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TITLE:

Feeding and Quantifying Animal-Derived Blood and Artificial Meals in *Aedes aegypti* Mosquitoes

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KEYWORDS:

Aedes aegypti, mosquito, blood-feeding, pharmacology, blood meal quantification, fluorescence reading, behavior

SUMMARY:

The goal of this protocol is to deliver animal-derived and artificial blood meals to *Aedes aegypti* mosquitoes through an artificial membrane feeder and precisely quantify the volume of meal ingested.

ABSTRACT:

Females of certain mosquito species can spread diseases while biting vertebrate hosts to obtain protein-rich blood meals required for egg development. In the laboratory, researchers can deliver animal-derived and artificial blood meals to mosquitoes via membrane feeders, which allow for manipulation of meal composition. Here, we present methods for feeding blood and artificial blood meals to *Aedes aegypti* mosquitoes and quantifying the volume consumed by individual females.

Targeted feeding and quantification of artificial/blood meals have broad uses, including testing the effects of meal components on mosquito behavior and physiology, delivering pharmacological compounds without injection, and infecting mosquitoes with specific pathogens. Adding fluorescein dye to the meal prior to feeding allows for subsequent meal size quantification. The meal volume consumed by mosquitoes can be measured either by weight, if the females are to be used later for behavioral experiments, or by homogenizing individual females in 96-well plates and measuring fluorescence levels using a plate reader as an endpoint assay. Meal size quantification can be used to determine whether changing the meal components

alters the meal volume ingested or if meal consumption differs between mosquito strains. Precise meal size quantification is also critical for downstream assays, such as those measuring effects on host attraction or fecundity. The methods presented here can be further adapted to track meal digestion over the course of days or to include multiple distinguishable markers added to different meals (like nectar and blood) to quantify the consumption of each meal by a single mosquito.

These methods allow researchers to singlehandedly perform high-throughput measurements to compare the meal volume consumed by hundreds of individual mosquitoes. These tools will therefore be broadly useful to the community of mosquito researchers for answering diverse biological questions.

INTRODUCTION:

We present a protocol for feeding modified blood meals to *Aedes aegypti* mosquitoes using an artificial membrane feeder and precisely measuring the meal volume consumed by each individual mosquito. This protocol can be flexibly adapted to alter the content of the meal or to compare the meal volume consumed by different experimental groups of mosquitoes.

The *Ae. aegypti* mosquito threatens global health by spreading pathogens that cause diseases including yellow fever, dengue fever, chikungunya, and Zika[1–5]. *Ae. aegypti* females are obligate blood-feeders; they must consume vertebrate blood to obtain the necessary protein for egg development, and each clutch of eggs requires a full blood meal from at least one host[6–8]. The female mosquito first bites her host by piercing the skin with her stylet and injecting saliva, which contains compounds that trigger the host's immune response[9]. She then feeds by pumping blood through her stylet into her midgut. While consuming a blood meal from an infected host, she may ingest blood-borne pathogens[6,8], which then migrate from the mosquito's midgut to her salivary glands[10]. Female mosquitoes infected in this manner can spread disease by injecting pathogens along with saliva when biting subsequent hosts[11,12]. Understanding and quantifying the mechanisms of blood-feeding behavior are crucial steps in controlling the transmission of mosquito-borne diseases.

Many laboratory protocols for mosquito rearing and experimentation use live animals including mice, guinea pigs, or humans as a blood source[13–16]. The use of live animals imposes ethical concerns as well as complex requirements for personnel training, animal housing and care, and compliance with Institutional Animal Care and Use Committee (IACUC) policies. It also limits the types of compounds that can be delivered to mosquitoes, which constrains the studies that can be carried out[17].

Artificial blood-feeding apparatuses, which typically use a membrane system to simulate host skin, are useful tools for studying blood-feeding behaviors that circumvent the need for the maintenance of live hosts. Whole blood can be purchased from a number of vendors and fed to mosquitoes using heated, water-jacketed artificial membrane feeders or similar devices[18, 19]. In this protocol, we demonstrate the use of small, disposable membrane feeders termed "Glytubes". This membrane feeder, previously published by Costa-da-Silva et al. (2013)[20], can be

easily assembled from standard laboratory equipment, making it ideal for delivering blood meals to moderate numbers of mosquitoes and straightforward to scale up for testing larger groups or multiple meal formulations. The Glytube is an inexpensive and efficient alternative to other commercial artificial feeders, which may require larger meal volumes and are more suitable for batch feeding large groups of mosquitoes on a single meal formulation[21].

This protocol includes three sections: preparing and delivering artificial meals and quantifying consumption. In the first section, Glytubes are used as an efficient means to deliver manipulated diets. Whole blood may be substituted with an entirely artificial meal to compare the effects of blood substitutes in lieu of a blood meal. A recipe adapted from Kogan (1990)[22] is presented here, although multiple artificial meal formulations have been developed[23, 24]. Furthermore, feeding is a less invasive and less laborious method to introduce pharmacological compounds than injection. Due to the low total volume required for each meal (1–2 mL), this protocol provides an attractive delivery method to reduce the amounts of expensive reagents. *Ae. aegypti* females readily consume protein-free meals of saline solution with adenosine 5'-triphosphate (ATP)[25, 26], which provides a baseline for measuring the effects of single meal components. For example, Neuropeptide Y-like receptor 7 (NPYLR7) in *Ae. aegypti* is known to mediate host-seeking suppression after a protein-rich blood meal, and when NPYLR7 agonists are added to a protein-free saline meal, female mosquitoes exhibit host-seeking suppression similar to those that have consumed whole blood[7].

In the third section, steps for quantifying the volume of each meal consumed by an individual female mosquito are presented. This assay is fluorescence-based and captures feeding status in higher resolution than methods in which females are classified as "fed" or "unfed" based on visual assessment of abdominal distension alone. By adding fluorescein to the meal prior to feeding, meal volumes ingested by individuals can be quantified by homogenizing each mosquito in a 96-well plate and measuring fluorescence intensity as a readout. This assay can measure differences in feeding vigor in response to variables such as meal composition or the mosquitoes' genetic background. Precise quantification is critical for intermediate meal sizes, for example when females are offered suboptimal meals containing feeding deterrents or when they consume sucrose meals of variable sizes[27]. If fed mosquitoes are required for subsequent behavioral assays after meal size quantification, meal size can instead be calculated by weighing anesthetized females in groups and estimating the average increased mass per individual. Although less precise than fluorescein marking, weighing still provides an aggregated estimate of meal volume and allows examination of the meal's effect on downstream processes, such as fecundity or subsequent host attraction. While blood meal size is variable and can be influenced by a myriad of factors[11, 28, 29], ingested meal sizes measured with the methods described here are consistent with previous quantifications[7, 30, 31].

PROTOCOL:

Blood-feeding procedures were not performed using live animals or human hosts and complied with the guidelines set by The Rockefeller University Institutional Animal Care and Use Committee (IACUC) and Institutional Review Board (IRB).

1. Meal preparation

1.1. Preparation of the phagostimulant, adenosine 5'-triphosphate

1.1.1. Prepare a 25 mM solution of aqueous $NaHCO_3$ (molecular weight, MW = 84.006 g/mol). For 100 mL of 25 mM $NaHCO_3$, add 210 mg of $NaHCO_3$ to a volumetric flask and fill with double-distilled water (ddH_2O) to a total volume of 100 mL. Using a magnetic stir bar, thoroughly mix the solution until all the $NaHCO_3$ is dissolved.

1.1.2. Reconstitute ATP disodium salt hydrate (MW = 551.14 g/mol) in the aqueous 25 mM $NaHCO_3$ to a final concentration of 200 mM ATP. For a total volume of 10 mL of 200 mM ATP in 25 mM $NaHCO_3$ buffer, add 1.1 g of ATP disodium salt hydrate to a volumetric flask and fill with 25 mM $NaHCO_3$ buffer to a total volume of 10 mL. Using a magnetic stir bar, thoroughly mix the solution until all the ATP is dissolved.

NOTE: To minimize hydrolysis of ATP, it must be buffered by a salt solution such as $NaHCO_3$.

1.1.3. Aliquot the ATP solution and store at -20 °C.

NOTE: This stock solution of ATP is typically made fresh every six months and is used for all meals described below. To prevent degradation, ATP aliquots should not undergo multiple freeze-thaw cycles or be heated along with other meal components.

1.2. Preparation of the fluorescent tracer solution, fluorescein

1.2.1. Prepare a 2% (w/v) stock solution of aqueous fluorescein. For a total stock solution volume of 10 mL, mix 0.2 g of fluorescein disodium salt with 10 mL of ddH_2O in a 15 mL conical tube wrapped in aluminum foil at room temperature. This stock solution of fluorescein can be used for dilution in all meals described below.

NOTE: As fluorescein is light-sensitive, avoid exposure to light by wrapping containers in aluminum foil.

1.3. Preparation of animal-derived blood meals

1.3.1. Calculate the number of meals needed to feed all mosquitoes; each Glytube holds a 2 mL meal and feeds approximately 25 mosquitoes. Prepare one additional meal to calibrate the standard curve for fluorescence readings. Unless stated otherwise, all steps in this section describe reagent amounts required to prepare one meal with a final volume of 2 mL.

1.3.2. For animal-derived blood meals, transfer 1.98–2 mL defibrinated sheep blood into a 15 mL conical tube (see step 3.3 for desired volume of blood).

NOTE: Commercially defibrinated sources of vertebrate blood, including from sheep, guinea pigs, and humans, may be used[13]. Prior to use, ensure that the purchased blood has not passed its expiry date and mix it well by inverting the bottle, especially if there is visible separation of blood components.

1.3.3. For optimal feeding, add ATP to a final concentration of 1–2 mM after the sheep blood has been warmed to 45 °C in a water bath. For a final concentration of 1 mM ATP, add 10 µL of the 200 mM ATP stock solution to 1.99 mL of pre-warmed blood and mix. For a final concentration of 2 mM ATP, add 20 µL of the 200 mM ATP stock to 1.98 mL of pre-warmed blood and mix. If ATP is not to be added, warm 2 mL of defibrinated sheep blood.

1.3.4. If fluorescence-based quantification of meal size is to be subsequently carried out, add fluorescein solution to a final concentration of 0.002% (2 µL of 2% fluorescein stock in 2 mL total meal volume). Reduce the volume of blood by the same amount as the fluorescein added. Retain 1 mL of the final meal formulation containing 0.002% fluorescein to generate the reference standard curve. Treat the retained volume identically to the meal that is being delivered to mosquitoes; expose to the same light and temperature conditions throughout the duration of the experiment, and subsequently freeze this along with the delivered meal.

