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Blood serum and BSA, but neither red blood cells nor hemoglobin can support vitellogenesis and egg production in the dengue vector *Aedes aegypti*

Kristina K Gonzales, Hitoshi Tsujimoto, Immo A Hansen

Aedes aegypti is the major vector of dengue, yellow fever and chikungunya viruses that put millions of people in endemic countries at risk. Mass rearing of this mosquito is crucial for strategies that use modified insects to reduce vector populations and transmission of pathogens, such as sterile insect technique or population replacement. A major problem for vector mosquito mass rearing is the requirement of vertebrate blood for egg production since it poses significant costs as well as potential health hazards. Also regulations for human and animal use as blood source can pose a significant obstacle. A completely artificial diet that supports egg production in vector mosquitoes can solve this problem. In this study, we compared different blood fractions as dietary protein sources for mosquito egg production. We also tested artificial diets made from commercially available blood proteins (bovine serum albumin (BSA) and hemoglobin). We found that *Ae. aegypti* performed vitellogenesis and produced eggs when given whole bovine blood, serum, or an artificial diet containing BSA. Conversely, egg production was impaired after feeding of the red blood cell fraction or an artificial diet containing only hemoglobin. Our results indicate that serum proteins, not hemoglobin, may replace vertebrate blood in artificial diets for mass mosquito rearing.

BLOOD SERUM AND BSA, BUT NEITHER RED BLOOD CELLS NOR HEMOGLOBIN CAN SUPPORT VITELLOGENESIS AND EGG PRODUCTION IN THE DENGUE VECTOR *AEDES AEGYPTI*

Authors:

[1]Kristina K. Gonzales

[1]Hitoshi Tsujimoto

[1,2,3,*]Immo A. Hansen

Affiliations:

[1]Department of Biology, New Mexico State University, Las Cruces, NM, U.S.A.

[2]Institute for Applied Biosciences, New Mexico State University, Las Cruces, NM, U.S.A.

[3]Molecular Biology Program, New Mexico State University, Las Cruces, NM, U.S.A.

*Corresponding author:

Immo A. Hansen: New Mexico State University, Las Cruces, NM, USA; +1 (575) 646-7719
immoh@nmsu.edu

Abstract

Aedes aegypti is the major vector of dengue, yellow fever and chikungunya viruses that put millions of people in endemic countries at risk. Mass rearing of this mosquito is crucial for strategies that use modified insects to reduce vector populations and transmission of pathogens, such as sterile insect technique (SIT) or population replacement. A major problem for vector mosquito mass rearing is the requirement of vertebrate blood for egg production since it poses significant costs as well as potential health hazards. Also regulations for human and animal use as blood source can pose a significant obstacle. A completely artificial diet that supports egg production in vector mosquitoes can solve this problem. In this study, we compared different blood fractions as dietary protein sources for mosquito egg production. We also tested artificial diets made from commercially available blood proteins (BSA and hemoglobin). We found that *Ae. aegypti* performed vitellogenesis and produced eggs when given whole bovine blood, serum, or an artificial diet containing BSA. Conversely, egg production was impaired after feeding of the red blood cell fraction or an artificial diet containing only hemoglobin. Our results indicate that serum proteins, not hemoglobin, may replace vertebrate blood in artificial diets for mass mosquito rearing.

Introduction

During the past decades several mosquito control strategies have been developed that require the release of large numbers of mosquitoes grown in culture: 1. Sterile Insect Technique (SIT) requires the production of large quantities of male mosquitoes that are sterilized either by ionizing radiation (Joint 1990; Helinski, Parker et al. 2009; Rodriguez, Brar et al. 2013) or that are genetically sterile (Alphey 2002; Alphey 2007). 2. Infection with endosymbiotic bacteria, *Wolbachia* can make mosquitoes refractory to viral infections. In order to drive these endosymbionts in field populations, large numbers of *Wolbachia* infected females are needed (Rainey, Shah et al. 2014). 3. For replacement of field populations with such modified mosquitoes that are refractory for diseases, both males and females can be released.

