experience increased fecundity in our study, regardless of the strength of the nutritive source.

Most of our knowledge on the role of MAG secretions comes from studies in another dipteran, *Drosophila*, where proteins from the MAG have been found to induce sexual refractoriness, increase egg production rate, alter feeding behavior, reduce survival, stimulate ovulation, and facilitate sperm binding and storage (reviewed in Avila et al. (2011)). Sex peptide is a MAG component found to promote female refractory behavior in *Drosophila melanogaster* and also reduces a female's lifespan (Wigby and Chapman, 2005). Our data indicate that the MAG components in *Ae. aegypti* differ significantly from *Drosophila melanogaster* not just in their composition (Sirot et al., 2011) but also in some of their functions, for example by promoting a female's survival rather than decreasing her lifespan.

Based on life-table analysis, females *Ae. aegypti* injected with seminal proteins experience a boost in cumulative net reproductive rate (R_0). Identification of the protein(s) responsible for promoting female survival post-mating could play an important role in control strategies focused on manipulating mosquito reproductive biology. Especially considering that arboviruses and other vector borne pathogens require an incubation period in the female mosquito (Alto and Bettinardi, 2013; Chan et al., 2012; Rigau-Pérez et al., 1998), and that females will feed multiple times both within and between gonotrophic cycles (Harrington et al., 2014; Scott et al., 1993b).

5. Conclusion

This work highlights an important effect of mating: increasing female survival is likely due to one or more male-derived seminal fluid molecules transferred to the female. Because protein composition in the male ejaculate is diverse across insect species, further analysis is necessary to determine which component of *Ae. aegypti*'s seminal fluid is responsible for the survival boost. Here, we have demonstrated an important influence of male mosquito ejaculate on female fitness traits that has the potential to increase pathogen transmission by female mosquitoes (Dye, 1986; Kramer and Ebel, 2003).

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