1.4. Preparation of artificial blood meals

1.4.1. Calculate the number of meals needed to feed all mosquitoes; each Glytube holds a 2 mL meal and feeds approximately 25 mosquitoes. Prepare one additional meal to calibrate the standard curve for fluorescence readings. Unless stated otherwise, all steps in this section describe reagent amounts required to prepare one 2 mL meal.

1.4.2. To prepare artificial blood (adapted from Kogan (1990)[22]), as in **Table 1**, first make a stock solution of 400 mM $NaHCO_3$. For a total volume of 10 mL of 400 mM $NaHCO_3$ (MW = 84.006 g/mol), add 336 mg of $NaHCO_3$ to a volumetric flask and fill with double-distilled water (ddH_2O) to a total volume of 10 mL. Using a magnetic stir bar, thoroughly mix the solution until all the $NaHCO_3$ is dissolved.

1.4.3. For the protein components of artificial blood, prepare stock solutions of 50 mg/mL of γ-globulins in 400 mM $NaHCO_3$, 35 mg/mL of hemoglobin in ddH_2O, and 300 mg/mL of albumin in ddH_2O. Protein stock solutions can be stored at 4 °C for up to 2 months. The final concentration of total human proteins in artificial blood is 125 mg/mL. This includes final concentrations of 15 mg/mL γ-globulins, 8 mg/mL hemoglobin, and 102 mg/mL albumin.

1.4.4. For each 2 mL meal, combine 600 µL of γ-globulins, 460 µL of hemoglobin, 680 µL of albumin, and 250 µL of ddH_2O from stock solutions listed in **Table 1**. Wait to add 10 µL of 200 mM ATP stock solution until after the meal has been warmed to 45 °C, immediately before presenting the meal.

1.4.5. If fluorescence-based quantification of meal size is to be subsequently carried out, add fluorescein solution to a final concentration of 0.002% (2 µL of 2% fluorescein stock in 2 mL total meal volume). Reduce the volume of ddH_2O in step 4.4 by the same amount as the fluorescein added. Retain at least 1 mL of the final meal formulation containing 0.002% fluorescein to generate the reference standard curve. Treat the retained volume identically to the meal that is being delivered to mosquitoes; expose to the same light and temperature conditions throughout the duration of the experiment, and subsequently freeze this along with the delivered meal.

1.5. Preparation of example protein-free saline meals (adapted from Duvall et al. (2019)[7])

NOTE: Protein-free saline meals can be prepared in multiple ways[7,27,32]. The saline meal presented here is a protein-free version of the artificial blood recipe described above.

1.5.1. Calculate the number of meals needed to feed all mosquitoes; each Glytube holds a 2 mL meal and feeds approximately 25 mosquitoes Prepare one additional meal to calibrate the standard curve for fluorescence measurements. Unless stated otherwise, all steps in this section describe reagent amounts required to prepare one 2 mL meal.

1.5.2. To prepare the saline meal, make a stock solution of 400 mM $NaHCO_3$. For a total volume of 10 mL of 400 mM $NaHCO_3$ (MW = 84.006 g/mol), add 336 mg of $NaHCO_3$ to a volumetric flask and fill with ddH_2O to a total volume of 10 mL. Using a magnetic stir bar, thoroughly mix the solution until all the $NaHCO_3$ is dissolved.

1.5.3. For each 2 mL meal, combine in a 15 mL conical tube 600 µL of 400 mM $NaHCO_3$ with 1.39 mL of ddH_2O. Wait to add 10 µL of the 200 mM ATP stock solution until after the meal has been warmed to 45 °C in a water bath.

1.5.4. If fluorescence-based quantification of meal size is to be subsequently carried out, add fluorescein solution to a final concentration of 0.002% (2 µL of 2% fluorescein stock in 2 mL of total meal volume). Reduce the volume of ddH_2O in step 5.3 by the same amount as the fluorescein added. Retain at least 1 mL of the final meal formulation containing 0.002% fluorescein to generate the reference standard curve. Treat the retained volume identically to the meal that is being delivered to mosquitoes; expose to the same light and temperature conditions throughout the duration of the experiment, and subsequently freeze this along with the delivered meal.

2. **Meal delivery to mosquitoes**

2.1. Setting up mosquito containers for feeding

NOTE: Mosquitoes can be fed in a variety of containers as long as the following criteria are met. Ensure that the container is large enough for mosquitoes to fly around in, but not so large that it will be difficult for the mosquitoes to locate the mesh surface and begin feeding. The mesh used

to cover the container can vary in material and hole size. The holes must be large enough for the female mosquito's stylet to pierce through, but not so large that the mosquito can escape. Secure the mesh firmly so that it is taut, and the Glytube can rest stably on its surface throughout the feeding period.

2.1.1. An example container (**Figure 1**) is a modified 946 mL (32 oz) high density polyethylene (HDPE) plastic bucket. To replicate this setup, use a razor blade to cut a central hole of ~10 cm diameter in the bucket lid. To assemble the container for occupation by mosquitoes, secure a ~400 cm^2 square piece of white 0.8 mm polyester mosquito netting on top of the bucket, securely pushing the perforated lid down over it to snap tightly.

2.1.2. Collect female mosquitoes that are at least 3 days post-eclosion to ensure that they are mature enough to blood-feed. Optimal feeding rates are observed after 7 days[33].

2.1.3. Place female mosquitoes into the container and cover with mesh. If the container is densely populated with mosquitoes, increase the number of Glytubes used. Optimal feeding is achieved with ~25 mosquitoes/Glytube. This reduces competition for access to the feeding membrane.

2.1.4. Set aside a control group of unfed mosquitoes that will not be offered a meal. In the weight measurement protocol, weigh the unfed group separately and use this weight to estimate weight gain in the experimental group that fed on a meal. In the fluorescence-based quantification protocol, add the unfed group of mosquitoes to the wells for the standard curve calculations and for negative controls. To match baseline mosquito tissue autofluorescence in the experimental group, ensure that the standard curve and negative control wells contain an unfed mosquito.

2.2. Constructing and setting up the Glytube (adapted from Costa-da-Silva et al. (2013)[20])

2.2.1. As depicted in **Figure 1**, to generate a heat source, fill a 50 mL conical tube with 40 mL of 100% glycerol. Seal the open conical tube with a 5 cm × 5 cm piece of parafilm and repeat with an additional piece of 5 cm × 5 cm parafilm to minimize the chance of leakage. Optionally, the parafilm can be held in place using rubber bands. Invert the tube to ensure that there are no holes or gaps.

2.2.2. To create the meal delivery device, cut a centered hole of 2.5 cm diameter in the screw cap of the conical tube using a sharp razor blade or, for better consistency, a lathe. Stretch a 5 cm × 5 cm piece of parafilm evenly so that it roughly doubles in size. The parafilm should be thin enough that mosquitoes can easily pierce through it, but there should be no leaks. Seal over the outer surface of the screw cap to fully cover the hole and set the cap aside.

NOTE: To increase attraction to the Glytube, prior to stretching the parafilm, perfume it with human odor by gently rubbing it on a patch of human skin with no cosmetics applied, taking care that no holes are created. This is recommended if the experiment is not aimed at investigating

the sensory cues required for mosquitoes to approach the meal.

2.2.3. Heat both the sealed tube of glycerol and the meal (with all components except ATP) in a 42–45 °C water bath for at least 15 min. Do not pre-heat ATP; add it immediately before starting the experiment.

2.2.4. Add ATP to the warmed meal and vortex thoroughly. Pipette 2 mL of the warmed meal into the inner chamber of the screw cap and gently place the inverted, warmed, glycerol-filled 50 mL conical tube in it. Partially screw the cap with the meal onto the glycerol-filled tube—just enough to prevent leakage of the meal or the glycerol.

NOTE: The meal volume used can range between 1 mL and 2.5 mL. Lower volumes may be especially useful when meals are used to deliver compounds that are scarce or expensive. It is important to work quickly at this step so that the meal does not cool down to ambient temperature and reduce the likelihood of maximal feeding. The rate of cooling will depend on the ambient temperature of the room where these steps are conducted, but they should typically be completed within 5 min at 25 °C.

2.2.5. Place the assembled Glytube on top of the mosquito container and allow the mosquitoes access to feed for at least 15 min to achieve maximal feeding rates.

2.2.6. For optimal feeding, place mosquito containers inside a chamber equipped with a CO_2 pad, and allow at least 15 min of acclimation at 25–28 °C and 70–80% humidity prior to delivering the meal. The assay chamber used here is a simple and low cost modification of a previously published setup[16]. It uses a translucent polypropylene storage box of size 36 cm L × 31 cm W × 32 cm H with a removable lid. A 1.5 cm diameter hole made in the chamber wall allows CO_2 delivery through silicone tubing. The CO_2 diffusion pad is affixed to the inner center of the lid for delivery of purified air and CO_2 to condition the chamber atmosphere during the trial.

NOTE: Make sure host cues (heat and CO_2, with optional host odor[16]) are present so that the mosquitoes are attracted to the membrane feeder. If mosquitoes are not crowding underneath the Glytube, check that CO_2 is properly delivered and that the meal and Glytube are sufficiently warm. If an external CO_2 source is not available, CO_2 can be delivered via puffs of exhaled human breath.

2.2.7. After feeding, the Glytube cap can be discarded as biohazard waste or reused after soaking in a low percentage bleach solution and thoroughly rinsing in water.

3. Quantification of consumed meals

3.1. Weighing mosquitoes to be used for further experiments

NOTE: Weighing mosquitoes to quantify meal size allows them to be used for further live experimentation, but this method requires taking weight measurements from a group of 5

mosquitoes. Since weights of individual mosquitoes are difficult to precisely measure using most laboratory balances, variability in individual meal size cannot be easily quantified by measuring weights. Weighing is only recommended for situations in which females visibly engorge on the meal.

3.1.1. Cold anesthetize mosquitoes by moving their container to a 4 °C cold room or placing it on ice.

3.1.2. Weigh groups of 5 females from the unfed cohort (i.e., mosquitoes that were never offered a meal) and calculate their average weight as the estimate of the "pre-feeding" weight. The average weight of an unfed mosquito depends on genotype, sex, and rearing conditions. Unfed female *Ae. aegypti* mosquitoes reared with *ad libitum* access to sucrose typically weigh approximately 2 mg each.

