Published in final edited form as:

Behav Ecol Sociobiol. 2012 August 1; 66(8): 1131–1140. doi:10.1007/s00265-012-1365-9.

The role of male harassment on female fitness for the dengue vector mosquito *Aedes aegypti*

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Abstract

Sexual harassment studies in insects suggest that females can incur several kinds of costs from male harassment and mating. Here, we examined direct and indirect costs of male harassment on components of female fitness in the predominantly monandrous mosquito *Aedes aegypti*. To disentangle the costs of harassment versus the costs of mating, we held females at a low or high density with males whose claspers were modified to prevent insemination, and compared these to females held with normal males and to those held with females or alone. A reduced longevity was observed when females were held under high density conditions with males or females, regardless if male claspers had been modified. There was no consistent effect of harassment on female fecundity. Net reproductive rate (R_0) was higher in females held at low density with normal males compared to females held with males in the other treatments, even though only a small number of females showed direct evidence of remating. Indirect costs and benefits that were not due to harassment alone were observed. Daughters of females held with normal males at high density had reduced longevity compared to daughters from females held without conspecifics. However, their fitness (R_0) was higher compared to females in all other treatments. Overall, our results indicate that *A. aegypti* females do not suffer a fitness cost from harassment of males when kept at moderate densities, and they suggest the potential for benefits obtained from ejaculate components.

Keywords

Aedes aegypti; monandry; harassment; longevity; net reproductive rate

Introduction

Males and females can have profoundly different stakes in mating. While male fitness generally increases with each successive mating, for females often there are trade-offs to consider when mating with multiple mates, thus creating room for sexual conflict (Parker 2006). One form of sexual conflict is male harassment, where repeated male copulation attempts can be costly to females (Clutton-Brock and Parker 1995). In insects, these costs for females can include depletion of energy reserves, reduced opportunity to feed, reduced

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Ethical standards All experiments comply with the current laws of the country in which they were performed.

Conflict of interests The authors declare that they have no conflict of interest.

longevity, and decreased fecundity (Arnqvist and Nilsson 2000). The act of mating itself can also be costly to females, including energetic costs (Watson et al. 1998), damage inflicted by male genital organs (Crudginton and Siva-Jothy 2000; Blanckenhorn et al. 2002), and toxic effects from accessory gland substances affecting longevity (Wolfner 1997). Longevity of the female dung fly *Sepsis cynipsea* was reduced when females were maintained with males (Muhlhauser and Blanckenhorn 2002), and this was largely due to the cost of copulation rather than precopulatory costs of assessment and rejection. In the field cricket *Gryllus bimaculatus*, female longevity was reduced as a result of courtship and copulation, which was not due to receipt of semen (Bateman et al. 2006). In contrast, *Drosophila* females do incur a longevity cost as a result of seminal fluid proteins transferred in the ejaculate (Chapman et al. 1995).

In spite of the costs associated with mating, females of many insect species mate multiply (Arnqvist and Nilsson 2000). Mechanisms by which females can directly benefit from polyandry include the replenishment of sperm, increased resources either through the transfer of a nuptial gift or seminal fluid proteins, and protection from other males (Arnqvist and Nilsson 2000). In addition, indirect genetic benefits to their offspring have been described (Jennions and Petrie 2000). A meta-analysis suggests that in many insects, females gain from a moderate rate of re-mating in terms of increased lifetime reproductive output (Arnqvist and Nilsson 2000). Recent evidence from a field population of crickets supports this notion and both sexes benefitted from mating with multiple partners (Rodriguez-Munoz et al. 2010). Even though female polyandry is common in insects, female monandry exists in a variety of orders (e.g. Lepidoptera, Diptera, Coleoptera (Arnqvist et al. 2000)). From an evolutionary perspective, female monandry could have resulted from an ongoing sexual conflict between the sexes, where males benefit from monandry and can manipulate female behavior to increase their own fitness while imposing costs on females (Morrow et al. 2003; Arnqvist and Rowe 2005). Alternatively, females might equally benefit from monandry if their fitness is maximized with a single insemination (Arnqvist and Andrés 2006). In the majority of medically important mosquito species, females are generally monandrous (Clements 1999; Yuval 2006), although there is some evidence that a low proportion of females engages in multiple mating (Tripet et al. 2003; Helinski et al. 2012). Monandrous behavior in aedine and anopheline mosquitoes studied to date is induced by male derived proteins transferred in the ejaculate (Craig 1967; Shutt et al. 2010). In this study, we investigated whether harassment and mating are costly to females of a predominantly monandrous mosquito species.

In many mating systems it is hard to discern the cost of harassment from the cost of mating. However, this can be investigated in species for which males have claspers that are necessary to grasp females during copulation (Eberhard 1985; Eberhard 2004). By modifying the claspers, one can create males that are able to harass but unable to mate with females, similar to the ablation of the aedeagus in Coleoptera (Sakurai and Kasuya 2008; den Hollander and Gwynne 2009). Here, we used this technique to test for a cost of harassment and a cost of mating to females of the dengue vector mosquito *Aedes aegypti*. The effect of male harassment on female fecundity and longevity was determined by comparing females exposed to males that could harass and mate to those exposed to males

that could only harass. Females were either exposed to a low or a high density of males. Although it is unlikely that females in the field are exposed to the same level of harassment observed in this study, our experiments were designed to reveal if conflict over harassment could occur in this species.

We tested the prediction that, if harassment is costly for females, it will lead to a decrease in reproduction or longevity. In addition, we predicted that costs would increase with male density. For the group of females housed with males that can harass but not mate, two scenarios could unfold. If the act of copulation is costly to females, females in these treatments may have improved fitness through no probability of mating. Alternatively, treated males may harass females more as their mating behavior cannot be completed, which could result in increased costs to the female. Besides the direct cost of harassment, for a subset of females we investigated indirect effects by determining the longevity and reproductive capacity of daughters of harassed females.