Mosquitoes are hematophagous insects, and only certain species require a blood meal to produce their first batch of eggs, they are termed anautogenous. In contrast autogenous mosquitoes can produce a first batch of eggs using nutrients acquired from their larval stage and require blood only to produce subsequent egg batches (Hansen, Attardo et al. 2014). Since anautogenous mosquitoes need vertebrate blood for egg development, and most vector species are anautogenous, a source of blood and an efficient feeding system has to be acquired at every facility mass rearing mosquitoes for the above mentioned strategies. This can pose a significant hindrance since local regulations, ethical concerns, and infrastructure vary greatly in different countries. In many countries protocols involving animal subjects have to undergo a review process and create (and implement) an animal care and use program before experiments are carried out (1996) which, consequently, results in significant delay in laboratory activity and added requirements, recordkeeping and personnel training. Therefore, the push for alternative vertebrate blood-free meals is attractive to mosquito rearing facilities all over the world. In order to replace vertebrate blood in mosquito rearing, an artificial blood meal has to meet the following requirements: 1) Mosquito females must readily take it in sufficient amounts 2) It must support vitellogenesis 3) It must support large egg batches and 4) The offspring should be fit.

Vertebrate blood is a mixture of erythrocytes, leucocytes and platelets suspended in an aqueous medium called plasma. Erythrocytes (red blood cells: RBCs) are the main component of blood cells comprising ~45% of total blood volume and the major protein in these cells is hemoglobin (Hb). The other 55% of blood consists of plasma, which is a water- and protein-rich formulation that has a balanced salt concentration acting as a buffer to maintain stable pH levels and other cellular components. Nutrients absorbed into the blood stream from digested food, dissolved gases and other blood proteins and lipids are also found in blood plasma (Farley, Hendry et al. 2012). Blood can be fractionated into packed red blood cells and plasma, using centrifugation protocols (Duarte, Carvalho Simões et al. 1999).

Ae. aegypti is the primary vector for Yellow fever, Dengue and Chikungunya viruses through saliva during blood feeding (Nile, Encepha- et al. 2014). *Ae. aegypti* is an invasive species in the Americas, preferring areas close to humans where blood is easily accessible (Powell and Tabachnick 2013). After a female takes a blood meal into their midgut, the blood proteins are enzymatically digested into amino acids, which are then released into the hemolymph (Pacey and O'Donnell 2014). The accumulated amino acids in the hemolymph are absorbed by the mosquito fat body, functionally similar to the vertebrate liver, which synthesizes yolk protein precursors (YPP), called vitellogenin. These YPPs are then secreted into the hemolymph and taken up by developing oocytes via a vitellogenin receptor (Sappington, Hays et al. 1995), a process called vitellogenesis (Hansen, Attardo et al. 2014; Hansen, Attardo et al. 2014).

A study on blood substitutes for *Ae. aegypti* found that feeding a mixture of blood proteins can support egg production in *Ae. aegypti*. Increasing the protein content from 60 mg/ml to 123 mg/ml of porcine albumin, Hb and γ-globulins in the artificial meal significantly increased the number of deposited eggs (Kogan 1990). Another recent study presents an artificial diet based on two concentrations, 100 and 200 mg/ml, of bovine serum albumin (BSA) that can support *Ae. albopictus* vitellogenesis and egg development. The total number of eggs produced from the 100 mg/ml BSA meal was statistically significantly smaller than the eggs produced from whole blood and 200 mg/ml BSA meal. The findings of these studies provide important considerations for the development of artificial blood meals for mosquitoes (Pitts 2014).

Phagostimulants are important components of artificial blood meals because they increase the proportion of females that take the meal and also the amount of meal taken. Studies focused on identifying phagostimulants found that mosquitoes were likely to fully engorge on meals containing adenyl nucleotides (adenosine monophosphate (AMP), adenosine diphosphate (ADP), and adenosine triphosphate) and the success rate increased with the number of attached phosphate groups (Clements 1992). A phagostimulant study produced similar results when bed bugs, *Cimex lectularius*, were offered 1 mM of AMP, ADP and ATP (Romero and Schal 2014). 1 mM of ATP has been shown to be the most effective in *Ae. albopictus* (Pitts 2014).

The aim of the present study was to develop a blood-free meal that supports egg development in *Ae. aegypti*. We have tested different protein components bovine serum albumin, Hb, serum and red blood cells and their effect on egg production. We found that neither RBC nor Hb, but blood serum proteins are sufficient to support egg production in *Ae. aegypti*.

Materials and Methods

Insect culture – *Ae. aegypti* (Rockefeller strain) eggs were submerged in tap water and connected to a vacuum pump for 20 minutes to deoxygenate water and induce hatching. Larvae were raised on cat food (Special Kitty) and water was changed as needed. Pupae were separated by hand using a handheld screen and placed in a separate container with clean water. Pupae containers were placed inside 30 cm × 30 cm × 30 cm cube-shaped mosquito cages for emergence with 20% sucrose solution as carbohydrate source. During the rearing process mosquitoes were held in an insect environmental chamber at 26.5 °C, 70% relative humidity (RH) and 16:8 hr (L:D) cycle. Adults were at least 4 days old before given a meal.