3.1.3. From the experimental cohort (i.e., mosquitoes that were offered a meal), sort females into "fed" and "not fed" piles based on the abdominal distention observable by eye[7]. Divide each of the "fed" and "not fed" piles, respectively, into groups of 5 mosquitoes for weighing. Mosquitoes within each group of 5 should be derived from the same experimental cohort for taking group weight measurements. Calculate the average weight per female from each of the "fed" and "not fed" piles of the experimental group.

3.2. Fluorescence measurement for end-point analysis[7,27,34]

NOTE: To obtain precise meal size measurements from individual mosquitoes that are no longer required for further live experimentation, store the mosquitoes and the remaining 1 mL of meal containing 0.002% fluorescein at -20 °C immediately after feeding. The experiment can be paused here. This method is outlined in **Figure 2**.

3.2.1. To generate a reference standard curve, prepare a serial dilution of the same meal containing 0.002% fluorescein that was offered to the experimental group of mosquitoes. There will be a total of 8 standard curve solutions. In each of these solutions, the final volume of meal containing 0.002% fluorescein will be 5, 2.5, 1.25, 0.625, 0.3125, 0.15625, 0.078125, or 0 µL, and each will be in 1 x phosphate-buffered saline (PBS) for a total volume of 100 µL (e.g., 5 µL of meal containing 0.002% fluorescein in 95 µL of 1x PBS).

3.2.2. To make the first solution of the standard curve, add 50 µL of meal containing 0.002% fluorescein to 950 µL of 1x PBS and vortex thoroughly (final volume: 5 µL of meal containing 0.002% fluorescein in 95 µL of 1x PBS). To make the rest of the standard curve solutions, perform a 2-fold dilution for each step by taking 500 µL from the previous tube and adding it to a new tube containing 500 µL of 1x PBS. Vortex well before preparing the next 2-fold dilution.

3.2.3. To prepare wells to be used for generating a reference standard curve, pipette 100 µL of each of the standard curve solutions into each of the 8 wells in the first column of a 96-well PCR plate. Add 1 unfed control mosquito to each of the same 8 wells in the first column of the plate.

Repeat in the second column of the plate for a replicate measurement.

NOTE: If experimental groups are offered different meal types, a separate reference standard curve must be prepared for each meal type.

3.2.4. Add 100 µL of 1x PBS in each remaining well for the unfed control and experimental groups. If tissue is to be disrupted in subsequent steps using a bead mill homogenizer or vortex, add one 3 mm borosilicate solid-glass bead to each well.

3.2.5. As a negative control, add 1 unfed mosquito to each well in the next 2 columns of the plate. The fluorescence measured in this group sets a baseline cutoff to account for tissue autofluorescence and will be used to determine whether a mosquito in the experimental group fed on the meal.

3.2.6. Add 1 mosquito per well to the remaining wells from the experimental groups that were offered a meal.

3.2.7. Seal the plate carefully and disrupt the tissue by manual grinding. The abdomen should be thoroughly homogenized to release the meal. Methods to disrupt tissue include using a bead mill homogenizer with 3 mm borosilicate solid-glass beads (30 Hz for 30 seconds), vortex mixer with 3 mm borosilicate solid-glass beads, or a pestle grinder without beads.

3.2.8. Centrifuge the plate at 2000 rpm for 1–2 min to collect the lysate.

3.2.9. Prepare a black 96-well plate with 180 µL of 1x PBS in each well.

3.2.10. Transfer 20 µL of lysate to each well with 180 µL of 1x PBS and mix. If available, use a multi-channel pipette at this step for increased speed and better consistency.

3.2.11. Measure fluorescence intensity of each well using a plate reader on the 485/520 excitation/emission channel. Generate the reference standard curve by plotting the known volume of meal against the corresponding fluorescence intensity measurement.

3.2.12. Using the reference standard curve generated, extrapolate the meal volume ingested by each of the experimental group mosquitoes. Subtract the average fluorescence intensity reading of the negative control group of unfed mosquitoes from the fluorescence intensity reading of each experimental group individual to correct for baseline tissue autofluorescence.

REPRESENTATIVE RESULTS:

Figure 1 presents a schematic for assembling the Glytube, whereas **Figure 2** shows an overview of the experimental design to measure meal size using the fluorescence-based assay described here. **Figure 3** provides representative fluorescein meal size measurements from a blood-feeding experiment. **Figure 4**, **Figure 5**, and **Figure 6** illustrate a sampling of biological questions that can be addressed using this protocol. Applications of the protocol are wide-ranging and include

altering blood meal composition, feeding pharmacological compounds, precisely quantifying sub-optimal blood meals or smaller nectar meals, and comparing feeding behavior across mosquito genotypes.

To generate a standard curve for meal volume calculations, fluorescence readings are plotted from the designated reference wells each containing an unfed mosquito and a known volume of the meal with 0.002% fluorescein (**Figure 3A**). Fluorescence readings from the remaining wells, which contain mosquitoes from either the negative control group of unfed mosquitoes or the experimental group of mosquitoes offered a meal, are compared to this standard curve to quantify the meal volume (µL) consumed by each mosquito (**Figure 3B**). To validate the baseline readings in this assay, it should be confirmed that mosquitoes from the unfed negative control group are not assigned a positive value of µL consumed (**Figure 3B**, **left**). Although all females in the experimental group were offered the blood meal, some mosquitoes fed (**Figure 3B**, **middle**) and some did not (**Figure 3B**, **right**). This result demonstrates that two types of data can be obtained from this protocol: 1) the percentage of total females that feed on a given meal, and 2) the volume ingested by the females that feed on a given meal.

This protocol can be used to deliver and quantify meals with various protein compositions. **Figure 4A,B** show data collected using meals with added fluorescein. The proportion of mosquitoes that fed and the meal volume they ingested, respectively, were calculated from the fluorescence readings. These readings are highly sensitive and allow for precise quantification of µL, but have the limitation that mosquitoes cannot be used for future live experiments. **Figure 4C,D** show data collected from an independent experiment with mosquitoes that were scored as fed or unfed by eye after they were offered meals without fluorescein. Meal size was calculated as average weight/female from groups of 5 mosquitoes. Although these weight measurements are less sensitive than fluorescence measurements, they allow the females to be recovered and used for further live experimentation. The proportion of mosquitoes that feed can vary across different experimental days, as reflected in **Figure 4A** and **Figure 4C**.

Figure 5 shows the volume consumed of meals containing drugs that regulate mosquito host-seeking behavior. In these experiments, females were offered saline + ATP meals with 100 µM of the human NPY Y2 receptor agonist, TM30338. This drug alters host-seeking behavior through activation of *Ae. aegypti* NPY-like receptor 7. Measuring meal sizes is critical for the interpretation of experiments to assess the effect of this drug on post-blood-feeding behavior because it allows the researcher to calculate the dose consumed by each female.

In the previous examples, females were fed either blood or substitute blood meals, all of which resulted in 3–5 µL meals (**Figure 3, Figure 4, Figure 5**). This fluorescence-based assay can also be used to measure smaller and/or more variable meal sizes that cannot be accurately discerned from average group weight measurements. In **Figure 6**, the same fluorescence quantification protocol was used to measure nectar-feeding behavior by exchanging the Glytube for a cotton ball saturated with 10% sucrose containing 0.002% fluorescein. Nectar sugars cannot be presented in the Glytube assay because females cannot detect the presence of nectar sugars with the stylet and do not initiate feeding[27]. These data allow the researcher to determine that sugar

meals are consistently smaller than blood meals, in agreement with previous work[34] (**Figure 6**).

FIGURE LEGENDS:

Figure 1: Setup of Glytube method used to feed meals to mosquitoes. (**A**) Schematic of a deconstructed Glytube used to feed blood and other meals to mosquitoes. (**B**) Schematic of a Glytube presented atop a container of mosquitoes with a mesh lid. Female mosquitoes can pierce through the mesh lid to feed. (**C**) Photographs of the Glytube (top), and female *Aedes aegypti* mosquitoes before, during, and after feeding (bottom, from left to right) on a Glytube-delivered meal. Mosquitoes are shown piercing through the mesh covering their container to access the membrane feeder. (**D**) Photographs showing the appearance of female *Ae. aegypti* mosquitoes that are unfed (left) and that have engorged on either an artificial blood meal (right, top) or a saline + ATP meal (right, bottom). The Glytube method was previously published in Costa-da-Silva et al. (2013)[20]. Photographs in (**C**) and (**D**) are courtesy of Alex Wild.

Figure 2: Schematic of how to quantify meal size after Glytube blood-feeding protocol. (**A**) Mosquitoes are offered a meal with fluorescein (top, experimental group) or no meal (bottom, unfed negative control group). (**B**) Individual mosquitoes are added to a 96-well plate after terminating the feeding experiment. (**C**) Standard curve is generated using known amounts of meal containing 0.002% fluorescein. (**D**) Mosquitoes are homogenized to release any consumed fluorescein, and fluorescence levels in each well are quantified using a plate reader. This fluorescence quantification method is modified from Liesch et al. (2013)[34].

Figure 3: Glytube blood-feeding experiment with fluorescein-based quantification. (**A**) Standard curve measurements obtained from the wells where a mosquito from the unfed control group was added to a known quantity of meal containing 0.002% fluorescein (y-axis scale = arbitrary units). (**B**) Meal volume calculated using fluorescence readings for females in the unfed control group (left, black, n = 40), the experimental group that fed on blood (middle, red, n = 37), and the experimental group that did not feed on blood (right, red, n = 23). Each point represents a measurement from an individual female. Data are shown as median with range. Letters indicate statistically distinct groups, Kruskal-Wallis test with Dunn's multiple comparison, $p<0.01$. These data were published in Jové et al. (2020)[27].

Figure 4: Quantification of meals with differing protein composition. Females were offered meals of either sheep blood (red), artificial blood with human blood proteins (Kogan (1990)[22]) (orange), or protein-free saline + ATP meal (aqua)[7]. (**A**) Percentage of females fed scored using fluorescence readings. Each point represents a group of 12–16 females. Data are shown as medians with ranges, n = 12. (**B**) Meal volume calculated using fluorescence readings. Each point represents a measurement from an individual female in a single trial from **Figure 4A**. Data are shown as medians with ranges, n = 12. (**C**) Percentage of females fully engorged after artificial membrane feeding, scored by eye. Each point represents the percent of females engorged from groups of 20–30 females. Data are shown as medians with ranges, n = 23. (**D**) Meal sizes scored as weight/female after feeding status was scored by eye. Weights were calculated as the average of groups of 5 mosquitoes. Data are shown as medians with ranges, n = 23. **A–D**: Letters indicate statistically distinct groups, Kruskal-Wallis test with Dunn's multiple comparison, $p<0.05$.