Material and Methods

Study organism and rearing

Aedes aegypti mosquitoes are important vectors of yellow fever and dengue viruses throughout the tropics. *A. aegypti* typically breed in human-made container habitats. The aquatic larval stage lasts on average between 7-12 days. Adult *A. aegypti* disperse relatively short distances (i.e. less than 500 meters (Harrington et al. 2005)), and tend to rest indoors (Perich et al. 2000). Females require a blood meal to develop eggs, and are highly anthropophilic in host choice (Scott et al. 1993). *A. aegypti* males are considered to be polygynous, and can mate with multiple females within one day (Helinski and Harrington 2011), although male mating frequency in the field has not been empirically determined. Normal sex ratios observed in the field are 1:1 with average numbers of males per house ranging from 0-20 and varying considerably by house and region of the world (Scott et al. 2000; Garcia-Rejon et al. 2008). *A. aegypti* is generally observed to mate around the host (Hartberg 1971; Cator et al. 2011). *A. aegypti* males identify females by their wing beat frequency and actively pursue females. Mating is initiated in flight, where males grasp the female with their legs and orientate themselves in a ventral position (Roth 1948; Spielman 1964). Once in the proper position, the male flexes his abdomen until he grasps the female's genitalia, holding onto the female cerci with his claspers, and rapid copulation, i.e. between 8-20 sec, follows (Roth 1948). In confined spaces such as laboratory cages, males harass flying females and attempt to mate with them. Females can resist these attempts by evasive flight, by kicking the male with her hind legs, or by pseudo-copulation (Jones 1974; Cator and Harrington 2011).

Two strains of *A. aegypti* mosquitoes were used in this study. Focal females were obtained from a Higgs White Eye (HWE) recessive white eye mutant strain. The phenotype is characterized by a lack of pigment in both the compound eyes and ocelli (Wendell et al. 2000), and has been in colony for > 30 years. Mosquitoes of the Mexico (MEX) strain were used as harassers. The MEX strain originated from Tapachula, Mexico (14° 54'N, 92° 15'W) and has been maintained in colony since 2006 at Cornell University. The strain was augmented in 2008 and 2009 with wild mosquitoes collected from the field. MEX

mosquitoes have normal dominant wild-type black eye color. The two phenotypically distinct strains were used to 1) determine if multiple mating had occurred, 2) to identify focal females from the harasser females (see below).

Eggs were vacuum hatched and transferred to rearing pans at a density of 200 larvae/ 1 L water. Larvae were fed a fixed diet of 1:1 Brewer's yeast: lactalbumin (2.45 mg/larva total) to obtain medium body sized adults (see Ponlawat and Harrington (2007) for rearing details). HWE pupae were isolated in individual test tubes for sexing upon eclosion to assure virginity. Pupae from the MEX strain were collected at 24 hrs intervals and placed in cages for eclosion. The following day, eclosed mosquitoes were anesthetized and the sexes were separated. Mosquitoes were maintained on a diet of 10% sucrose solution until the start of experiments. Experiments were conducted in a climate chamber set at 25.9 ± 0.8 °C and $83.5 \pm 9.2\%$ RH, and L10, D10, with 2 hr increments simulating dusk and dawn.

Direct effect of harassment on female fitness

The goal of the experiment was to determine the effect of male harassment on female longevity and reproduction. To untangle the effects of mating from those of harassment (i.e. males pursuing females, holding on to females, attempting body alignment for copulation), females were either held with males that were able to harass but not mate (i.e. 'treated', see below) or with normal males that could harass and mate. The effect of density was determined by using a low or a high number of potential harassers.

Prior to being placed in their treatment groups, mated focal HWE females were obtained by placing 200 2-4 day old virgin females with 200 HWE males aged 4-7 days in a large cage (60 cm²) for 48 hrs. Females were removed and placed in a smaller cage for blood feeding (human forearm MEHH/ LCH, in accordance with Cornell University's Institutional Review Board approved protocol #0906000050); females that did not feed were discarded. The following day, females were transferred to individual cartons (1.57 L) and assigned to treatment groups.

Two replicates of the experiment using different cohorts of mosquitoes were performed. Replicates differed only in the type of control treatments used. Individually held mated and blood fed HWE females were randomly assigned to treatments, and the MEX harasser mosquitoes (age 5-10 days) were introduced. In replicate one, females were either held with normal males (N) or treated males (T) at a low (N= 2) or high density (N= 10). Two female control treatments (F) were set up to account for density by adding in 2 or 10 MEX females. Thus in total, six treatments were set up: N2, N10, T2, T10, F2 and F10. Each treatment had 24 females with the exception of the female control treatments that had 21 females. In replicate two, the female harasser treatments were removed, and a single female was maintained without any harassers (C). This change of control treatment was necessary to reduce handling of the focal female and to allow for blood feeding taking place in the presence of males. Thus, in total 5 treatments were established: N2, N10, T2, T10, C. Each treatment had 29 females with the exception of the C treatment which had 25 females.

Every 3-4 days females were offered a human blood meal for 3-5 min, until a total of 10 blood meals were offered. An oviposition cup (90 ml capacity filled halfway with water and

lined with paper towel) was provided inside the carton, and females were supplied with 5% sucrose solution on a sugar wick (i.e. a low percent of sugar was given to stimulate blood feeding). One day before the blood meal, sugar wicks were removed to encourage feeding, and returned after blood feeding. In replicate one, on the morning of blood feeding, the focal female was aspirated from her carton and placed in a smaller cup (i.e. 0.5 L), to prevent the harasser females from taking a blood meal. After blood feeding, the female was returned to her carton (i.e. this took on average 4 hrs). In replicate two, control females were held alone and all females were fed directly in their carton. Blood feeding frequency was monitored after each blood meal. Cartons were examined every day for mortality (i.e. of harassers and focal female) during blood feeding and every 1-3 days thereafter. Dead harasser mosquitoes were replaced initially with mosquitoes from the same cohort. However, as the mortality rate of the harassers was relatively high due to a restricted diet, an additional cohort of mosquitoes was used to replace harassers. Treated harasser males had a significantly reduced longevity compared to normal males ($U=7,288.5$, $df=1$, $P<0.001$). In addition, significantly more harasser males and females died at high density than at low density ($U=7,146$, $df=1$, $P<0.001$).

