Journal of Insect Physiology 108 (2018) 1–9

Contents lists available at ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

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

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ARTICLE INFO

Keywords:
Post-mating
Seminal fluid
Blood feeding
Fecundity
Survival
R_0
Life table analysis

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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https://doi.org/10.1016/j.jinsphys.2018.05.001
Received 20 August 2017; Received in revised form 30 April 2018; Accepted 1 May 2018
Available online 03 May 2018

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