Meal treatments - Prior to feeding experiments, sucrose solution was withheld from mosquitoes for at least 16 hr. Starved females were separated from males and equally distributed into smaller 15 cm × 15 cm × 15 cm cube-shaped mosquito cages and fed using an artificial lab-made feeding system. RBCs or blood serum was fractionated from whole defibrinated bovine blood (Hemostat Labs, Dixon, CA). The whole blood and serum was given alone and RBCs were washed with phosphate buffered saline (PBS). Whole bovine blood was centrifuged and the serum supernatant was pipetted off without disturbing packed RBCs. Serum was collected and stored at −20 °C. RBCs were washed 3 times by resuspending in one volume of PBS, centrifuging and pipetting off supernatant. Washed RBCs were stored at 4 °C. All centrifugations were done at ~1275 × *g* for 20 min at 4 °C. Females were each fed a meal consisting of different protein components. Bovine serum albumin (BSA, Research Products International, Mt. Prospect, IL) or Hb (Sigma-Aldrich, St. Louis, MO) [200 mg/mL] meal was dissolved in either PBS, *Aedes* physiological saline

(APS), sodium PBS (NaPBS) or potassium PBS (KPBS). Chemical concentrations and components are listed in Table 1. A 1 mM ATP (Sigma-Aldrich) solution was made and immediately stored at −20 °C. ATP solution was thawed right before feeding experiment and kept on ice during preparation.

Membrane feeding system - 1 mL of each solution was added to a lab-constructed feeding receptacle made from a 50 mL centrifuge tube cut at the 45 mL line. Parafilm was stretched to near breaking point and sealed over the cap-end of the receptacle. The parafilm membrane was rubbed on sweaty human skin to stimulate host-seeking behavior. Each prepared solution was heated in a 37°C water bath for 15 min, then pipetted into feeding receptacle and placed on top of each cage. 1 mM ATP was used as a phagostimulant in all treatment meals. Thawed freeze packs were microwaved for 2 min and placed on top of each feeding receptacle to keep solution warm and aid in attracting mosquitoes. Females were provided with a meal for 1 hr then immediately collected with a battery operated aspirator and anesthetized on ice. Fully engorged females were counted and weighed together on a balance. The females were then transported to a 15 cm × 15 cm × 15 cm cube-shaped mosquito cage and each provided a water-soaked cotton ball and a 20% sucrose-soaked cotton ball and kept at 26.5 °C, 69% RH for 48 hr. Each female was then individually placed into a 50 mL centrifuge tube containing a water-soaked cotton ball and filter paper substrate for egg deposition for additional 24 hr. 72 hr post-fed dissections were carried out and the number of developed and retained oocytes and the number of deposited eggs were counted.

Data Analysis – The proportion of females that fed was calculated by dividing the number of fully engorged females by the total number of females given the meal. The Kruskal-Wallis test was performed to determine significant differences among multiple groups, and the Mann-Whitney test was used to compare pairwise treatment meals. In the analysis of egg deposition numbers of all treatment meals, the high percentage of zero values for the RBC and Hb treatment, and consequent high number of ties, precluded the use of Mann-Whitney U test for pairwise comparisons. Therefore, data for whole blood control and all other treatments were recoded as "yes" for individual females that laid eggs and "no" for individual females that did not. A contingency table analysis, Chi-squared and Fisher's exact test, was used to determine significant differences between each meal against the whole blood control.

Results & Discussion

Mosquitoes prefer buffered BSA solution and serum over red blood cells – We offered several diet formulations containing different protein sources to *Ae. aegypti* females: RBCs, BSA, and Hb all diluted in PBS, serum, and whole blood as control. All solutions contained 1 mM ATP. We determined the proportion of fully engorged females after one hour (Figure 1). The highest proportion of females fully engorged was found with whole blood (control) and this value was significantly different from the number of females engorged on RBCs. The percentage of mosquitoes engorged on serum, BSA, and Hb solutions was not quite significantly different from the control. However, a strong trend was found that suggests that BSA solution is the preferred

meal, when compared to the other protein sources (P=0.057). The RBC and Hb formulation had the lowest engorgement response. However, independent from the diet formulations offered, mosquitoes were in all cases actively probing the membranes of the feeding apparatus. It is apparent that mosquito females when they come in physical contact with the diet solution make a decision either to continue feeding or reject the meal and continue probing. One possible explanation for the reduced engorgement rates we observed with some diet formulations is that red blood cells and Hb somehow made the phagostimulant ATP inaccessible for the mosquito to sense either by binding it or by chemical conversion. Another, and in our opinion more likely explanation is that mosquitos can taste the solution with chemoreceptors that are located in the labella or in the cibarium. In general, our results support the idea that mosquitoes have a system to judge the quality of a blood/food source and based on this information accept or reject it. The nature of the sensing system is unknown and an interesting topic for further studies.

