FACTORS INFLUENCING THE FEEDING RESPONSE OF LABORATORY-REARED *AEDES AEGYPTI*

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Abstract. We evaluated the effects of membrane surface area (cm^2), female density, and container/cage size on feeding response in laboratory-reared *Aedes aegypti*. Female density did not affect feeding rates at low surface areas, but higher density did significantly increase feeding as surface area increased. Females in large, cloth cages fed less compared to those in large, plastic cups. The rate of feeding was higher when using live, anesthetized mice versus a membrane feeding system. The AFRIMS Insectary will continue to look for innovative ways to improve our membrane feeding system.

Keywords: *Aedes aegypti*, feeding response, surface area, female density

INTRODUCTION

Reported cases of dengue and dengue hemorrhagic fever have experienced an exponential increase over the last 30 years with the number of cases reported to the WHO between 2000 and 2007 over doubling those in the previous decade; Southeast Asian and Western Pacific countries bear the brunt of global disease burden due to dengue (WHO, 2009). While

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dengue-like illness has been reported from tropical urban centers since 1780, the current Asian pandemic probably began with the wide-spread habitat disruption and consequent transportation of the mosquito, *Aedes aegypti* throughout Southeast Asia during World War II (Gubler, 1998, 2002).

The study of various factors relating to disease transmission of *Aedes aegypti* mosquitoes is facilitated by using lab-reared mosquitoes. Managing mosquito colonies to produce 100,000s of female mosquitoes monthly is a technically challenging endeavor and those who successfully maintain these colonies are consulted for advice. The Department of Entomology, Armed Forces Research Institute of Medical Sciences (AFRIMS) receives many requests for advice on colony management. While trying to provide the best information available on rearing techniques, we were surprised to find

little published documentation on some husbandry factors that support highly productive mosquito colonies. Many elements potentially involved in the feeding response of mosquitoes are well studied: temperature (Cosgrove and Wood, 1995), type of meal proposed (Cosgrove and Wood, 1996; Harre *et al*, 2001), type of feeding membrane and mosquito species (Blackwell *et al*, 1994). However, no investigations into the relationship between container size, available feeding surface area, and female density on feeding success could be found. This is surprising given the long history of artificial feeding methods (Rutledge *et al*, 1964) and is reflected in the frequency of questions we receive regarding these particular aspects of mosquito rearing.

Direct host blood feeding was the initial method used in mass rearing mosquitoes (Morlan *et al*, 1963). Using animals can be expensive, inconvenient, and is increasingly unacceptable in terms of animal welfare (Nasirian and Ladonni 2006), however logistical constraints on available blood supplies often require their use and any impact on colony maintenance needs to be investigated. Membrane feeding techniques quickly developed to manage the ever increasing demand for laboratory-reared mosquitoes to assist in research and control methods (Tarshis, 1956). However, differences in mosquito feeding response between direct animal and membrane feeding (Bunner *et al*, 1989) necessitate re-evaluation of any methodological changes.

This study was initiated to investigate the affects of membrane surface area, container size and female density on artificial membrane feeding rates of female *Aedes aegypti* (L.). The optimum combination of these three factors was then used to test for differences in feeding rates between artificial membrane and live host assays.