Figure 5: Quantification of meals with pharmacological compounds. Females consume meals of the same size of sheep blood (red), saline + ATP (aqua), and saline + ATP + 100 µM dose of human NPY Y2 receptor agonist TM30338 (dark blue). Meal volume calculated using fluorescence readings. Each point represents a measurement from an individual female. Data are shown as medians with ranges, n = 12. Letters indicate statistically distinct groups, Kruskal-Wallis test with Dunn's multiple comparison, $p<0.05$.

Figure 6: Quantification of smaller nectar meals. (**A**) Schematic of nectar-feeding assay. (**B**) Meal volume calculated using fluorescence readings for wild-type females offered meals of either water (blue, n = 36) or 10% sucrose (green, n = 53), each with 0.002% fluorescein, in the nectar-feeding assay. Each point represents a measurement from an individual female. Data are shown as medians with ranges. Letters indicate statistically distinct groups, Mann-Whitney test, $p<0.05$. These data were published in Jové et al. (2020)[27].

Table 1: Recipe for preparing artificial blood meals (adapted from Kogan (1990)[22]
Artificial blood consists of protein and non-protein components regularly found in human blood and provides the option to vary the ratios of these components. Mosquitoes can produce eggs after feeding on artificial blood[7, 22].

Table 2: Recipe for saline meal with ATP (adapted from Duvall et al. (2019)[7]
Protein-free saline meals can be used to deliver compounds of interest to mosquitoes while still mimicking the abdominal distension that occurs after blood-feeding, but without triggering the egg development that occurs when proteins are ingested.

DISCUSSION:

For many laboratory applications, artificial membrane feeders offer distinct benefits compared to live hosts by allowing researchers the ability to directly manipulate the contents of the meal. Although multiple methods are available for artificial membrane feeding, the method described here offers advantages in flexibility, cost, and throughput. In comparison to other commercial membrane feeders, the Glytube assay requires a small meal volume, making it an efficient delivery mechanism for costly reagents, including drugs or pathogens, by minimizing the total volume required[7,35]. As both the protein-free saline and artificial blood meals promote engorgement, compounds or pathogens can be added to either meal as a high-throughput and non-invasive alternative to injections. Additionally, each component of the Glytube can easily be washed, replaced, or scaled up to deliver and quantify multiple meal types without cross-contamination of the feeding apparatus.

To quantify meal volumes consumed by mosquitoes, the fluorescence-based method enables more precise meal size quantification than weighing the mosquitoes before and after feeding. It should be noted that this method is an end-point assay. In contrast, weighing allows the mosquitoes to be kept alive for further experimentation. By using a plate reader, the fluorescence-based method can be easily scaled up for high-throughput quantification of meals consumed by hundreds of individual females.

To achieve high feeding rates, a combination of sufficient host cues must be present to activate female host-seeking behavior and attract females to the feeder. If mosquitoes are not crowding underneath the Glytube, the meal may not be properly warmed, or CO_2 delivery may not be sufficient. Addition of human odor to the membrane surface reliably increases attractiveness of the artificial membrane. If mosquitoes are observed underneath the Glytube but fail to feed, the meal composition may be at fault. Females may not feed if the meal itself is not warm, the blood is too old, or if the additives to the meal are intrinsically aversive or cause an undesirable chemical reaction[36]. Additional ATP also reliably increases feeding rates and can be scaled up to a final concentration of 2 mM in each of the recipes provided. Females may not feed if the parafilm is not pulled taut across the Glytube cap; the parafilm should be uniformly transparent and should not buckle, as this prevents the female from being able to effectively pierce the parafilm with her stylet. If the meal leaks through the Glytube onto the mesh, the parafilm may have torn during the stretching process and should be replaced.

Changing the meal composition can also allow researchers to manipulate the length of time needed to clear the meal from the midgut as well as the subsequent host-seeking behavior. The meals presented here require 24–36 h for digestion[7] similar to animal-derived blood. After feeding on any of these meals, females will suppress host-seeking during the digestion time window. Since the saline meal lacks protein, females return to host-seeking after the meal is cleared. If a faster return is desirable, researchers can choose alternate "quick clearing" saline meals that are excreted in approximately 6 h[27]. While the composition of the saline meal presented here is matched to directly compare results with the artificial blood meal, the "quick clearing" meal more closely matches physiological salt levels found in vertebrate blood.

The methods described here have limitations that should be considered before selecting the assay that is most suited to the researcher's experimental goals. The fluorescein measurements described do not allow mosquitoes to be used again for additional experimentation. However, weight measurements can be taken prior to meal size quantification using the fluorescein assay. If weight and meal size are consistent across multiple trials for a given meal, weight can be used as a proxy in future experiments. Moreover, this protocol does not distinguish between deficits in host-seeking versus blood-feeding behavior; mosquitoes that show impairments in finding the membrane feeder will have a reduction in feeding rates and/or meal size. By adding a camera to record behavior throughout the assay, researchers can determine whether the females cannot find the Glytube, or whether they find the Glytube, but do not feed.

The assay described here can be adapted to explore many outstanding questions related to feeding behavior in mosquitoes. For example, the contribution of specific blood proteins can be explored by altering the ratio of constituent proteins or total protein concentration in the artificial blood meal. To evaluate meal sizes from multiple feeding events, dyes with distinct fluorescence spectra can be added to differentiate meals from unique sources[37]. This protocol can also be modified to separately stimulate the internal mouthparts that detect blood and that are used for ingestion (i.e., stylet), and the chemosensory appendages that contact skin (i.e., labium, legs) as the mosquito lands to begin blood feeding[36]. For example, if ligands are added

directly to the meal, they do not contact the labium and legs, since the membrane is pierced only by the stylet. If ligands are added to the outer surface of the parafilm instead, they remain separated from the meal and may be contacted by the labium and legs[36]. Finally, the detailed kinetics of blood-feeding behavior are not well understood and the method presented here could be modified to combine high-resolution tracking with machine learning tools to extract behavioral readouts of locomotion, posture, and feeding dynamics[38].

This protocol is aimed at being user-friendly and cost-effective, with the ability to serve researchers employing pharmacological and genetic manipulations to study mosquito blood-feeding and post-blood-feeding behavior.

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DISCLOSURES:

The authors have nothing to disclose.

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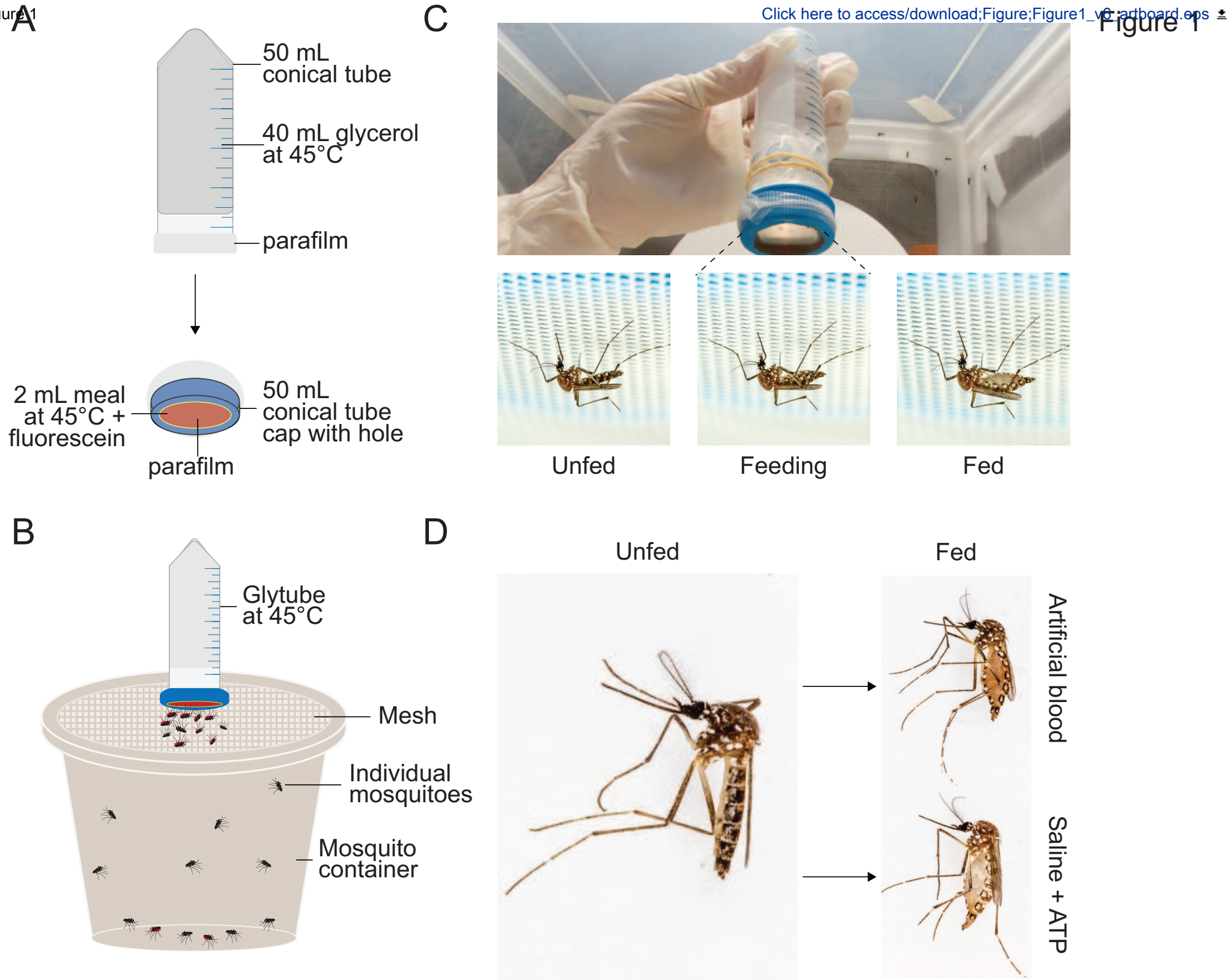