Red blood cells or hemoglobin do not support egg production - Next, we tested the effects of feeding different diets on egg deposition rates. We compared whole blood (positive control), against its fractionated components, serum and RBCs, and both BSA and Hb in PBS (Figure 2). An initial Kruskal-Wallis analysis of variance test revealed a significant difference in egg deposition numbers among the five treatments (DF=4, H=61.735, $P < 0.001$). Each treatment was compared against the whole blood control using a contingency table analysis (chi-squared and Fisher's exact test). We found a statistical significant difference between the whole blood control and the RBC ($P <0.001$) and Hb ($P <0.001$) treatment meals. While we did not find significant differences in egg numbers between whole blood and serum-fed females, no eggs were deposited by females fed on the RBC or Hb meal. Figure 3 shows some representative ovaries and attached alimentary canals of mosquitoes 72 hrs after they were fed on different diets. No full ovary development was observed in the RBC-fed females, while most of the ovaries in whole-blood fed females developed fully. Partial ovary development was observed in two females that fed on the RBC formulation and in one female that fed on the Hb formulation. We also observed what appears to be a mostly undigested bolus of RBCs and Hb in the midgut of the RBC-fed and Hb-fed females. Our results indicate that a diet of pure RBCs and Hb causes a delayed or early aborted digestion compared to whole blood, serum, or BSA- fed females. The nature of this constipation phenotype is unknown.

Effect of different buffers on egg production after a BSA meal – After a blood meal, *Ae. aegypti* mosquitoes excrete up to 80% of the meals volume within the first hours (Drake, Boudko et al. 2010; Drake, Price et al. 2012). The meal becomes more concentrated and therefore easier to digest and the mosquito loses weight which restores its flight capabilities. We hypothesized that an efficient concentration process after an artificial BSA meal depends on the ion concentrations of the buffer and can be optimized. In order to determine a good buffer solution for a blood-free meal we offered BSA meals in four different buffer solutions: Phosphate-buffered saline (PBS), *Aedes* physiological saline (APS), Sodium-enriched PBS (NaPBS) and Potassium-enriched PBS (KPBS). We used 200 mg/ml BSA in the various buffers since this concentration supported *Ae. aegypti* vitellogenesis in the experiments described above and also has been shown to support egg development in *Ae. albopictus* (Pitts 2014). We found no significant difference between the

formulations on egg numbers (Figure 4). We also offered Hb meals in the four different buffer solutions to compare with BSA meals. Hb did not support egg production in any buffer solution (Table 2).

Conclusion

The goal of this study was to identify potential alternative blood-free and vertebrate-free meals that supports egg production in the dengue vector, *Ae. aegypti*. We were able to show that vitellogenesis and egg production can be supported on serum fractions from whole blood and PBS-buffered BSA solutions. The egg numbers produced by females fed on such diets are comparable to whole blood. Further studies are necessary to determine the relative fitness of mosquitoes raised on such diets. Our findings are important for further development of blood meal alternatives in order to eliminate traditional vertebrate feeding protocols and optimize mosquito egg production.