Every 5-6 days oviposition cups were examined for eggs; if eggs were detected, the cup was removed and replaced with a new cup. After blood feeding ended (day 25-26), cartons were continuously supplied with 5% sucrose. Oviposition cups were removed 7-8 days after the last blood meal was offered. At this moment, all harasser mosquitoes were removed, and one last egg cup was given for any additional eggs. Mortality was recorded until day 96 when the majority of females had died, and a wing was removed to estimate body size. No significant differences were observed in female wing lengths between treatments (GLMs, Rep 1: $F_{5,86}=0.75$, $P=0.59$; Rep 2: $F_{4,87}=0.18$, $P=0.95$). Females from replicate two had significantly greater body size (wing length) than females from replicate one (t-test= -3.03 , $df=182$, $P=0.003$), although differences were small (Rep 1: 2.78 ± 0.01 (SEM) mm, Rep 2: 2.84 ± 0.02 mm).

The number of eggs laid per female was counted before conditioning and storage. Egg batches were individually hatched and subsamples of larvae screened for presence of wild-type larvae (i.e. indicating female mated with a second male). In replicate 1, egg papers were flooded with water to hatch; while in replicate 2 egg papers were vacuum hatched for 30 min to obtain a higher and more synchronous hatch rate. A total of 7,527 (i.e. approx. 36% of total amount eggs) and 14,644 larvae (i.e. approx. 54%) were screened for eye color in replicates 1 and 2, respectively.

Preparation of treated males—We created males that were unable to mate but capable of harassment by modifying their claspers. Males were anesthetized on ice for 15-30 min and the distal segment of each clasper was removed with a razorblade (See Supplement Fig S1). Longevity, insemination capability, and harassment rate of treated males were compared with normal males (Supplement 1). Briefly, there were no significant differences in longevity of treated or normal males when held without females. In addition, none of the females held with treated males were inseminated, indicating that the cutting procedure successfully prevented males from inseminating females. When males were allowed to harass females for 5 min, there was no difference in the ability of treated males to pursue

and contact females (without assuming the copulation position) compared to normal males (i.e. on average 5.0 ± 0.3 (SEM) events per female). However, treated males had a moderately lower number of mating attempts (4.7 ± 0.3 events per female) leading to a copulation position (venter-to-venter) than normal males (8.2 ± 0.5). We do not believe this impacted our findings as females were confined with males for an extended period of time. In addition, treated males spent significantly more time in attempted copulation than normal males because they could not establish firm genital contact with the female (Supplement 1).

Indirect effects of harassment on female offspring

To determine if there were any indirect effects of harassment, egg batches from females in replicate two were used to compare longevity and reproduction of daughters across treatments. The eggs collected 7-8 days after the last blood meal were used in this assessment as any harassment-related effects would have culminated at this stage. Egg papers with more than 30 eggs were randomly selected from ten females in every treatment and eggs were vacuum hatched. Individual families consisting of 19-30 first instar stage larvae were placed in a container ($10.2 \times 10.2 \times 7.5$ cm) in 0.5 l water and a fixed amount of food was added to each container on days 1, 3, 4, 5 and 6 (i.e. 6.8- 10.7 total mg food/larva). All pupae were counted and placed in individual tubes for emergence. The first three daughters that eclosed in each family were selected. All daughters were white-eyed and thus fathered by HWE males. Daughters underwent the same experimental protocol as their mothers, however, without harassment. Daughters were placed individually in a carton, and three to five days after eclosion, two MEX males aged 2-3 days were introduced for 2 days for mating and then removed. Females were offered a blood meal every 2-4 days on ten occasions (3 min; MEHH) and eggs were collected every 5-6 days. Females were maintained on 5% sucrose solution between and after blood feeding. Female longevity was monitored every 1-2 days until day 96. A wing was taken to determine female size.

Data analysis

All data analyses were performed in SPSS (v18, SPSS Inc, Chicago, IL, USA). Prior to analysis, data were checked for normality. When data were not normal, non-parametric tests were performed. All means presented in the text are $\pm$ SEM. The alpha value was set at 0.05. Benjamini-Hochberg (B-H) corrections on P values for multiple comparisons were applied to control for the false discovery rate (Benjamini and Hochberg 1995).

Survival data of females and daughters were analyzed separately with Cox regression analysis (Cox 1972). Females that escaped or were accidentally killed were censored in the analysis. Blood feeding frequency (time-dependent), host (LCH/MEHH), insect density (0, 2, 10), treatment (treated, normal, or female), and replicate (1 or 2) were initially used as covariates and non-significant terms were removed. Combined results are only described when there was no replicate effect. Survival graphs were derived from Kaplan-Meier survival analysis. Additionally, between-treatment comparisons were made using B-H corrected P values. The number of harasser mosquitoes that had died per cup was compared between treated and normal males, and between high and low density treatments with Mann-Whitney U tests. Replicates were combined. For comparison with low density treatments, the number of mosquitoes in the high density treatment was divided by 5.

Fecundity data were obtained by dividing the total number of eggs laid over a female's lifetime by the number of blood meals taken. General linear models (GLMs) were performed with host, density, male treatment, and replicate as factors, and their interactions. In addition, within replicates, treatments were compared using unplanned contrasts (Tukey HSD). Females with zero fecundity (most likely the result of non-insemination in the first mating, $n = 5$ for replicate 1 and 2 combined, and $n = 5$ for daughter experiment), and one female initially not mated by a HWE male in replicate 1 were removed from analysis. A Mann-Whitney U test was used to compare overall fecundity between mothers and daughters.

