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A blood-free protein meal supporting oogenesis in the Asian tiger mosquito, *Aedes albopictus* (Skuse)

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ABSTRACT

Female mosquitoes require blood meals to complete oogenesis, or egg development. Current methods of maintaining laboratory colonies of mosquitoes generally rely on the use of whole blood to feed females. Blood feeding protocols require special handling techniques, impart numerous potential health hazards, involve significant costs, and are widely variable in terms of their success rates. In this study, a simple protein formulation was provided to *Aedes albopictus* using a membrane feeding system. Under the experimental conditions tested, females readily accepted the blood-free meal and produced eggs in greater numbers than cohort females that were fed with whole human blood. Moreover, fertility was comparable between treatments and survivorship of hatched larvae was equal among feedings. This implies that a readily available blood-free meal could be utilized in the laboratory rearing of this species. The elimination of blood handling, reduced cost, and consistency of blood-free meals would potentially be advantageous to mosquito rearing facilities generally, and in terms of scale, to mass rearing facilities specifically.

1. Introduction

Females of anautogenous mosquito species require an ingested blood meal to complete ovarian follicle development, which remains in a suspended state (Clements, 1992). Various studies have concluded that protein is the major, if not exclusive, nutrient in the blood that is necessary for oogenesis (Clements, 1992). However, not all blood meals are equivalent and host species can affect female egg production. In some studies, *Aedes aegypti* (L.) females have produced fewer eggs after taking a human blood meal than after taking an animal blood meal (Chang and Judson, 1979; Greenberg, 1951). Fertility and fecundity were both higher in *Culex pipiens quinquefasciatus* (Say) that were fed chicken blood compared with bovine blood (Richards et al., 2012). In another study, oviposition rate was highest in *Anopheles gambiae* (Patton) after feeding on human or cow blood, but was lowest on chicken or dog blood (Lyimo et al., 2012). A low level of free isoleucine in human plasma relative to other species has been shown to account for this difference in fecundity, highlighting the importance of this essential amino acid in female mosquito oogenesis (Briegel, 1985; Chang and Judson, 1977; 1979; Greenberg, 1951). Moreover, proteins that are low in isoleucine content, like hemoglobins (human hemoglobin lacks isoleucine), fail to stimulate oogenesis while those that are high in isoleucine, such as globulins, support oogenesis (Spielman, 1971; Spielman and Wong, 1974). However, differences in fecundity between human blood and mouse blood feedings were negated in *Ae. aegypti* when sugar was withheld, and in this context females imbibed less fully, but more frequently on human blood than on mouse blood (Harrington et al., 2001). A recent study using radiolabeled tracers showed that free plasma isoleucine and protein-bound phenylalanine were incorporated at the highest rates into *Ae. aegypti* egg proteins compared to other essential amino acids, again demonstrating the importance of specific amino acids in oogenesis (Zhou and Miesfeld, 2009). Interestingly, *Ae. aegypti* were able to produce viable eggs when fed a meal consisting of only 12 amino acids, including isoleucine (Lea et al., 1956). Amino acids also act as signals for numerous metabolic processes in the mosquito midgut and stimulate a hormone-signaling cascade that initiates vitellogenesis in several culicine species (Uchida et al., 2001, 1998).

While the gonotrophic requirement for blood protein is well established, blood-free meals for mosquito colony maintenance have not been widely tested for egg development or adopted for laboratory use. Successful oogenesis in *Ae. aegypti* can be supported by formulations made from protein sources like milk and egg powder; washed erythrocytes, supplemented with various proteins; and purified blood protein components, supplemented with isoleucine (Greenberg, 1951; Lea et al., 1956; Kogan, 1990). Absent from the published literature are studies that utilize blood

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meal substitutes to support egg production in anopheline species. One study specifically stated that *An. gambiae* fed inconsistently on an artificial meal and produced viable eggs in only 1 of 6 trials (Kogan, 1990). Another study described successful oviposition by *An. quadrimaculatus* resulting from artificial meals, but did not provide data regarding egg numbers or comparisons with controls (Lea et al., 1956). Few studies have provided detailed information about important parameters such as percentage of females fed on artificial meals, hatch rates of eggs, or survivorship to adult stage as compared to mosquitoes fed on live animals or whole blood delivered via artificial feeding apparatus. It thus appears that blood meal substitutes have the potential to be used successfully for laboratory mosquito colony rearing, but that more study is required in order to establish the utility of artificially delivered meals for laboratory colony maintenance in species other than *Ae. aegypti*. The current study was conducted to investigate the effects of a blood-free meal on fecundity of *Ae. albopictus*, an invasive species that is a competent vector for numerous arboviruses and has been implicated in several outbreaks of Chikungunya in various geographic locations within the past decade (Gratz, 2004; Pialoux et al., 2007; Paupy et al., 2009; Lambrechts et al., 2010). *Ae. albopictus* is a potential target for vector control programs that depend on the release of large numbers of sterile or genetically modified individuals (Benedict, 2003; Labbé et al., 2010; Alphey et al., 2011; Iturbe-Ormaetxe et al., 2011; Oliva et al., 2012). Such programs would depend upon consistent, high volume, cost effective, and pathogen free blood sources (Benedict et al., 2009). Here, a simple protein formulation supported significantly higher egg production in *Ae. albopictus* females compared with whole human blood and at a much lower cost. This outcome provides a framework for the development of blood-free formulations that will be useful for large-scale rearing of mosquitoes and potentially other hematophagous insects.