Acknowledgements

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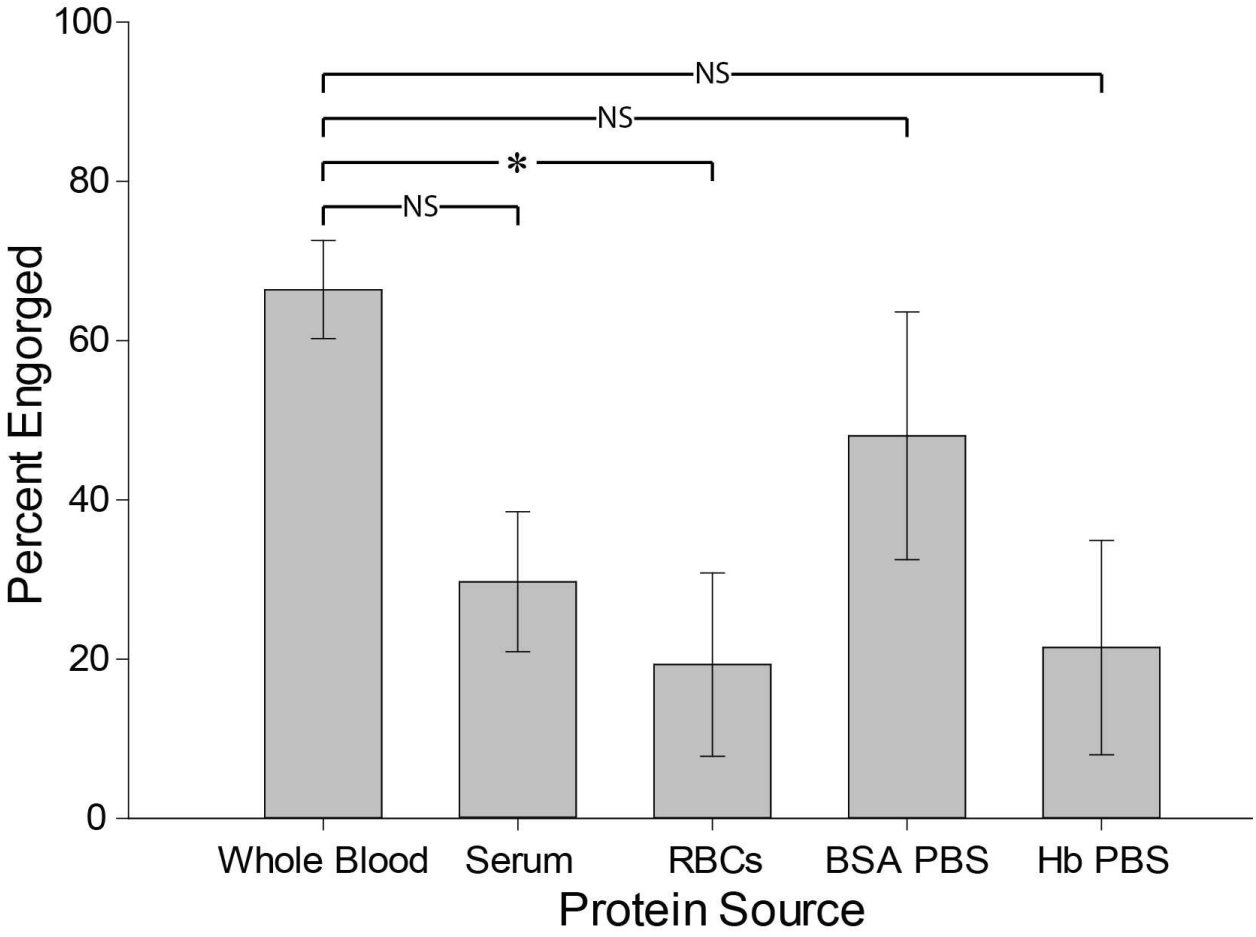


Figure 1: Proportion of fully engorged females fed on whole blood, serum, RBCs, BSA in PBS and Hb in PBS. Data is representative of at least three independent trials. Graph represents mean ± SEM. The Mann-Whitney test was performed to determine statistical differences in comparison to whole blood: * indicates significant difference at $P < 0.05$.