Blood feeding analysis only included females that were alive during the whole period of blood feeding. One female not mated initially by a HWE male in replicate 1 was excluded from analysis. The number of blood meals females took between treatments and replicates were analyzed using GLMs for direct and Kruskal-Wallis for indirect cost experiments.

Several life-table parameters were calculated for each treatment and replicate including survival (l_x), expected number of daughters (m_x) assuming a 1:1 sex ratio, net reproductive rate $R_0 (= l_x m_x)$, and generation time $T_c (= x l_x m_x / R_0)$ (Begon et al. 1996; Harrington et al. 2001). The intrinsic rate of increase (r) was calculated iteratively by solving Euler's equation (Begon et al. 1996). Females that escaped or were accidentally killed were not included in the analysis. Because eggs were not collected daily, egg production was averaged over the number of days included in the collection interval. Differences in R_0 curves between treatments were analyzed with Kaplan-Meier survival analysis within replicates and Cox regression analysis for combined results. B-H corrections to P values were applied.

Differences in larval survival (i.e. number of pupae divided by initial number of larvae added) between treatments for the indirect effects experiment were analyzed using GLMs with initial number of larvae added, and treatment, as variables. The relationship between initial number of larvae added and female body size (wing length) was analyzed with Pearson correlation. An independent t-test was used to compare body sizes between females and daughters.

Results

Direct effect of harassment on female fitness

Longevity—Females that co-habited with insects at a high density had a significantly reduced longevity compared to females that were maintained alone when replicates were combined ($W = 19.66$, $df = 2$, $P < 0.001$; Fig. 1a). In addition, there was a non-significant reduction in longevity compared to females maintained at a low density with male or female harassers (Fig. 1a). Male clasper modification had no effect and similar longevity was observed for females held with treated or normal males ($W = 0.84$, $df = 2$, $P = 0.66$). Between individual treatments, longevity of females housed with ten treated males was significantly reduced compared to females housed alone or at low densities with males or females (B-H corrected P values between < 0.001 - 0.016).

Fecundity—When replicates were combined, neither density nor male treatment had an effect on female fecundity, and similar levels of egg production were found between treatments ($F_{21,251} = 1.26$, $P = 0.21$; Fig. 2). Within replicate one, females held with 10 normal males (N10) had a significantly reduced fecundity compared to females held with 10 treated males (T10; $F_{5,129} = 2.72$, $P = 0.023$; Fig. 2). In replicate two, no significant differences were observed ($F_{4,133} = 1.57$, $P = 0.19$). Females took an equal numbers of blood meals in all treatments or replicates (i.e. 5.4 ± 1.2 ; $F_{21,207} = 1.18$, $P = 0.27$).

Twenty-one percent of females held with normal males at low density (N2) produced eggs with mixed offspring in replicate one, while for the high density treatment (N10) only 4% of females showed evidence of re-mating. In replicate two, 10% of females with mixed offspring were observed in the low density treatment (N2; Table 1). The low level of remating we observed could have had implications on the degree to which T and N treatments differed from one another as discussed below.

Net reproductive rate (R_0)—Females held with two normal males (N2) had significantly higher net reproductive rate (R_0) than females in all other treatments when replicates were combined (P values between < 0.001 - 0.008 ; Fig 3a), with the exception of females held with harasser females at high density (F10). Within replicate 2, similar results were obtained, and females held with 2 normal males had increased R_0 compared to females in the other treatments (Table 2). In replicate one, no statistically significant differences in R_0 were observed between treatments.

Indirect effect of harassment on female offspring

Larval survival—Female harassment treatment had no statistically significant effect on the larval survival of her offspring ($F_{4,35} = 2.27$, $P = 0.08$). However, a non-significant increase in survival of larvae from control females (C) compared to the other treatments was observed (Table 3). The initial number of larvae added did not affect pupation rate ($F_{7,35} = 0.69$, $P = 0.68$), and was not correlated to adult female size ($r = -0.17$, $P = 0.25$).

Longevity—Daughters from females that were held with 10 normal males (N10) had significantly reduced longevity compared to daughters from females that were held alone (C; $W = 6.92$, $df = 1$, $P = 0.036$; Fig. 1b).

Fecundity—Daughter fecundity was similar across all treatments ($F_{4,138} = 1.91$, $P = 0.11$; Table 3). There were no differences in blood feeding frequencies between daughters of the different treatments ($\phi^2 = 1.68$, $df = 4$, $P = 0.794$), and on average females took 4.3 ± 0.1 blood meals. There were no significant differences in female body size between treatments ($F_{4,102} = 1.43$, $P = 0.231$). Daughters had significantly greater body size (wings, 2.97 ± 0.01 mm) than their mothers (2.84 ± 0.02 mm; $t = 5.85$, $df = 197$, $P < 0.001$) due to different rearing conditions, and therefore daughters were more fecund than their mothers ($U = 5,517$, $df = 193$, $P < 0.001$).

Net reproductive rate (R_0)—Net reproductive rate (R_0) was significantly higher in daughters of females held with 10 normal males (N10) compared to daughters of all other females (B-H corrected P values between 0.015 - 0.040 , Table 2; Fig. 3b). In addition, there

was a non-significant increase in R_0 for daughters of females held with two males (normal or treated) compared to daughters from females held alone (B-H corrected $P=0.063$, Fig. 3b).

Discussion

In this study we examined direct and indirect effects of male harassment on female fitness in the predominantly monandrous mosquito *A. aegypti*. Females held with males at a high density had a reduced longevity compared to females held alone or at low density. Females housed with normal males at low density had increased reproductive rate (R_0) compared to females held with males in the other treatments. These results were largely consistent within replicates, despite the use of different controls because of handling and feeding considerations. Indirect effects were observed, and daughters of females housed with normal males at high density had shorter lifespans but the highest R_0 . These findings are discussed in more detail below.