Figure 2

Figure 2

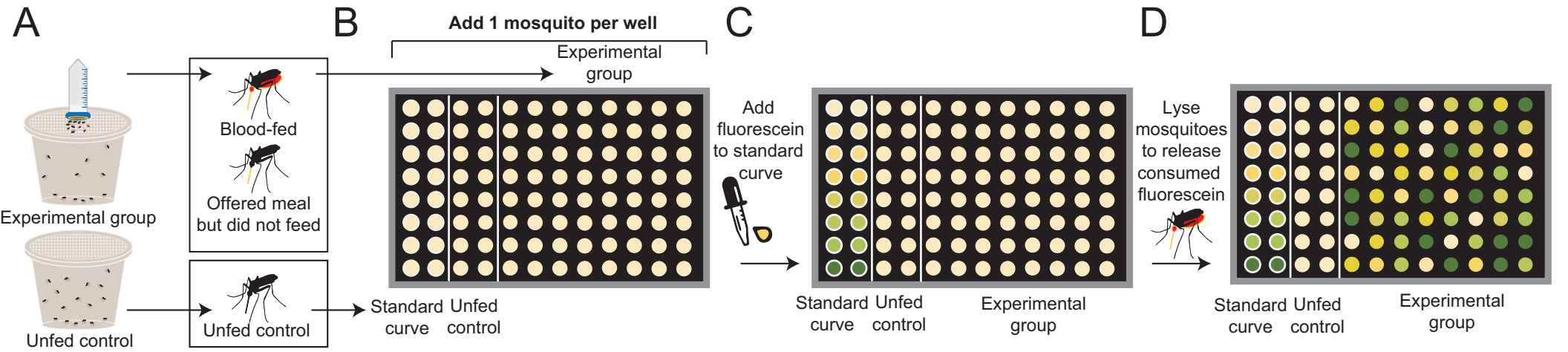


Figure 3

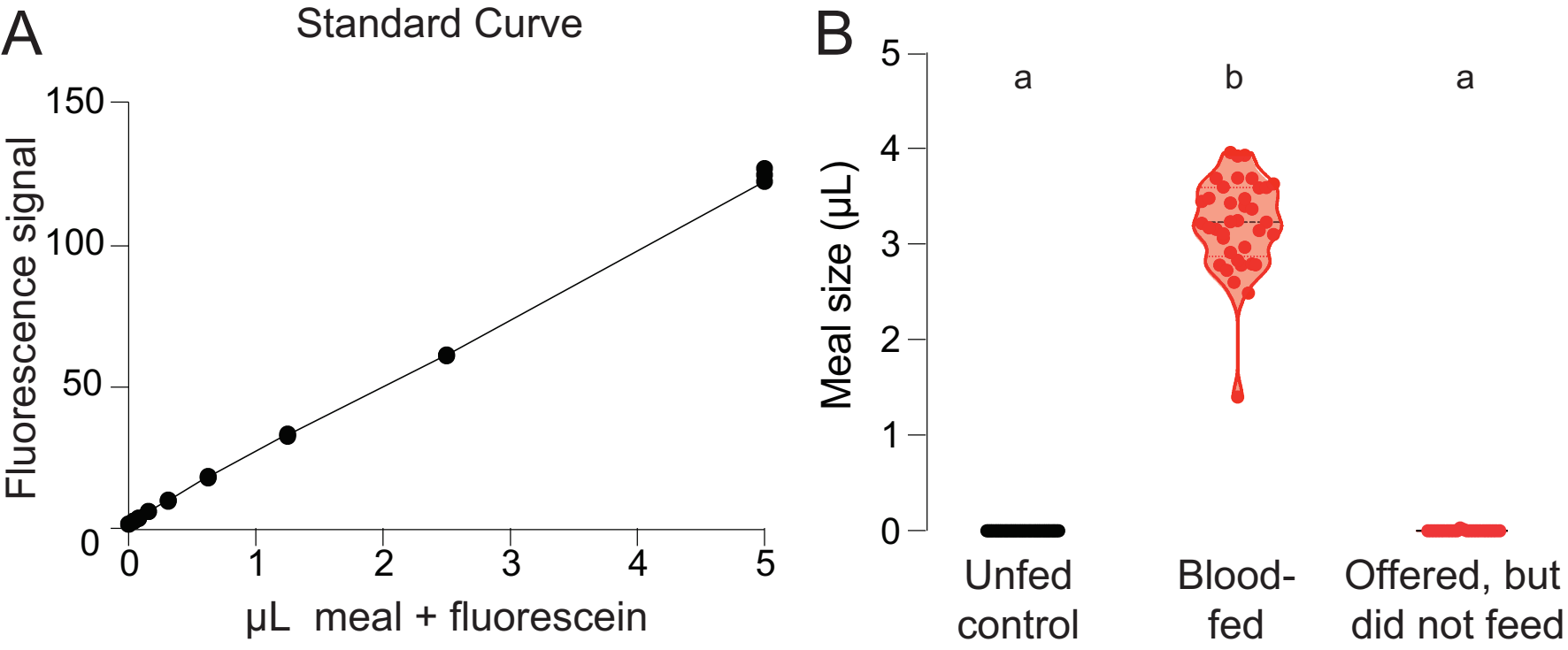


Figure 4

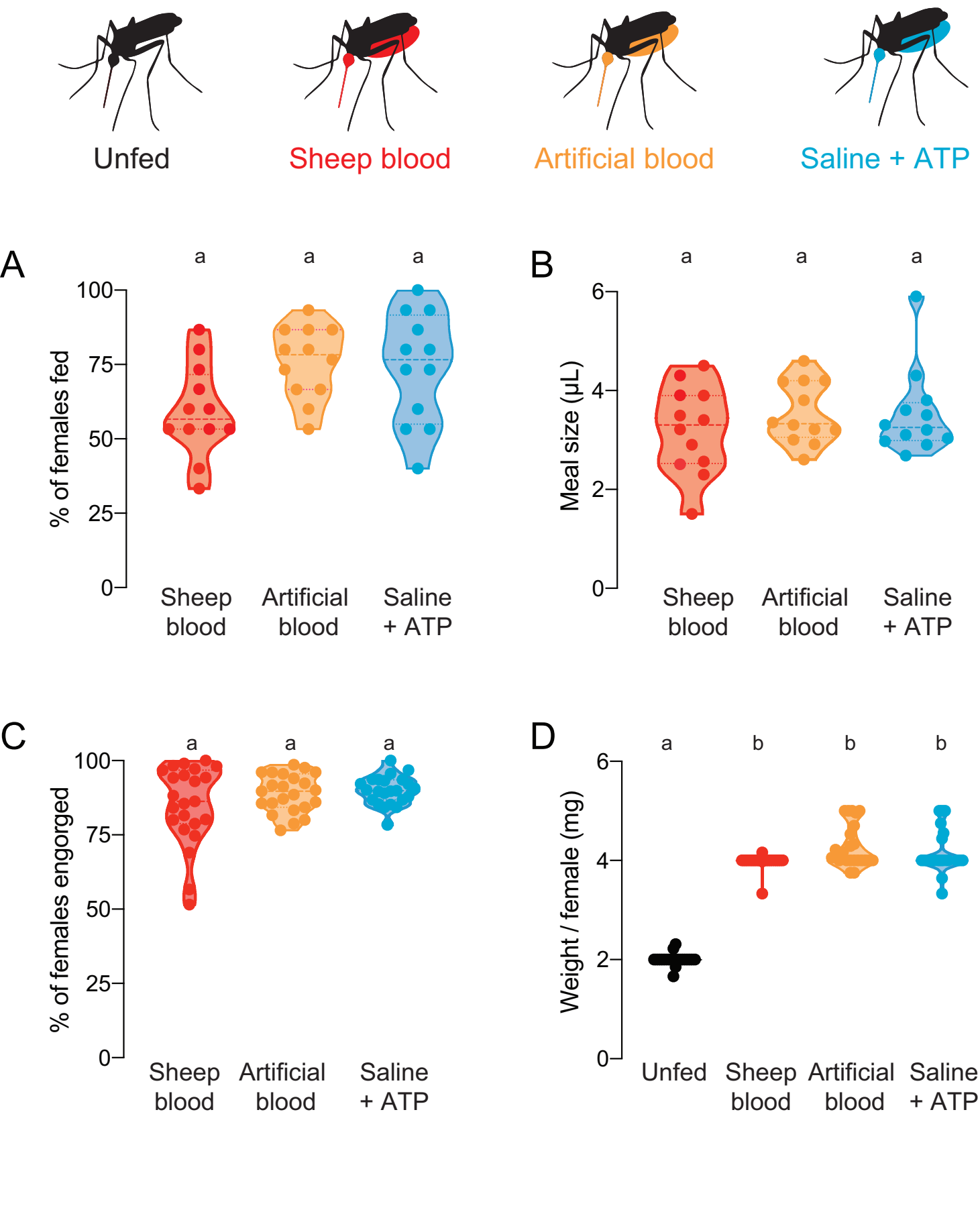
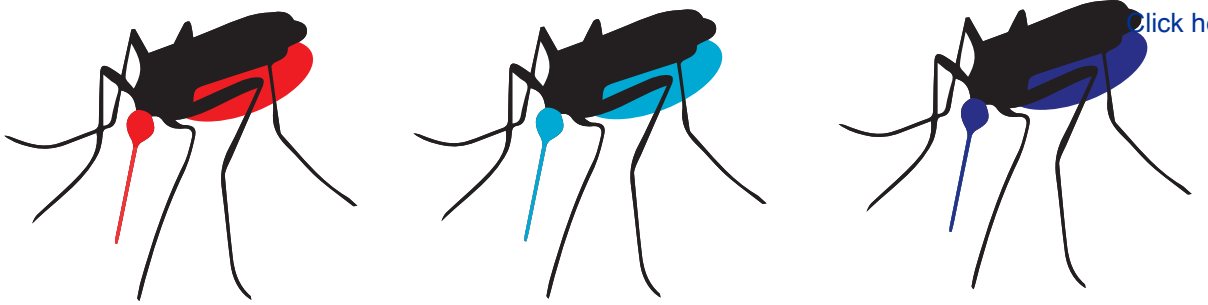