MATERIALS AND METHODS

Aedes aegypti are colonized and maintained by standardized rearing procedures in the insectary of the Department of Entomology, Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok. The eggs were hatched in a glass jar with 250 ml of water. We then transferred batches of 200 larvae to white plastic trays (30 x 35 x 5 cm). Fish food (HIPRO®) was added (0.1 g for 1st and 2nd instar larvae, 0.3 g for 3rd instar larvae, and 0.5 g for 4th instar larvae at 08:00 AM and 04:00 PM each day) to each tray for the successive two weeks until pupation of all larvae. The pupae were collected weekly and kept in a holding cage (size 30 x 40 x 30 cm) for emergence. Adult mosquitoes were reared at 25±2ºC, and a relative humidity of 80±10% with a photoperiod of 12 hours light followed by 12 hours dark (12L:12D). We provided adults with soaked cotton balls containing a 5% multivitamin solution. Adult female *Ae. aegypti*, 5 to 7 days post-eclosion, were used in these experiments. The night before being exposed to the blood meal they were deprived of the multivitamin solution and supplied with water pads only. Mice (*Mus musculus*) for direct feeding were obtained from the Department of Veterinary Medicine (DVM), Armed Forces Research Institute of Medical Sciences (AFRIMS), while commercial defibrinated sheep blood for the membrane feeding system was purchased from the National Laboratory Animal Center, Mahidol University (NLAC-MU), Nakhon Pathom, Thailand.

We evaluated three levels of female density, three container sizes and four different membrane surface areas in the initial experiment. Fifty, 100 or 150

female mosquitoes were placed in mesh covered small plastic cups (8 cm dia. x 8 cm high), large plastic cups (16 cm dia. x 16.5 cm high) or cloth covered cages (30 cm x 40 cm x 30 cm). For the membrane feeding system, mosquitoes were fed on sheep blood through a sausage casing skin membrane stretched over a standard membrane feeder (Rutledge *et al*, 1964) with surface area of 1.8 cm^2, 2.5 cm^2, 9.6 cm^2 or 30.2 cm^2 and secured with a rubber band. Water temperature was maintained at a temperature of 37ºC (±2ºC) and was circulated through the feeder water jacket before and during feeding. Blood volumes of 1 ml, 1.5 ml, 2.5 ml and 10 ml were added to the glass feeder cones with larger blood volumes paired with larger membrane surface areas. The mosquitoes were allowed to feed for 15 minutes through the membrane, after which time the membrane feeder was removed from the container. Then, all mosquitoes that failed to engorge were removed and the numbers of fully engorged mosquitoes were counted. For the comparison between direct and membrane feeding, mice were anesthetized by intraperitoneal injection (using 1ml syringe with a 23-39 g x - 1" needle in accordance with AFRIMS Animal Use Protocol VET-VC-101-00; Giving Intraperitoneal Injections to Small Animals and Non-huma Primates) with a combination of Ketamine HCl, Xylazine, and Atropine (40 mg-ketamine, 2-mg xylazine, 0.06-mg atropine) at a dose of 0.1 ml per 100 g body weight in accordance with AFRIMS IACUC approved Animal Use Protocol VET-VC-201-00: Rodent Injectable Anesthesia) and placed on the large cup (16 cm dia. x 16.5 high) after which they were fed upon by 50, 100, or 150 females mosquitoes for 15 minutes. Only the large plastic cup and 9.6 cm^2 surface area feeders were compared to the live feeding, otherwise membrane feeding was performed as described above.

All experiments were replicated 5 times. Differences in percent blood feeding were compared by analysis of deviance of a generalized linear model (GLM) with quasibinomial error structure and logit link function with an alpha = 0.05 considered significant for this test. We used a quasibinomial distribution to account for overdispersion in the model resulting in overly conservative estimates of the model coefficient standard errors. We estimated the scale parameter $ø = X^2 / df$, as the value of Pearson's X^2 from the model divided by the model's residual degrees of freedom. All model coefficient standard errors were then multiplied by $\sqrt{\hat{ø}}$ (Fox and Weisberg, 2011). A measure of model performance, $R2 = 1 - \frac{logeL_1}{logeL_\circ}$, where L_1 and L_0 are the log likelihoods for the full and intercept-only models, was also calculated (Fox, 2008). Post-hoc comparisons of significant affects were calculated using the Holm procedure to control the familywise error rate (Bretz *et al*, 2011). Our analyses were performed in R version 3.0.1 (R Core Team, 2013).

Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 2011 edition.