Whether or not the observed reduction in longevity of females housed at high density with males was solely due to male harassment is unclear, as females housed with a high density of harasser females also experienced a reduction in longevity. Exposure to low densities of males did not impact female longevity. Male treatment (i.e. clasper modification or not) was not a significant factor in the model describing longevity. This suggests that the observed reduction in longevity was not the result of insemination *per se*. However, evidence of remating was found only for a small number of females in the normal treatments, thus it is unclear how different the normal and treated groups were. Other factors, such as energetic costs due to repeated contact, or a reduced opportunity to rest and sugar feed, could have mediated survival. Females in this study had to fly short distances to sugar feed and oviposit, and were subject to male harassment in these instances. In addition, females in replicate two which were fed directly in the cup were subjected to harassment during blood feeding. Although direct observations were not used in this study, harassment of females by males was regularly observed during blood feeding and egg collection. Styer et al (2007) observed no effect of the presence of males on female survival or reproduction in *A. aegypti*, however a 1:1 ratio of insects was used in this study. Co-habiting with males can exact a survival cost on females in other insects. In the dung fly *S. cynipsea*, females had a reduced longevity when co-habiting with males, and the costs of rejecting a male were minor compared to the costs of copulation (Muhlhauser and Blanckenhorn 2002). On the other hand, studies with *Callosobruchus* seed beetles suggested that the observed reduction in longevity was mostly due to the energetic costs a female sustained in resisting male harassment, and not due to the cost of copulation *per se* (Ronn et al. 2006). In the field cricket *G. bimaculatus*, significant reductions in female longevity were observed under various levels of male courtship, however, this was not due to compounds in the ejaculate (Bateman et al. 2006). Female *A. aegypti* can reject mating attempts by evasive flight and kicking (Jones 1974; Cator and Harrington 2011), however, the relative costs of rejection versus copulation remain to be determined. In addition, laboratory mated females are frequently observed to engage in pseudo-copulation, which results in brief genital contact without semen transfer (Gwadz et al. 1971). It is not clear whether pseudo-copulation occurs in nature.

Co-habiting with males had no consistent effect on female fecundity. In other insects, negative effects on female fecundity due to male harassment and copulation have been reported. In the adzuki bean beetle *C. chinensis*, male harassment significantly reduced female fitness (measured by mean number of offspring), due to a loss of time for oviposition and/or energetic costs to avoid male harassment (Sakurai and Kasuya 2008). In other *Callosobruchus* species, females had a reduced fecundity when co-habiting with males (Ronn et al. 2006). In *C. maculatus*, females held with males had lower lifetime reproductive rates than females held alone (den Hollander and Gwynne 2009), and male harassment led to a reduction in fecundity (Gay et al. 2009). In the leafcutting bee *Megachile rotundata* male harassment decreased female foraging behavior and fecundity (Rossi et al. 2010). In our investigation using the horizontal life table analysis approach, which incorporates longevity, females benefitted from access to normal males at moderate densities (N2) as they had overall highest net reproductive rate (R_0) compared to all other male treatments. However, their R_0 was similar to females housed with other females at high density (F10). Females held with treated males did not show the same increase in R_0 as N2 females, which could suggest that benefits were mediated through components transferred in the ejaculate, although only a small proportion of females definitely received this additional ejaculate. Further studies should be conducted to more definitively determine the role of additional ejaculates and other factors such as good genes effects on female fitness.

Male claspers were modified to determine the effects of harassment in the absence of mating. Treated males had a reduced longevity compared to normal males when kept with females, but not when kept with males alone in the control experiment. This could indicate that treated males spent more effort in harassment because their behavior could not be completed, leading to a reduction in their longevity. Interestingly, the greatest reduction in female longevity was observed when females were housed with ten treated males.

In this study we observed polyandry in 0-21% of females held with normal males, which is similar to the range observed when this species is tested in field enclosures (Helinski et al. 2012). Females in the low density treatment had higher rates of multiple mating compared to females in the high density treatment, which is counterintuitive to the assumption that crowding is largely responsible for polyandry in laboratory cage studies (Mahmood and Reisen 1980). On the other hand, males in the high density treatment could have interfered more with one another compared to males in the low density treatment which could have interrupted mating. In this study, polyandry was only detected if a female used the second male's sperm for fertilization. In *Aedes*, sperm use dynamics are poorly understood and it is thus possible that we underestimated re-mating rate if second male sperm is not frequently used for fertilization. Monandry in female *A. aegypti* is induced by seminal fluid proteins (SFPs) in the ejaculate (Craig 1967). Interestingly, *A. aegypti* females have three spermathecae, which in polyandrous species may be used to exercise cryptic female choice (Eberhard 1996). It has been suggested that the existing number of spermathecae and the effects of SFPs on females are evidence of ongoing sexual conflict (Yuval 2006). As there appears to be no major cost of additional copulations to females and even a potential benefit in the form of increased R_0 for the highest mating females (N2), the current situation of monandry seems to reflect primarily male benefits. In the monandrous house fly, *Musca*

domestica, female monandry was maintained despite potential beneficial effects of multiple mating on female fitness (Arnqvist and Andrés 2006). Indirect evidence of a beneficial effect of SFPs in *A. aegypti* comes from a study examining the effects of male mating history on female longevity. Females mated to semen-depleted males had reduced longevity compared to females mated to virgin males (Helinski and Harrington 2011).

Besides direct effects, maternal environmental conditions can have indirect effects on offspring fitness (Bernardo 1996; Mousseau and Fox 1998; Wolf and Wade 2009). Daughters of females held without males had increased longevity compared to daughters of females held with ten normal males. However, fitness (R_0) was highest in daughters of females held with ten normal males, indicating that females produced more eggs over their shorter life-span. Whether or not longevity was shortened due to the increased reproductive rate or due to mother holding conditions cannot be determined. Maintaining females on a diet of sugar only (i.e. without the possibility to oviposit) would clarify this question further. There have been few studies in mosquitoes where parental conditions have been followed through to the offspring. In the malaria mosquito *Anopheles stephensi*, offspring of parents reared at low food conditions produced more eggs than the offspring from parents reared at high food conditions (Grech et al. 2007).