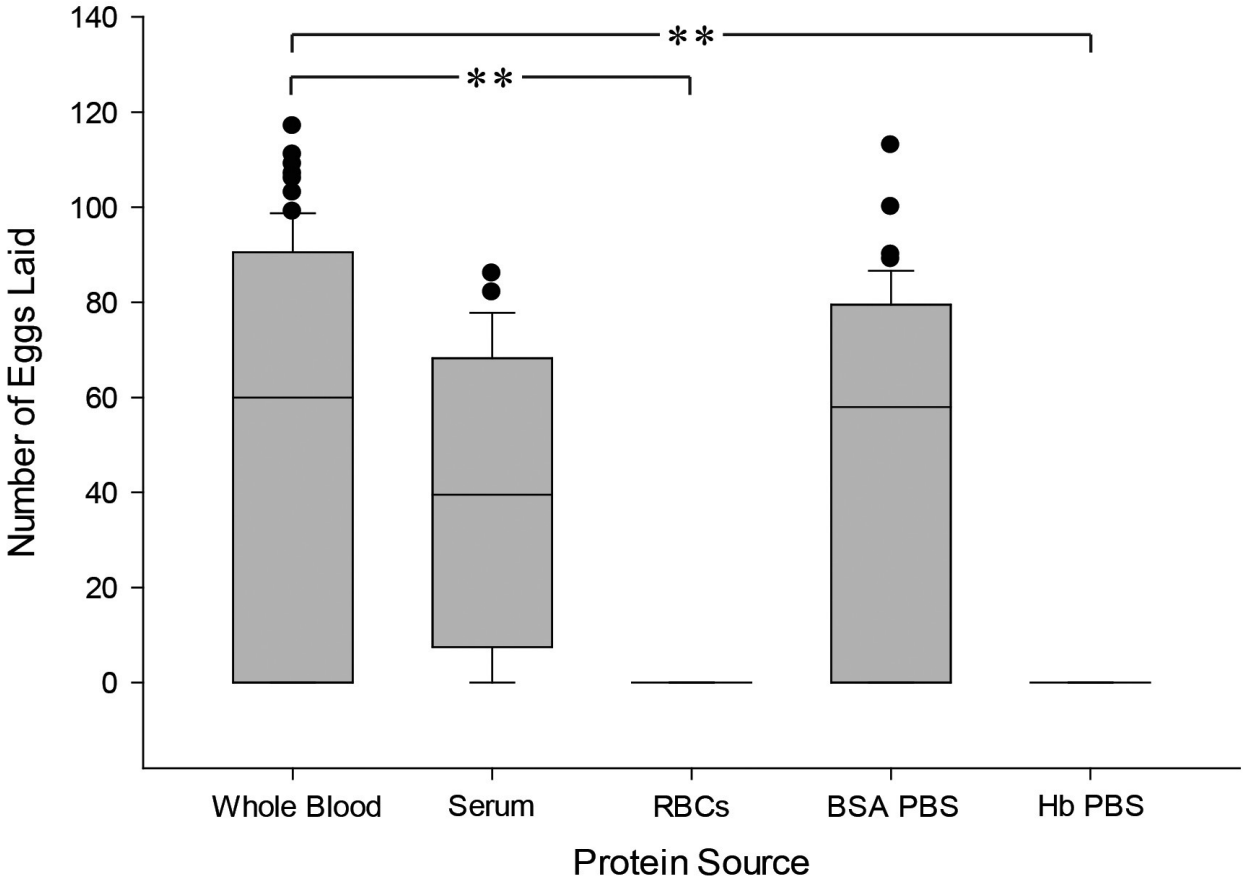