RESULTS

There were significant container, membrane surface area, female density, and membrane surface area x female density interaction effects (Table 1). The significant interaction is explained by the larger proportion of mosquitoes feed at densities

Table 1

Analysis of deviance examining the effects of membrane surface area (cm^2), female density, and container size on feeding rates of adults, female *Ae. aegypti*.

Source	SS	df	F	Pr(<F)
Container	180.37	2	5.9	0.003
Membrane surface area	902.09	3	19.67	<0.001
Female density	104.4	2	3.42	0.0356
Container x surface area	116.67	6	1.27	0.2737
Container x female density	41.43	4	0.68	0.6086
Surface area x female density	408.65	6	4.46	<0.001
Container x surface area x female density	125.14	12	0.68	0.7664
Residuals	2,200.89	144		

ø = 14.96; R^2 = 0.38.

Table 2

Analysis of deviance examining using live mice versus a membrane feeding system on the feeding rates of adult, female *Ae. aegypti*.

Source	SS	df	F	Pr(<F)
Female density	1.62	2	0.2	0.8179
Feeding method	87.02	1	21.75	<0.001
Female density x feeding method	13.63	2	1.7	0.2032
Residuals	96.01	24		

ø = 4.0; R^2 = 0.44.

of 150 females and membrane surface area of 9.6 cm^2 (mean ±SE; 72.0±3.3) than for the lowest density at both 1.76 cm^2 and 2.54 cm^2 (36.8±7.2, p=0.007 and 46.5±7.1, p=0.001, respectively) (Fig 1). More mosquitoes feed to engorgement when in the large plastic cup (49.7±2.7) compared to the large cloth cages (37.1±2.7, p=0.002); however, feeding rates in the small plastic cup (42.9±2.7) did not for either the large cup (p=0.152) or cloth cage (p=0.240) (Fig 2). Female density showed no effect on the proportion of mosquitoes engorged between direct feeding and membrane feeding (Table 2). However, a significantly higher proportion of females feed on the live animal (91.2±1.3) then from membrane feeding (79.2±2.1, p<0.001) (Fig 3).

Overdispersion is a common occurrence in binomial GLMs which can result for several reasons, including: correlated or clustered observations, model-misspecification, or unmodeled heterogeneity in the form of excessive variability in the proportions of engorged females across each factor combination. Looking at boxplots of individual proportions across all factor levels in each model suggests the last mentioned reason is responsible for the high dispersion values.

DISCUSSION

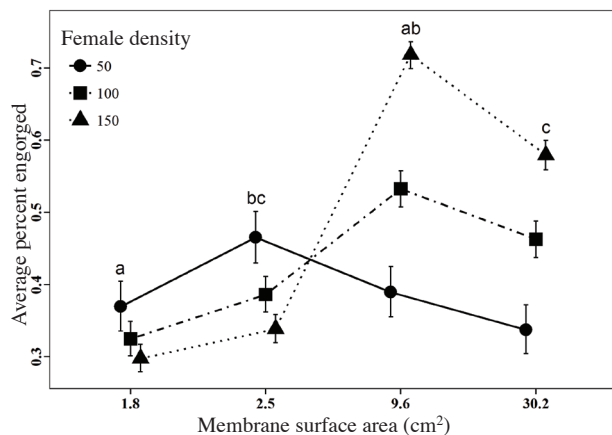That female mosquito density would affect feeding success seems rather intuitive. Host defensive responses increase as