Figure 5



Blood

Saline
+ ATP

Saline + ATP +
NPY receptor
agonist

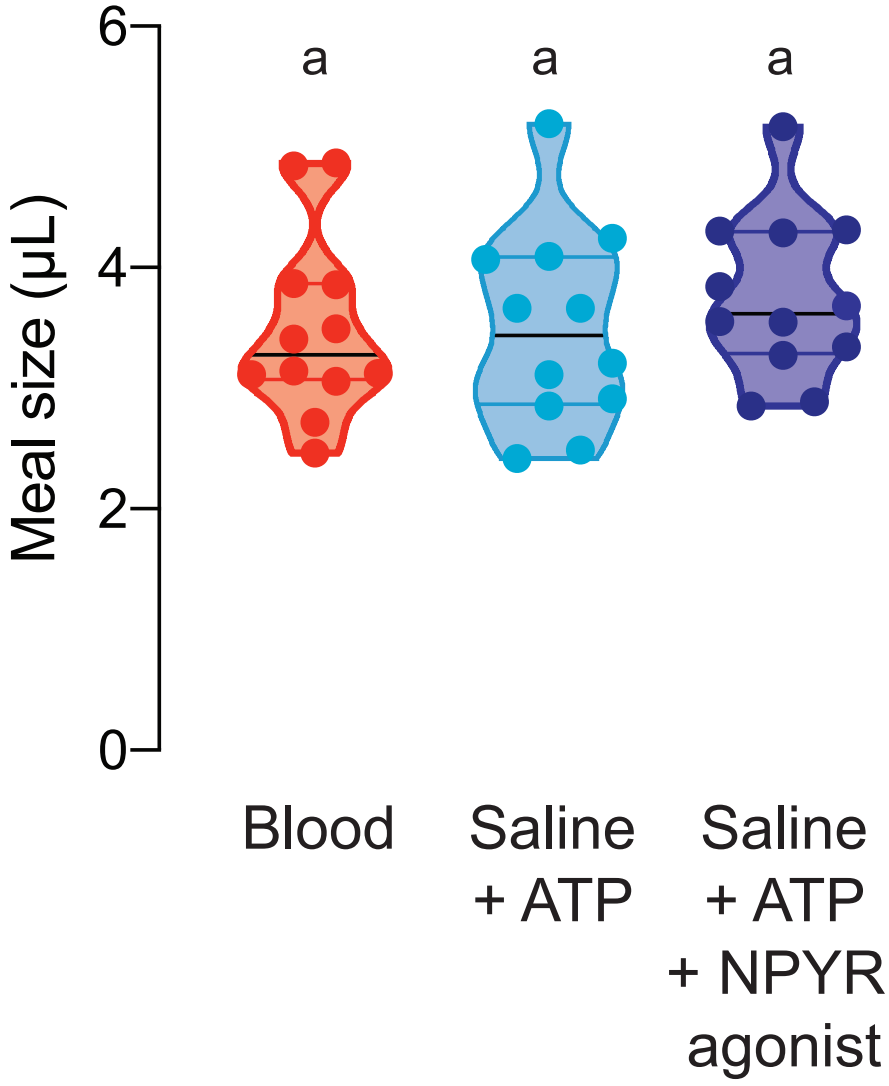
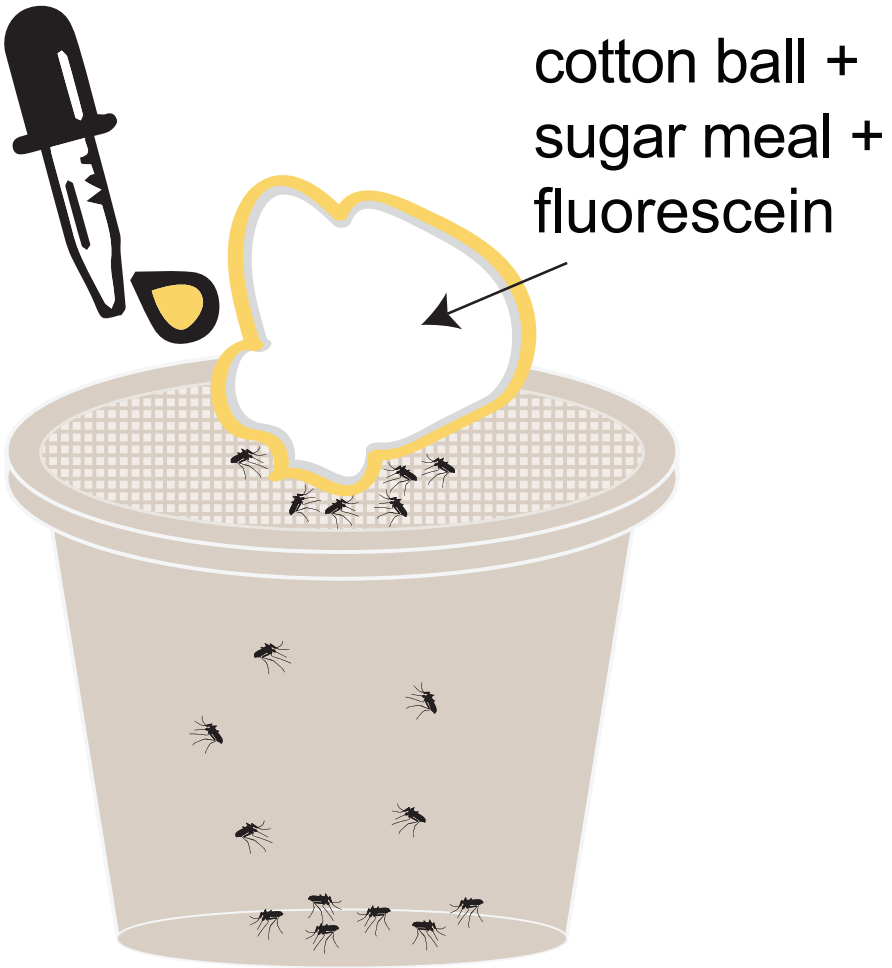


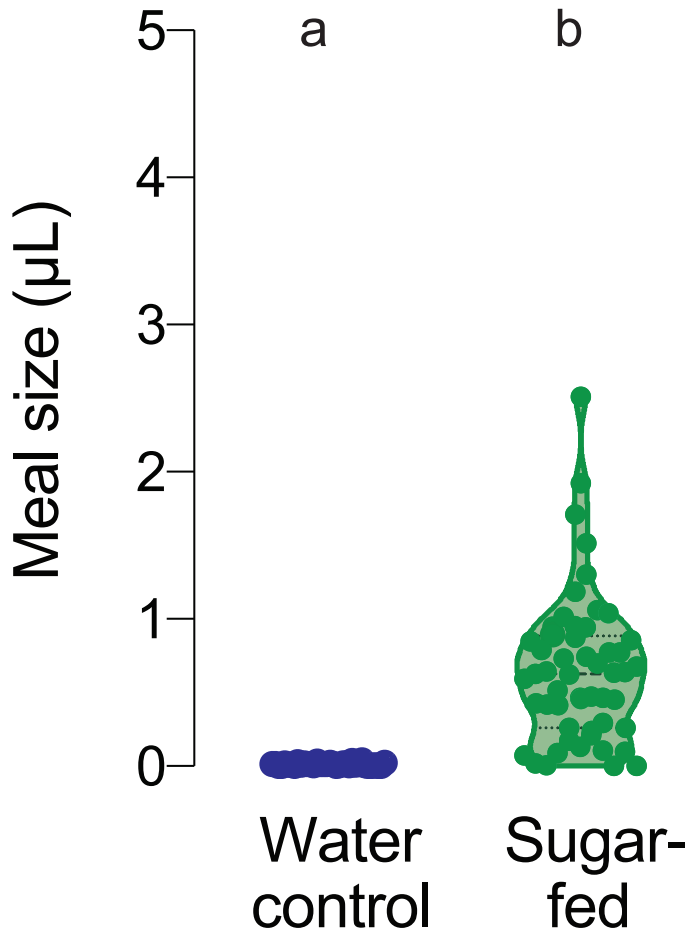
Figure 6

A

Nectar-feeding assay



B



Artificial Blood Meal

	Concentration of Stock Solution (mg/mL)	Volume of Stock Solution in Meal (µL/mL)	Final Meal Concentration (mg/mL)
Protein Components*			
γ-Globulins	50	300	15
Hemoglobin	35	230	8
Albumin	300	340	102
Total Protein	-	-	**125**
Non-Protein Components			
	Concentration of Stock Solution (mM)	Volume of Stock Solution in Meal (µL/mL)	Final Meal Concentration (mM)
NaCl	In γ-globulin stock	-	5-10
$NaHCO_3$	In γ-globulin stock	-	120
ATP	200	5	1
Water	-	125	-

*Protein components are prepared in stock solution of double-distilled water, except for γ-Globulins, which are dissolved in 400 mM $NaHCO_3$ and include a variable amount of NaCl (2-4%) in the product.

Saline Meal

Component	Concentration of Stock Solution (mM)	Volume of Stock Solution in Meal (µL/mL)	Final Meal Concentration (mM)
NaCl	-	-	-
$NaHCO_3$	400	300	120
ATP	200	5	1
Water	-	695	-

Name of Material/ Equipment	Company	Catalog Number
15 mL conical tubes	Fisher Scientific	14-959-70C
3 mm diameter borosilicate solid-glass bead	MilliporeSigma	Z143928
32 oz. high-density polyethylene (HDPE) plastic cup	VWR	89009-668
50 mL conical tubes	Fisher Scientific	14-959-49A
96-well black polystyrene plate	ThermoFisher	12-566-09
96-well PCR plate sealing film	Bio-Rad	MSB1001
96-well PCR plates	Bio-Rad	HSP9621
Adenosine 5'-triphosphate (ATP) disodium salt hydrate	MilliporeSigma	A6419
Albumin (human serum)	MilliporeSigma	A9511
Aluminum foil	Fisher Scientific	01-213
Balance	Fisher Scientific	01-911
Bead mill homogenizer	Qiagen	85300
Cotton ball	Fisher Scientific	22456880
Defibrinated sheep blood	Hemostat Laboratories	DSB100
Drosophila CO_2 fly pad	Tritech Research	MINJ-DROS-FP
Fluorescein	MilliporeSigma	F6377
Fluorescence plate-reader	ThermoFisher	VL0000D0
Gamma-globulin (human blood)	MilliporeSigma	H7379
Glycerol	MilliporeSigma	G7893
Hemoglobin (human)	MilliporeSigma	G4386
Laboratory wrapping film - parafilm	Fisher Scientific	13-374
Magnetic stirrer	Fisher Scientific	90-691
Microcentrifuge for 96-well plate	VWR	80094-180
Microcentrifuge Tubes	MilliporeSigma	2236412
Pellet pestle grinder	VWR	KT749521-1500
Phosphate buffered solution (PBS)	Fisher Scientific	BW17-516F
Razor blades	Fisher Scientific	12-640
Rubber bands		
Silicone tubing	McMaster Carr	
Sodium bicarbonate ($NaHCO_3$)	Fisher Scientific	S233

Sodium chloride (NaCl)	MilliporeSigma	S9888
Stir bars	Fisher Scientific	14-512
Translucent polypropylene storage box with removable lid		
Vortex mixer		
Water bath		
White 0.8 mm polyester mosquito netting	American Home & Habit Inc.	F03A-PONO-MOSQ-M008-WT

Comments/Description

For use for bead mill homogenizer; not required if using pellet pestle grinder

Example mosquito container used for feeding assays shown; alternate options can be used

Alternate options can be used
Alternate options can be used

Alternate options can be used to block light entering fluorescein container
Alternate options can be used
Not required if using pellet pestle grinder
For nectar-feeding; alternate options can be used
Alternate options can be used
Alternate options can be used

Alternate options can be used

Alternate magnetic stirrers can be used
Alternate options can be used
Alternate options can be used
Not required if using bead mill homogenizer
Optional
Alternate options can be used, such as a lathe for better consistency of cutting

Needed if using a fly pad for CO_2 delivery

Alternate magnetic stir bars can be used
Example box used for feeding assays shown

Alternate heating device may be used

Alternate options can be used

Dr. Vineeta Bajaj
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Dear Dr. Bajaj,

Thank you for the opportunity to revise our manuscript, titled "**Techniques for feeding and quantifying animal-derived blood and artificial meals in *Aedes aegypti* mosquitoes**" as part of "The study of mosquito biology" methods collection.