For disease-transmitting mosquitoes, little is known with regard to mating behavior and the determinants of male and female mating success, in spite of their importance as disease vectors (Ferguson et al. 2005; Yuval 2006; Howell and Knols 2009; Ferguson et al. 2010). In nature, mated females are likely to encounter males around the blood host (Hartberg 1971). However, the rate at which females will be harassed is expected to be much lower than what was observed in our study where insects were confined in space. On the other hand, females in nature experience many more challenges not encountered under laboratory conditions such as energetic, environmental, and predation stress that might exacerbate the effects of harassment. We conclude that females of this species do not suffer a fitness cost from prolonged harassment of males when kept at moderate densities, and that there is some indication that females benefit from access to males, potentially mediated through components in the ejaculate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We are thankful to all members of the Harrington laboratory at Cornell for their support, to Elizabeth Glennon and Melissa Hardstone for assisting with egg collection and counting, and to Laura Sirot, Lauren Cator, and anonymous reviewers for constructive comments on our manuscript. This study was supported by the Foundation for the National Institutes of Health through the Grand Challenges in Global Health Initiative (GC7 #316), and by NIH/NIAID grant R01AI095491.

References cited

Arnqvist G, Andrés JA. The effects of experimentally induced polyandry on female reproduction in a monandrous mating system. *Ethology*. 2006; 112:748–756.

- Arnqvist G, Edvardsson M, Friberg U, Nilsson T. Sexual conflict promotes speciation in insects. *P Natl Acad Sci USA*. 2000; 97:10460–10464.
- Arnqvist G, Nilsson T. The evolution of polyandry: multiple mating and female fitness in insects. *Anim Behav*. 2000; 60:145–164. [PubMed: 10973716]
- Arnqvist, G.; Rowe, L. *Sexual Conflict*. Princeton University Press; Princeton, New Jersey: 2005.
- Bateman PW, Ferguson JWH, Yetman CA. Courtship and copulation, but not ejaculate, reduce the longevity of female field crickets (*Gryllus bimaculatus*). *J Zool*. 2006; 268:341–346.
- Begon, M.; Townsend, CR.; Harper, JL. *Ecology: from individuals to ecosystems*. Blackwell; Oxford: 1996.
- Benjamini Y, Hochberg Y. Controlling the false discovery rate: A practical and powerful approach to multiple testing. *J Roy Stat Soc B*. 1995; 57:289–300.
- Bernardo J. Maternal effects in animal ecology. *Amer Zool*. 1996; 36
- Blanckenhorn WU, Hosken DJ, Martin OY, Reim C, Teuschl Y, Ward PI. The costs of copulating in the dung fly *Sepsis cynipsea*. *Behav Ecol*. 2002; 13:353–358.
- Cator LJ, Arthur BJ, Ponlawat A, Harrington LC. Behavioral observations and sound recordings of free-flight mating swarms of *Ae. aegypti* in Thailand. *J Med Entomol*. 2011; 48:941–946. [PubMed: 21845959]
- Cator LJ, Harrington LC. The harmonic convergence of fathers predicts the mating success of sons in the yellow fever mosquito. *Anim Behav*. 2011; 82:629–633.
- Chapman T, Liddle LF, Kalb JM, Wolfner MF, Partridge L. Cost of mating in *Drosophila melanogaster* females is mediated by male accessory gland products. *Nature*. 1995; 373:241–4. [PubMed: 7816137]
- Clements, AN. *The Biology of Mosquitoes*. CABI Publishing; Wallingford: 1999.
- Clutton-Brock TH, Parker GA. Sexual coercion in animal societies. *Anim Behav*. 1995; 49:1345–1365.
- Cox DR. Regression Models and Life Tables (with Discussion). *J Roy Stat Soc B*. 1972; 34
- Craig GB. Mosquitoes: Female monogamy induced by male accessory gland substance. *Science*. 1967; 156:1499–1501. [PubMed: 5611023]
- Crudgington HS, Siva-Jothy MT. Genital damage, kicking and early death. *Nature*. 2000; 407:855–6. [PubMed: 11057654]
- den Hollander M, Gwynne DT. Female fitness consequences of male harassment and copulation in seed beetles, *Callosobruchus maculatus*. *Anim Behav*. 2009; 78:1061–1070.
- Eberhard, WG. *Sexual selection and animal genitalia*. Harvard University Press; Cambridge, MA: 1985.
- Eberhard, WG. *Female control: sexual selection by cryptic female choice*. Princeton University Press; Princeton, New Jersey: 1996.
- Eberhard WG. Male-female conflict and genitalia: failure to confirm predictions in insects and spiders. *Biol Rev Camb Philos Soc*. 2004; 79:121–86. [PubMed: 15005176]
- Ferguson HM, Dornhaus A, Beeche A, Borgemeister C, Gottlieb M, Mulla MS, Gimnig JE, Fish D, Killeen GF. Ecology: a prerequisite for malaria elimination and eradication. *PLoS Med*. 2010; 7:e1000303. [PubMed: 20689800]
- Ferguson HM, John B, Ng'habi K, Knols BG. Redressing the sex imbalance in knowledge of vector biology. *Trends Ecol Evol*. 2005; 20:202–9. [PubMed: 16701369]
- Garcia-Rejon J, Lorono-Pino MA, Farfan-Ale JA, Flores-Flores L, Del Pilar Rosado-Paredes E, Rivero-Cardenas N, Najera-Vazquez R, Gomez-Carro S, Lira-Zumbardo V, Gonzalez-Martinez P, Lozano-Fuentes S, Elizondo-Quiroga D, Beaty BJ, Eisen L. Dengue virus-infected *Aedes aegypti* in the home environment. *Am J Trop Med Hyg*. 2008; 79:940–50. [PubMed: 19052309]
- Gay L, Eady PE, Vasudev R, Hosken DJ, Tregenza T. Costly sexual harassment in a beetle. *Physiol Entom*. 2009; 34:86–92.
- Grech K, Maung LA, Read AF. The effect of parental rearing conditions on offspring life history in *Anopheles stephensi*. *Malar J*. 2007; 6:130. [PubMed: 17892562]
- Gwadz RW, Craig GB Jr, Hickey WA. Female sexual behavior as the mechanism rendering *Aedes aegypti* refractory to insemination. *Biol Bull*. 1971; 140:201–14. [PubMed: 5552355]