Fig 1–Plot of average percent engorged (± SE) females examining the effects of membrane surface area (cm^2) and female density on female feeding rates. Values with the same letters are significantly different at α = 0.05.
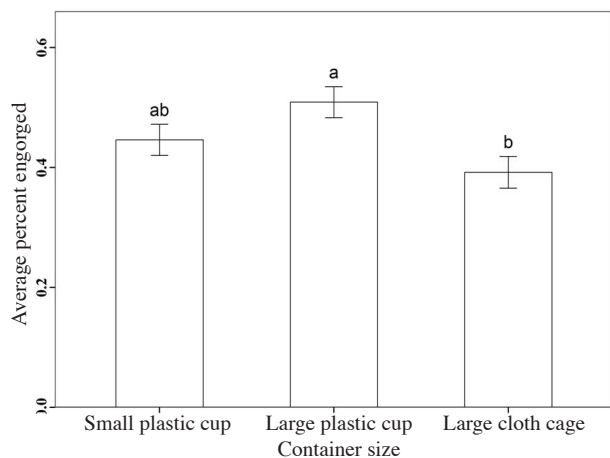

Fig 2–Effects of container size on average percent engorgement (± SE) of adult, female *Ae. aegypti*. Values with the same letters are significantly different at α = 0.05.

biting pressure intensifies leading to reduced feeding success (Klowden and Lea, 1978). Alternatively, it makes sense that agitation among females as available biting surface area decreases would also lead to decreasing feeding opportunities. We found better feeding success from larger surface areas at higher female densities. Several reasons could account for this observation. Available feeding area is very constrained at the two lowest surface areas leading to disruption in feeding as females compete for the limited space so even at lower female densities feeding rates were suppressed. However, while higher surface area seemed to alleviate crowding, rather than seeing a general increase in feeding across all densities, only higher female densities benefited. One difficulty we encountered using the large surface membrane feeders was blood clotting. A mosquito's capacity to maintain blood flow is limited to approximately the size of its blood meal (Stark and James, 1995). This suggests that while lower densities eased crowding, the inability to keep unclotted blood along the membrane/blood interface resulted in poorer feeding overall.

Container size can be a contributing factor in successful feeding (Barnard *et al*, 1998), although container composition may also be a contributing factor. Female density was not a contributing factor in feeding rate success even though available space went from 240 cm^3 in the cloth cage/low density treatment to 2.7 cm^2 small plastic cup/high density treatment. Rather, both the large plastic (29.8%) and small plastic (13.7%) containers had higher feeding success rates, even if the small, plastic cups were not significantly greater than the cloth cages. Given the mosquito's habit of loitering on verticle surfaces, this suggests that the smooth walls of the plastic containers provided an unsatisfactory resting surface leading to a higher encounter rate with the feeding membrane.
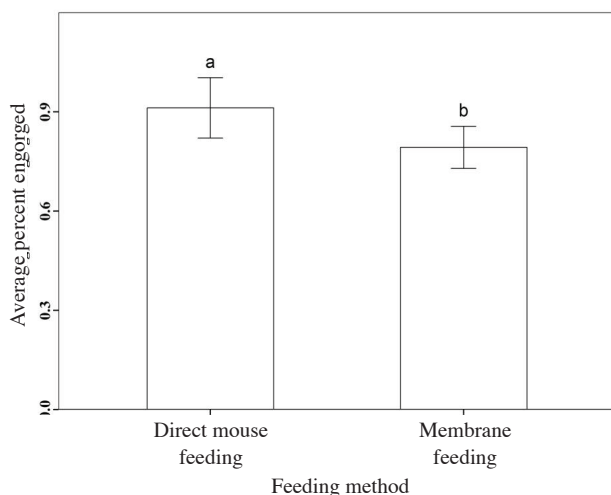
Fig 3–Average (± SE) percent engorgement of female *Ae. aegypti* when fed on anesthetized mice versus a membrane feeding system.

Direct feeding was the most effective means of providing a blood meal to mosquitoes. We estimated a 15% increase in feeding with anesthetized mice versus membrane feeding. Successful feeding is the product of a cascading series of events from detection of a viable host to probing for the blood meal itself. While no membrane system may ever out-perform a living organism as the best blood source, a 79% rate of feeding can easily maintain our high production rate of female *Ae. aegypti*. The AFRIMS Insectary will continue to look for innovative ways to improve our membrane feeding system.

ACKNOWLEDEMENTS

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