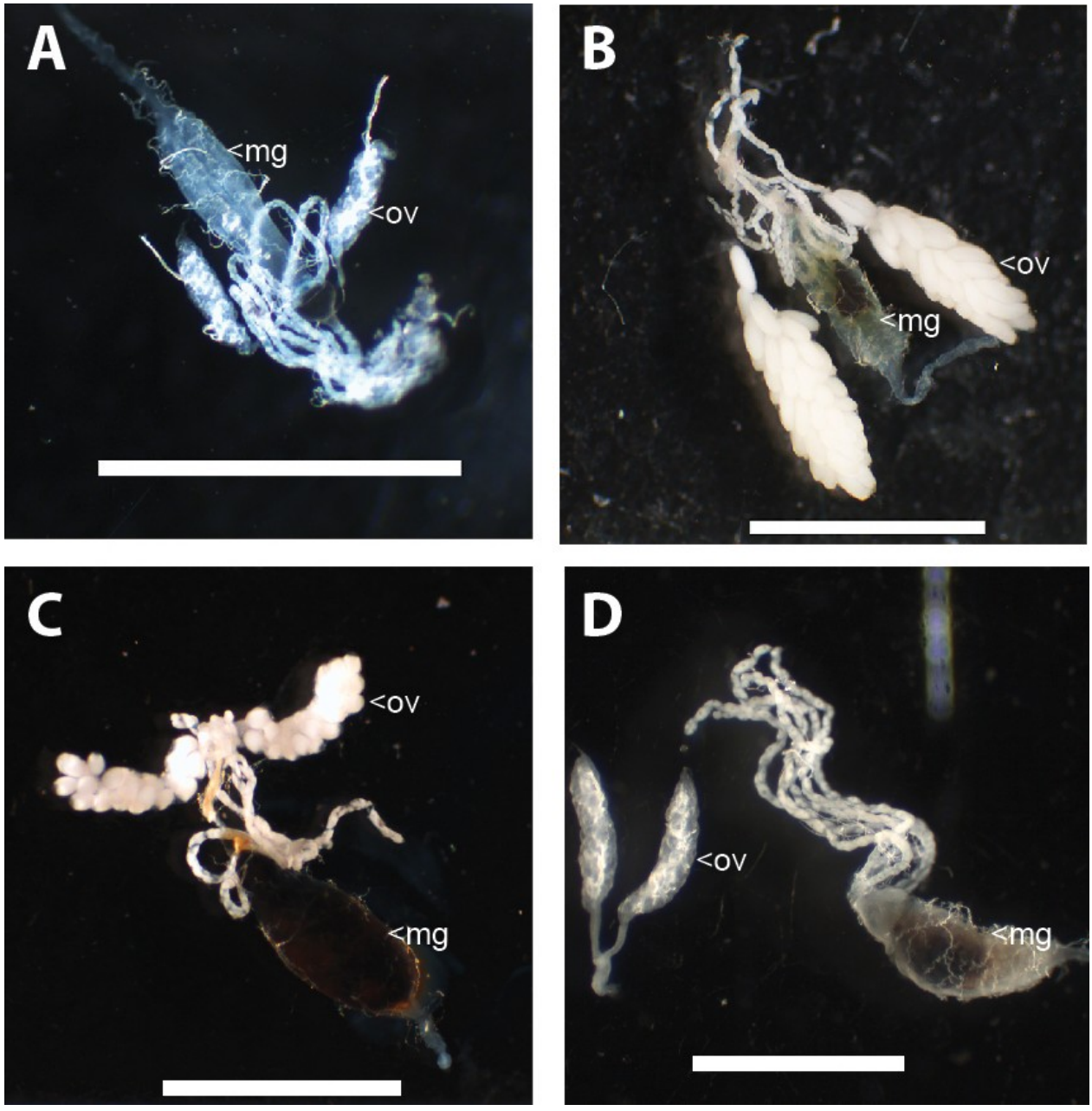