- Harrington LC, Edman JD, Scott TW. Why do female *Aedes aegypti* (Diptera: Culicidae) feed preferentially and frequently on human blood? *J Med Entomol.* 2001; 38:411–22. [PubMed: 11372967]
- Harrington LC, Scott TW, Lerdthusnee K, Coleman RC, Costero A, Clark GG, Jones JJ, Kitthawee S, Kittayapong P, Sithiprasasna R, Edman JD. Dispersal of the dengue vector *Aedes aegypti* within and between rural communities. *Am J Trop Med Hyg.* 2005; 72:209–20. [PubMed: 15741559]
- Hartberg WK. Observations on the mating behaviour of *Aedes aegypti* in nature. *Bull World Health Organ.* 1971; 45:847–850. [PubMed: 5317018]
- Helinski MEH, Harrington LC. Male mating history and body size influence female fecundity and longevity of the dengue vector *Aedes aegypti*. *J Med Entomol.* 2011; 48:202–211. [PubMed: 21485355]
- Helinski MEH, Valerio L, Facchinelli L, Scott TW, Ramsey J, Harrington LC. Evidence of polyandry for *Aedes aegypti* in semifield enclosures. *Am J Trop Med Hyg.* 2012; 86:635–641. [PubMed: 22492148]
- Howell PI, Knols BG. Male mating biology. *Malar J.* 2009; 8(Suppl 2):S8. [PubMed: 19917078]
- Jennions MD, Petrie M. Why do females mate multiply? A review of the genetic benefits. *Biol Rev Camb Philos Soc.* 2000; 75:21–64. [PubMed: 10740892]
- Jones TM. Sexual activities during single and multiple co-habitations in *Aedes aegypti* mosquitoes. *J Entomol.* 1974; 48:185–194.
- Mahmood F, Reisen WK. *Anopheles culicifacies* the occurrence of multiple insemination under laboratory conditions. *Entomol Exp Appl.* 1980; 27:69–76.
- Morrow EH, Arnqvist G, Pitnick S. Adaptation versus pleiotropy: why do males harm their mates? *Behav Ecol.* 2003; 14:802–806.
- Mousseau TA, Fox CW. The adaptive significance of maternal effects. *Trends Ecol Evol.* 1998; 13:403–407. [PubMed: 21238360]
- Muhlhauser C, Blanckenhorn WU. The cost of avoiding matings in the dung fly *Sepsis cynipsea*. *Behav Ecol.* 2002; 13:359–365.
- Parker GA. Sexual conflict over mating and fertilization: an overview. *Phil Trans R Soc B.* 2006; 361:235–259. [PubMed: 16612884]
- Perich MJ, Davila G, Turner A, Garcia A, Nelson M. Behavior of resting *Aedes aegypti* (Culicidae: Diptera) and its relation to ultra-low volume adulticide efficacy in Panama City, Panama. *J Med Entomol.* 2000; 37:541–6. [PubMed: 10916294]
- Ponlawat A, Harrington LC. Age and body size influence male sperm capacity of the dengue vector *Aedes aegypti* (Diptera: Culicidae). *J Med Entomol.* 2007; 44:422–6. [PubMed: 17547226]
- Rodriguez-Munoz R, Bretman A, Slate J, Walling CA, Tregenza T. Natural and sexual selection in a wild insect population. *Science.* 2010; 328:1269–72. [PubMed: 20522773]
- Ronn J, Katvala M, Arnqvist G. The cost of mating and egg production in *Callosobruchus* seed beetles. *Anim Behav.* 2006; 72:335–342.
- Rossi BH, Nonacs P, Pitts-Singer TL. Sexual harassment by males reduces female fecundity in the alfalfa leafcutting bee, *Megachile rotundata*. *Anim Behav.* 2010; 79:165–171.
- Roth LM. A study of mosquito behavior. An experimental laboratory study of the sexual behavior of *Aedes aegypti* (Linnaeus). *Am Midl Nat.* 1948; 40:265–352.
- Sakurai G, Kasuya E. The costs of harassment in the adzuki bean beetle. *Anim Behav.* 2008; 75:1367–1373.
- Scott TW, Amerasinghe PH, Morrison AC, Lorenz LH, Clark GG, Strickman D, Kittayapong P, Edman JD. Longitudinal studies of *Aedes aegypti* (Diptera: Culicidae) in Thailand and Puerto Rico: blood feeding frequency. *J Med Entomol.* 2000; 37:89–101. [PubMed: 15218911]
- Scott TW, Chow E, Strickman D, Kittayapong P, Wirtz RA, Lorenz LH, Edman JD. Blood-Feeding Patterns of *Aedes aegypti* (Diptera: Culicidae) Collected in a Rural Thai Village. *J Med Entomol.* 1993; 30:922–927. [PubMed: 8254642]
- Shutt B, Stables L, Aboagye-Antwi F, Moran J, Tripet F. Male accessory gland proteins induce female monogamy in anopheline mosquitoes. *Med Vet Entomol.* 2010; 24:91–4. [PubMed: 20377737]
- Spielman A. The mechanics of copulation in *Aedes aegypti*. *Biol Bull.* 1964; 127:324–344.