2. Materials and methods

2.1. Mosquito rearing

Ae. albopictus eggs were initially obtained through the Malaria Reseacrh and Reference Reagent Resource Center (ALBOPICTUS F4, MRA-804). Eggs were submerged in an aqueous oak leaf infusion and placed in a vacuum chamber for ~30 min to deoxygenate and stimulate hatching. Larvae were allowed to develop in clean tap water and fed ground koi fish food *ad libitum*. Pupae were collected by hand with an eyedropper and placed into clean water. Adults were allowed to emerge in cube-shaped cages measuring 30 cm^3 (Megaview Science Co, Ltd.) and had access to a 10% sucrose solution *ad libitum*, except for ~1 h prior to feeding experiments when females were separated from males and sucrose was withheld. All stages were reared at a temperature of 26 °C and 75% relative humidity (RH) in an upright incubator with a 12:12 light:dark cycle.

2.2. Feeding treatments

Starved females were fed using a membrane delivery system (Hemotek, Ltd.) using 2 ml of whole human blood or artificial meal formulation, which was pipetted into a holding disc, sealed with stretched parafilm, and heated to 37 °C prior to delivery. An odor blend consisting of ammonium hydroxide, lactic acid, isovaleric acid, geranyl acetone, and butylamine, each at [−5logM] concentrations in aqueous buffer was applied to parafilm membranes as a host-seeking stimulant. Whole human blood, collected from individual male subjects (blood type O^-) and stored in lithium heparin was purchased from a biological supply company (BioChemed Services), kept refrigerated at 4 °C for no longer than 1 week and used only one time per experiment. Blood meal substitute consisted of bovine serum albumin (fraction V; Research Products International Corp.) [100 or 200 mg/mL] dissolved in a phosphate buffered saline solution (NaCl [137 mM], Na_2HPO_4 [10 mM], KH_2PO_4 [1.76 mM], KCl [2.68 mM], pH7.2) by heating briefly at 42 °C. Adenosine triphosphate (Sigma–Aldrich Co., LLC), was serially diluted in BSA solution from a 100 mM stock solution just prior to membrane feed at final concentrations of [1 mM], [0.1 mM], [10 μM], [1 μM] and [0 μM]. After feeding, females were provided 10% sucrose and were kept at 26 °C/75% RH for 72 h. Gravid females were placed in small cups containing ~100 mL of distilled water with a partially submerged filter paper liner as a substrate for oviposition. For blood meal volumes, groups of unfed or fully fed females were cold anesthetized and weighed together on an electronic balance to the nearest milligram. Estimated mean mass was derived using the formula: total weight/# females.

2.3. Statistical treatment of data

Ovaries were hand dissected from gravid females 72 h after feeding and teased apart using fine forceps. Stage V oocytes or oviposited eggs were placed under a dissecting microscope and counted with the aid of a handheld counter. Arithmetic mean, median, and standard deviation were determined for egg counting data. The Kruskal–Wallis analysis of variance was used to test for significant differences in distribution among all 3 groups. When significant differences were identified, the Mann–Whitney U statistic was calculated as a measure of differences in pairwise distributions. Interquartile range (IQR) was calculated by subtracting the highest value in quartile 3 (Q3) from the lowest value in quartile 1 (Q1). Whiskers were graphed as the highest or lowest data points falling within 1.5 * IQR of Q3 and Q1, respectively. Data points with values higher or lower than whiskers were plotted individually as outliers. The chi-square test was applied to the adult sex ratio data to determine significant deviation from the expected 1:1.

3. Results and discussion

3.1. Feeding responses

Feeding responses of *Ae. albopictus* and subsequent oogenesis were compared separately using either whole human blood or a buffered formulation containing bovine serum albumin (BSA) as a protein source supplemented with adenosine triphosphate (ATP). Blood-free formulations induced qualitatively similar feeding responses at BSA concentrations of 200 mg/ml and 100 mg/ml (BSA 200 and BSA100, respectively; Fig. 1). ATP was added to these formulations because it is a known phagostimulant in *Ae. aegypti*, where labral sensilla are sensitive to ATP and other soluble compounds (Galun et al., 1984, 1985; Werner-Reiss et al., 1999). To address the question of whether ATP was necessary for *Ae. albopictus* feeding, a series of trials was conducted in which the final ATP concentration in the BSA 200 meal ranged from a low of 0 mM to a high of 1 mM. A clear requirement for ATP was observed as no females fed on the BSA formulation lacking ATP (Table 1), although vigorous probing of the membrane was observed. The proportion of females that had fed on BSA improved with successively higher concentrations of ATP, reaching a maximum of 69% at 1 mM ATP, which outpaced the proportion feeding on whole human blood (Table 1). These observations strongly suggests that ATP is a phagostimulant for *Ae. albopictus* and may imply that this trait is a general feature of aedines. Additionally, groups of females were weighed immediately after feeding as a way of estimating ingested