We appreciate the careful review and constructive suggestions, which have substantially improved our manuscript after making the suggested edits.

The reviewers major suggestions were to focus on fewer examples to clarify the objective of the methodologies presented. We agree, and have now removed former Figures 6A and Figure 7. These changes simplify the protocol by removing the multiple saline meal formulations and redundant examples of applications of the method. We have edited highlighted sections for filming to focus only on the critical steps to clarify the protocol.

Our responses to all of the editors' and reviewers' comments are found in the rebuttal document below.

The resulting modifications and edits have greatly improved our original paper and we hope that yourself and the reviewers find them appropriate. Thank you for your consideration.

Thank you for your assistance in handling our submission.

Sincerely,

Laura B. Duvall, Ph.D.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

3. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Hemotek, Hemotek Limited, Great Harwood, UK, etc.
Response: Thank you for this note. We have replaced all commercial language from the manuscript with general terms.

4. Is Glytubes commercial? If yes, please use generic term throughout.
Response: 'Glytubes' is not a commercial term – it is the term given to the device by authors in their original publication, Costa-da-Silva et al., (2013).

5. We cannot have non numbered steps in the protocol. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary.
Response: All steps of the protocol have been numbered according to the outlined format.

6. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution. This is required for the use of animal derived blood meal.
Response: This is an important point - we did not harm animals during these experiments and we have added an Ethics Statement.

7. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?
Response: We have amended the protocol to include more detailed steps, especially to explain how tasks should be carried out.

8. 3.1. How do you defibrinate the blood?
Response: We purchase commercially available defibrinated blood and have clarified this in section 3.2 of the protocol 'Preparation of animal-derived blood meals'. The text now reads: "For animal-derived blood meals, transfer 1.98 - 2 mL defibrinated sheep blood into a 15 mL conical tube (see step 3.3 for desired volume of blood). Commercially defibrinated sources of vertebrate blood, including from sheep, guinea pigs, and humans, may be used[13]."

9. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s) even in the protocol section.
Response: We have corrected this – all reference numbers now appear as numbered superscripts throughout the text.

10. In the JoVE Protocol format, only one note can follow one step "Notes" should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.
Response: Thank you for clarifying the format. We have made sure only a single note follows one step and have instead incorporated the detail into steps/sub-steps, or the discussion section as appropriate for maximum clarity.

11. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight

3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
Response: We have edited the manuscript to highlight only the essential steps of the protocol.

12. Notes cannot be filmed so please remove the highlighting.
Response: We have removed all highlighting of notes.

13. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
Response: Our revised figures do not contain any reprinted or modified figures from previous publications.

14. Please include all the Figure/Table Legends together at the end of the Representative Results in the manuscript text. Each Figure Legend should include a title and a short description of the data presented in the Figure and relevant symbols.
Response: We have now corrected this.

15. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
Response: Thank you. We have rewritten the Discussion to explicitly address these goals.

16. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials and sort the table in alphabetical order.
Response: We have made these changes.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
This manuscript by Jové and colleagues offers a useful protocol for feeding mosquitoes using a Glytube setup, as well as methods to analyze feeding amounts. The method is simple, and can be adapted for use by many research labs.

Major Concerns:
I have no major concerns.

Minor Concerns:
Line 27: Change "Female mosquitoes spread disease by biting vertebrate hosts" to "Female mosquitoes of certain species may spread diseases while biting vertebrate hosts"
Response: We agree that this is a more accurate statement and have changed the text accordingly. This sentence now reads "Females of certain mosquito species can spread diseases while biting vertebrate hosts to obtain protein-rich blood meals required for egg development."

Line 57: Please clarify what aspects of "feeding behavior" can be compared? Engorgement, probing, other?
Response: Thank you - we have clarified this by replacing "feeding behavior" with "meal volume consumed".

Lines 59-67: Please modify the text- currently it gives the reader a false impression that all Ae. aegypti mosquitoes are infected with pathogens, that females infect new hosts with each biting (they cannot infect during the first blood meal, and they only can infect a host if they themselves carry the pathogen and it is present in their salivary glands), and that females are preferentially attracted and bite only uninfected hosts (if that's the case, please provide references).
Response: Thank you for pointing this out. This paragraph has been re-written to address these points, and this section now reads as follows:
"While consuming a blood meal from an infected host, she may ingest blood-borne pathogens[6, 8], which then migrate from the mosquito's midgut to her salivary glands[10]. Female mosquitoes infected in this manner can spread disease by injecting pathogens along with saliva when biting subsequent hosts[11, 12]."

Line 62: please define the difference between biting and blood-feeding
Response: We have included the following sentence to clarify:
"The female mosquito first bites her host by piercing the skin with her stylet and injecting saliva, which contains compounds that trigger the host's immune response[9]. She then feeds by pumping blood through her stylet into her midgut."

Line 105: Typo. Remove "The"
Response: Thank you for catching this, we have removed the typo.

Line 110: Genetic background of the host or the mosquito?
Response: We have included wording to specify that it is the genetic background of the mosquito in question: "This assay can measure differences in feeding vigor in response to variables such as meal composition or the mosquitoes' genetic background."

Line 135, Step 1.3. How long can ATP stock aliquots last at -20C?
Response: We have added recommendations for how long to store ATP stock aliquots at -20°C: "Aliquot the ATP solution and store at -20°C. This stock solution of ATP is typically made fresh every six months and is used for all meals described below. To prevent degradation, ATP aliquots should not undergo multiple freeze-thaw cycles or be heated along with other meal components."

Line 138: Please clarify if ATP is already buffered by following the procedure in steps 1.1-1.3, or if additional steps need to be taken.
Response: Thank you for pointing out this confusion. We have modified the procedure steps 1.1-1.3 ('Preparation of the phagostimulant: adenosine 5'-triphosphate (ATP)' section) to improve clarity. This section now reads:
"Reconstitute ATP disodium salt hydrate (MW = 551.14 g/mol) in the aqueous 25 mM $NaHCO_3$ to a final concentration of 200 mM ATP. To minimize hydrolysis of ATP, it must be buffered by a salt solution such as $NaHCO_3$. For a total volume of 10 mL of 200 mM ATP in 25 mM $NaHCO_3$ buffer: add 1.1 g of ATP disodium salt hydrate to a volumetric flask and fill with 25 mM $NaHCO_3$ buffer to a total volume of 10 mL. Using a magnetic stir bar, thoroughly mix the solution until all the ATP is dissolved."

Line 155, Step 3.1. Is there a method by which one can check the quality of the blood? Quality control steps?
Response: We have added detail to step 3.1 ('Preparation of animal-derived blood meals' section), describing ways to check the quality of blood.
This text now reads: "Prior to use, ensure that the purchased blood has not passed its expiry date and mix it well by inverting the bottle, especially if there is visible separation of blood components."

Line 159, Step 3.2. Warmed to what temperature? Please mention here. Using a water bath or bead bath?
Response: We have included the recommended temperature for the water bath used to warm meal components other than ATP.
The text now reads: "For optimal feeding, ATP can be added to a final concentration of 1 - 2 mM after the sheep blood has been warmed to 45°C in a water bath."

Line 159: How is 1 mM- 2mM achieved from the 200mM stock made in step 1.2? I.e. how much should be added to the 2mL of blood?
Response: Thank you for this suggestion. We have added these details to step 3.2 ('Preparation of animal-derived blood meals' section).
"For a final concentration of 1 mM ATP, add 10 μL of the 200 mM ATP stock solution to 1.99 mL of pre-warmed blood and mix. For a final concentration of 2 mM ATP, add 20 μL of the 200 mM ATP stock to 1.98 mL of pre-warmed blood and mix."

Lines 167-169: How much (volume) of these stocks needs to be prepared
Response: Great question - we have clarified meal volume per experiment at the beginning of each section. This now reads: "Calculate the number of meals needed to feed all mosquitoes; each Glytube holds a 2 mL meal and feeds approximately 25 mosquitoes. One additional meal must be prepared to calibrate the standard curve for fluorescence readings. Unless stated otherwise, all steps in this section describe reagent amounts required to prepare one 2 mL meal."

Line 185: Is half of the 2mL meal kept aside, or is much larger volume prepared?

Response: Thank you for highlighting this confusion. We have reworded these instructions for improved clarity at all respective steps.
This now reads: "Retain 1 mL of the final meal formulation containing 0.002% fluorescein to generate the reference standard curve. Treat the retained volume identically to the meal that is being delivered to mosquitoes; expose to the same light and temperature conditions throughout the duration of the experiment, and subsequently freeze this along with the delivered meal."

Line 190: Titles use a different reference format (than numbers which are used throughout main text).
Response: We thank the review for pointing this out and have now updated the titles to match the reference formatting used throughout the text.

Line 195: How long does it take for a blood-meal to be digested in comparison?
Response: The blood meal is digested at a rate comparable to the artificial blood and saline meals. We have rewritten the discussion to clarify this point. This text now reads: "Changing the meal composition can also allow researchers to manipulate the amount of time needed to clear the meal from the midgut and as well as the subsequent host-seeking behavior. The meals presented here require 24 – 36 hours for digestion[7] similar to animal-derived blood."