- Styer LM, Minnick SL, Sun AK, Scott TW. Mortality and reproductive dynamics of *Aedes aegypti* (Diptera: Culicidae) fed human blood. *Vector Borne Zoonotic Dis.* 2007; 7:86–98. [PubMed: 17417961]
- Tripet F, Toure YT, Dolo G, Lanzaro GC. Frequency of multiple inseminations in field-collected *Anopheles gambiae* females revealed by DNA analysis of transferred sperm. *Am J Trop Med Hyg.* 2003; 68:1–5. [PubMed: 12556139]
- Watson PJ, Arnqvist G, Stallmann RR. Sexual conflict and the energetic costs of mating and mate choice in water striders. *Am Nat.* 1998; 151:46–58. [PubMed: 18811423]
- Wendell MD, Wilson TG, Higgs S, Black WC. Chemical and gamma-ray mutagenesis of the white gene in *Aedes aegypti*. *Insect Mol Biol.* 2000; 9:119–25. [PubMed: 10762419]
- Wolf JB, Wade MJ. What are maternal effects (and what are they not)? *Philos Trans R Soc Lond B.* 2009; 364:1107–15. [PubMed: 19324615]
- Wolfner MF. Tokens of love: functions and regulation of *Drosophila* male accessory gland products. *Insect Biochem Mol Biol.* 1997; 27:179–92. [PubMed: 9090115]
- Yuval B. Mating systems of blood-feeding flies. *Ann Rev Entomol.* 2006; 51:413–440. [PubMed: 16332218]

Fig. 1.

Survival of females for A) Direct harassment (both replicates combined), and B) Indirect harassment (daughters from harassed females). Females were maintained on a diet of blood and sugar. Females were kept with normal (N) or treated (T) males, females (F), or no mosquitoes (C) at low (2) or high (10) densities. Different letters indicate significance at $P < 0.05$ (Cox regression, Benjamini-Hochberg corrections on P values were applied). In A, different letters indicate significance between density treatments, while in B treatments were compared against the control treatment (C).

Fig. 2.

Mean fecundity $\pm$ SEM (number of eggs/ number of blood meals) of harassed females from replicate 1 (white bars), replicate 2 (grey bars) and replicates combined (black bars). Different letters/numerals indicate significant differences ($P < 0.05$, GLMs with Tukey HSD) between treatments, separately analyzed for each series (i.e. rep 1, rep 2, or replicates combined).

Fig. 3.

Cumulative net reproductive rate (R_0) for females from A) Direct harassment (averaged over both replicates), and B) Indirect harassment (daughters from harassed females). Females were blood fed for the first 25-27 days of their life. Different letters/numerals indicate significant differences between treatments for each graph at $P < 0.05$ with Cox regression (A) or Kaplan-Meier Log Rank (B); Benjamini-Hochberg corrections on P values were applied.

Table 1

Phenotype of larvae from females in the direct harassment experiment. The number of larvae classified as white eyes (HWE) or wild-type (WT) is shown for replicates 1 and 2. The percent of females with mixed offspring, and between brackets the number of females producing egg batches with mixed offspring over the total number of females in the treatment, is given.

Rep	Larval phenotype	Treatment				
		N2	N10	T2	T10	Control ^I
1	HWE	1575	1733	1993	1732	494
	WT	46	89 [†]	0	0	0
	% ♀ mixed offspring	21	4	-	-	-
	(♀ mixed offspring/ total ♀)	(5/24)	(1/24)			
2	HWE	4053	3643	2581	3225	1142
	WT	104	0	0	0	0
	% ♀ mixed offspring	10	0	-	-	-
	(♀ mixed offspring/total ♀)	(3/29)	(0/29)			

[†] 82 of these eggs larvae were laid by a single female who only produced wild-type larvae.

^I Control treatments are F2 and F10 combined for replicate 1, and C for replicate 2.

Table 2

Horizontal life-table characteristics of females in direct and indirect harassment experiments. Net reproductive rate (R_0), generation time (T_c), and intrinsic rate of increase (r) are displayed. Females were kept with normal (N) or treated (T) males, females (F), or no mosquitoes (C) at low (2) or high (10) densities. Daughters were the offspring of females from replicate 2, and were held without any harasser males or females (Indirect). Differences in R_0 over time were analyzed between treatments for each row with Kaplan Meier Log rank tests. Different letters indicate significance at $P < 0.05$ (Benjamini-Hochberg corrected).

Direct	Parameters	N2	N10	T2	T10	F2	F10	C
Rep 1	R_0	137.9A	128.9A	131.8A	133.1A	130.2A	136.5A	-
	T_c	14.4	14.8	14.7	14.4	14.7	14.2	-
	r	2.04	1.93	1.93	2.11	1.87	2.04	-
Rep 2	R_0	136.8a	121.0b	120.6b	123.1b	-	-	123.8b
	T_c	13.9	13.2	16.0	13.6	-	-	15.5
	r	2.19	2.11	1.81	2.11	-	-	2.11
Indirect	R_0	138.2B	158.2A	140.1B	128.6B	-	-	131.6B
	T_c	15.4	16.4	16.0	14.8	-	-	17.1
	r	1.97	1.95	1.85	2.03	-	-	1.67

Dashes indicate data were not collected.

Table 3

Mean $\pm$ SEM larval survival (number of pupae / number of L1 stage larvae) of egg batches from harassed females, and mean fecundity $\pm$ SEM (number of eggs/ number of blood meals) of daughters of harassed females.

Parameter	Treatment				
	N2	N10	T2	T10	C
Larval survival	0.58 $\pm$ 0.06 a	0.69 $\pm$ 0.05 a	0.69 $\pm$ 0.05 a	0.52 $\pm$ 0.07 a	0.80 $\pm$ 0.05 a
Fecundity	67.8 $\pm$ 4.8 a	75.9 $\pm$ 2.5 a	77.2 $\pm$ 3.9 a	69.6 $\pm$ 4.4 a	64.1 $\pm$ 3.9 a

Different letters indicate significant differences between treatments for each row at $P < 0.05$ (GLMs).