Figure 2: *Ae. aegypti* egg deposition between 48 and 72 hr after whole blood, serum, RBCs, BSA and Hb meals. Horizontal bars represent the median with 25th and 75th percentiles represented by boxes. Whiskers extend to the 5% and 95% confidence level and individual circles are outliers. All treatments are significantly different from each other as determined by Kruskal-Wallis rank test ($P< 0.001$). Each treatment meal was analyzed against the whole blood control using a contingency table analysis (chi-squared and Fisher's exact test). **denotes statistical significant difference ($P< 0.001$) as determined by a contingency table analysis.



223 Figure 3: The effect of RBCs and whole blood meal on ovary development 72 hr after feeding.
224 Midguts with attached Malpighian tubules and ovaries were dissected; mg – midgut, ov – ovary.
225 Scale bars = 2 mm. (A) Unfed control female, (B) Fully developed ovaries from a female given
226 whole blood (control). (C) Partial ovary development in female given RBC as sole protein
227 source. (D) No ovary development in females fed with PBS-buffered Hg diet.

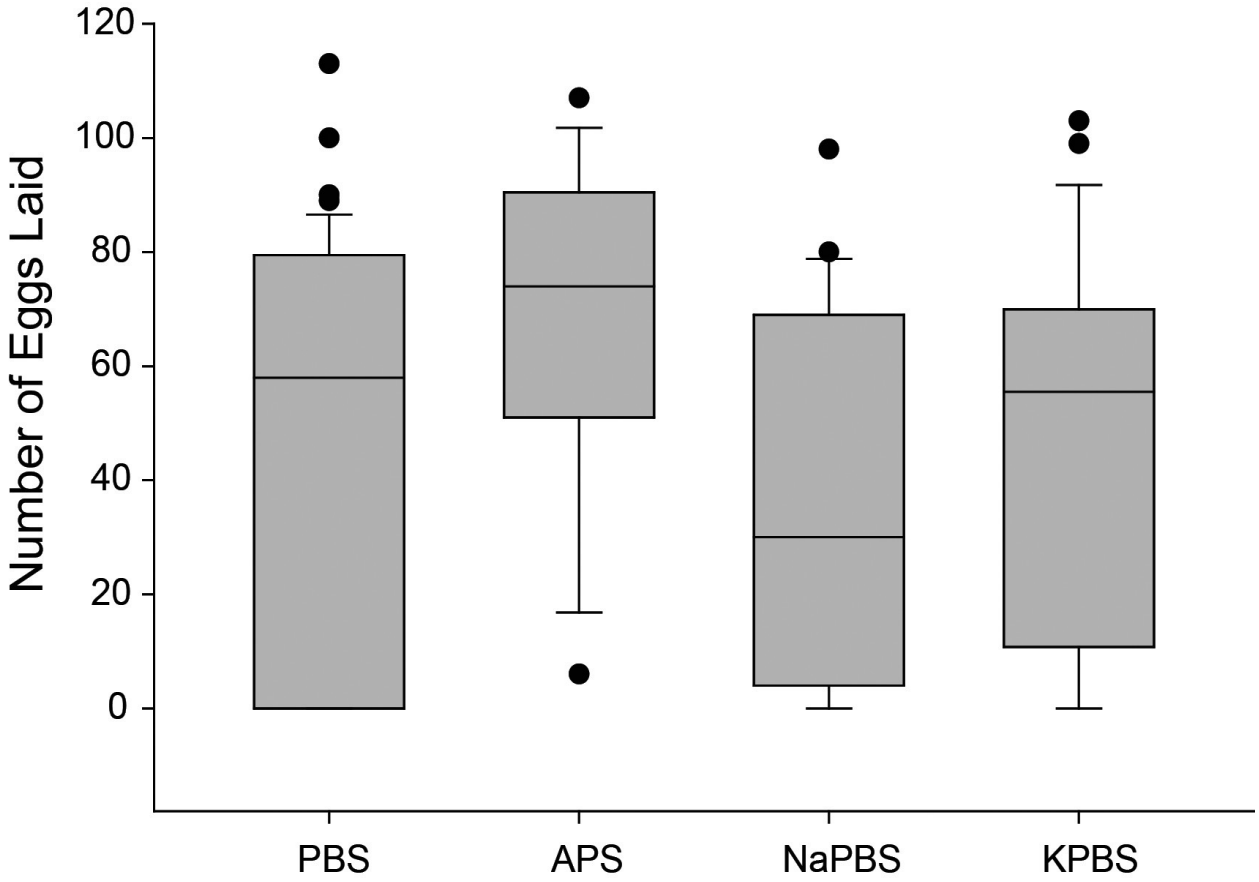


Figure 4: The effect of different buffer solutions on egg deposition provided a 200 mg/ml BSA meal. Box plot showing the number of eggs laid 72 hr post-meal. Each column represents BSA delivered in a different buffer. Horizontal bars represent the medians with 25th and 75th percentiles represented by boxes. Whiskers extend to the 5% and 95% confidence level and individual circles are outliers.

233 Table 1: Chemical components and pH of each buffer used in BSA and Hemoglobin feedings.
234 Phosphate buffered saline (PBS), *Aedes* physiological saline (APS), Sodium phosphate buffered
235 saline (NaPBS), and potassium phosphate buffered saline (KPBS). All concentrations are in mM.

	PBS	APS	NaPBS	KPBS
Sodium Chloride (NaCl)	137.0	150.0	137.0	2.7
Potassium Chloride (KCl)	2.7	4.0	2.7	137.0
Disodium Phosphate (Na_2HPO_4)	0.0	0.0	10.0	2.0
Sodium Bicarbonate ($NaHCO_3$)	0.0	0.1	0.0	0.0
Magnesium Chloride ($MgCl_2$)	0.0	0.6	0.0	0.0
Potassium Dihydrogen Phosphate (KH_2PO_4)	193.0	0.0	2.0	10.0
Calcium Chloride ($CaCl_2$)	0.0	1.7	0.0	0.0
Sodium Carbonate (Na_2CO_3)	73.4	0.0	0.0	0.0
HEPES buffer	0.0	25.0	0.0	0.0
pH	7.4	7.0	7.5	7.5

Table 2. *Aedes aegypti* egg production response to BSA and hemoglobin meal in four different buffers. Mean number of eggs laid and retained in the ovaries ±SEM are shown. Representative of three independent replicated feedings. n = number of engorged females for each meal.

		PBS	APS	NaPBS	KPBS
Bovine Serum Albumin	n	26	9	18	6
	Eggs Laid	42±8.01	66±10.80	37±7.98	52±17.02
	Eggs in Ovaries	23±7.96	21±10.29	6±3.85	25±11.34
Hemoglobin	n	18	7	17	17
	Eggs Laid	0±0.00	0±0.00	0±0.00	0±0.00
	Eggs in Ovaries	0±0.00	0±0.00	1±1.00	0±0.00

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