Line 200, Step 5.1 What are the details for warming the saline meal?
Response: We have added the water bath temperature for warming the saline meal.
The updated text for step 5.3, reads: "For each 2 mL meal, combine in a 15 mL conical tube: 600 µL of 400 mM $NaHCO_3$ with 1.39 mL ddH_2O. Wait to add 10 µL of the 200 mM ATP stock solution until after the meal has been warmed to 45°C in a water bath."

Line 223: change "fly around in it" to "fly around in"
Response: We have modified the text with this suggestion.

Line 224: What does difficult mean in this context? Also clarify that it's difficult to approach by the mosquito not the investigator.
Response: We have reworded the sentence to improve clarity.
The revised text now reads: "The container should be large enough for mosquitoes to fly around in, but not so large that it will be difficult for the mosquitoes to locate the mesh surface and begin feeding."

Line 249: Accuracy is how close measurements are to each other- is that what the authors mean here? Would it be better to say "not correct"?
Response: This is an important point – thank you. We have corrected our error.

Line 250: Even though they cannot be used for direct comparison, are wells with only fluorescein or only meal with fluorescein still included in the assay?
Response: The Reviewer is correct that these meals cannot be used for direct comparison, therefore we do not include them in the assay. We have rewritten this section of the protocol to clarify.

Line ~283. Rubber bands are shown on the conical tubes in Figure 1C. Is this recommended/necessary? Rubber bands are not mentioned in the protocol.
Response: We have added that rubber bands are optional in the protocol.

Line 284: How much cooling is considered significant? How much time (on average) does it take for the meal to cool down enough so that mosquitoes refuse to feed?
Response: Thank you for suggesting these important details. We have added them to the text which now reads: "It is important to work quickly at this step so that the meal does not cool down to ambient temperature and reduce the likelihood of maximal feeding. The rate of cooling will depend on the ambient temperature of the room where these steps are conducted, but they should typically be completed within 5 minutes in a 25°C room."

Line 292: is it "method" or "set up"
Response: We have replaced "method" with "set up".

Line 317, Step 1.1. What are the specifications required for the balance? What range is required? What is the expected average weight for a single mosquito?
Response: Unfed female *Aedes aegypti* mosquitoes reared with constant food access typically weigh approximately 2 mg each. However, the average weight of an unfed mosquito depends on genotype, sex, and rearing conditions. This section (now 1.2) now reads: "Weigh groups of 5 females from the unfed

cohort, i.e. mosquitoes that were never offered a meal, and calculate their average weight as the estimate of the "pre-feeding" weight. The average weight of an unfed mosquito depends on genotype, sex, and rearing conditions. Unfed female *Aedes aegypti* mosquitoes reared with *ad libitum* access to sucrose typically weigh approximately 2 mg each."

Line 318: remove "and" after "cohort"
Response: We have corrected this typo.

Line 320: groups are a bit unclear. Consider: "two groups (fed and unfed) with 5 mosquitoes each per weighing"
Response: We have reworded the respective sentence for better clarity.

Line 351: How much of the borosilicate beads are added and does this change the final volume in the well?
Response: We have revised the text to clarify that one 3 mm bead is added to each well.

Line 362, Step 2.5. "…in the next 2 columns of the plate." 3 columns are shown in Figure 2? Please clarify.
Response: Good point - we have adjusted the Figure to match the text.

Line 370: how are the tissues disrupted?
Response: We have included several of our suggestions for ways to disrupt the mosquito tissue.
This text now reads: "Seal the plate carefully and disrupt the tissue by manual grinding. The abdomen should be thoroughly homogenized to release the meal. Methods to disrupt tissue include bead mill homogenizer with 3 mm borosilicate solid-glass beads (30 Hz for 30 seconds), vortex mixer with 3 mm borosilicate solid-glass beads, or a pestle grinder without beads."

Line 400: These figure headings makes it seem like the sections are figure captions.
Response: This is an important point- we have removed figure headings from this section.

Line 402: change "wells that each contain" to "wells each containing"
Response: This has been corrected.

Line 415: differing or various?
Response: We replaced differing with various.

Line 430: for context, what does the NPY Y2 do?
Response: We have edited the text to provide this context: "In these experiments, females were offered saline + ATP meals with the human NPY Y2 receptor agonist TM30338 added at a concentration of 100 μM. This drug alters host-seeking behavior through activation of *Ae. aegypti* NPY-like receptor 7. Measuring meal sizes is critical for the interpretation of experiments to assess the effect of this drug on post-blood-feeding behavior because it allows the researcher to calculate the dose consumed by each female."

Line 437: Figure 6A shows meal size, not engorgement
Response: Thanks for point this out, we have edited this text for accuracy.

Line 449: some context for NPYLR7 mutant would be useful
Response: Based on feedback from the reviewers, we have now removed this figure.

Line 471 paragraph, discussion. Please discuss parafilm parameters. Is there a way to know when it is stretched thin enough? If it's not stretched thin enough, what happens? Or too thin?
Response: This is important – we have added additional information about parafilm parameters to the discussion. This text now reads: "Females may not feed if the parafilm is not pulled taut across the Glytube cap – the parafilm should be uniformly transparent and should not buckle, as this prevents the female from being able to effectively pierce the parafilm with her stylet. If the meal leaks through the Glytube onto the mesh, the parafilm may have torn during the stretching process and should be replaced."

Line 637: see comment for line 415
Response: We have corrected this.

Line 662: might want to explain Ir7a>TRPV1
Response: We agree with the reviewer that these data were somewhat confusing and have simplified Figure 6 to remove this panel.

Figures: All figures are a blurry. Please check the resolution and guidelines (dpi)
Response: We thank the reviewers for catching this – we have increased the quality of the exported figures.

Figure 4D: put a space between weight/ and female in the y axis
Response: We thank the reviewer for pointing this out – we have added an additional space on the y axis legend and agree that this improves legibility.

Figure 6B: what are the 2 gray color dots representing?
Response: We agree with the reviewer that these data were somewhat confusing and have simplified Figure 6 to remove this panel.

Reviewer #2:
Manuscript Summary:
The manuscript describes two methods for feeding and quantifying Aedes aegypti meal size. A simple fluorescent-based methodology to quantify the volume of a meal taken by a mosquito, that can be used for blood-based meals or any other artificial meal; and Glytubes, which is an apparatus to deliver diets, based on a modification of a common 50ml laboratory tube. Both methods very useful and offer several advantages over commonly used methods and can be used on a variety of applications. The methodologies are well explained. However, in the introduction the 2 methodologies are a bit mingled and the objectives of the manuscript only become clear on the protocol.
Response: We agree that the delineation between methodologies could be clearer. To that end, we have updated the introduction with wording to clarify what the two methodologies are as we discuss them. "This protocol includes 2 sections: delivering artificial meals and quantifying consumption…"

Major Concerns:
My major concern is that no comparison with existent methods used to quantify blood meal sizes are made. Were the meal volumes obtained consistent with those described in the literature for Aedes aegypti? Is it comparable to other methods such as quantification of haemoglobin to infer mosquito blood meal volume?
Response: We have updated the text to include the following statement and citations to address other methods of quantifying meal volume.
"While blood meal size is variable and can be influenced by a myriad of factors[11, 28, 29], ingested meal sizes measured with the methods described here are generally consistent with previous quantifications[7, 30, 31]."

The fluorescence-based method for quantification of blood meal volume is terminal. If live mosquitoes are needed afterwards, the alternative is to weight mosquito that were fed and un-fed, "but this method is less sensitive than the fluorescence-based quantification".
Were the methods compared? If so, could you add the data. Why is it less sensitive?
Response: We have explained this in a note at the beginning of the section, 'Weighing mosquitoes to be used for further experiments' in the protocol. To weigh mosquitoes, they must be pooled into groups of 5 females each – i.e. they cannot be weighed singly to discern differences between individuals. Fluorescence-based quantification allows the experimenter to take readings from individual females, therefore can be used to quantify variability in feeding between individuals.

The manuscript uses several examples for applications of the methodologies. This is good. However, it feels they are too many and some lack adequate explanation.
Response: We agree with the reviewer and have cut several examples (former Figure 6A and Figure 7) to improve the clarity of the protocol.

Figure 6. What are suboptimal meals? Why capsaicin appears here without any explanation? Some editing of the figure to retain the essential would increase the MS clarity.
Response: We agree with the reviewers that this panel was confusing and have deleted it for clarity.

Figure 7. Does not add anything extra to the manuscript.
Response: We agree with the reviewer and have cut this figure.

Table 2 and point 5. (line 190). Why two recipes? This is not the object of the protocol, one would be sufficient for demonstrating the use of the protocols for non-blood feeding, it is confusing.
Response: The Reviewer is correct that comparing saline meals is not the main focus of the protocol. We have re-written the manuscript to show a single recipe that is a protein-free version of the artificial blood recipe provided.

Minor Concerns:
Point 4.3.(line 174)
600 µL γ-globulins, 460 µL hemoglobin, 680 µL albumin and 10 µL ATP adds up to 1750. To prepare a total of 2ml 250ul of water should be added (not 240)
Response: Great point- we have fixed this is the text.

Point 2.5.(line 286)
For how long do mosquitoes feed? Please add.
Response: Thank you for this important question – we have added this so that the text now reads "Place the assembled Glytube on top of the mosquito container and allow the mosquitoes to access to feed for at least 15 minutes to achieve maximal feeding rates."

Point 2.5.(line 291)
Is the tube screwed up into the cap? Please clarify
Response: We have clarified this and explained how to perform this step in more detail. "Partially screw the cap with the meal onto the glycerol-filled tube – just enough to prevent leakage of the meal or the glycerol."

Point 1.1.(line 318)
Weigh groups of 5 females from the unfed cohort (and) to calculate the average "pre-feeding" weight.
Response: We have incorporated these changes as part of step 1.2 in the 'Weighing mosquitoes to be used for further experiments' section.

Point 1.2.(line 324)
How the pre-fed mosquitoes weight enters the calculations?
Response: Thank you for pointing out this confusion – we have reworded the respective steps of the 'Weighing mosquitoes to be used for further experiments' section to explain the difference between the "unfed" cohort that is never offered a meal, and the "not fed" pile of the experimental cohort, which does not consume a meal when offered.

Figures: The quality is poor. The medians are not visible. This is probably due to the quality of my pdf. If not, please increase quality of figures.
Response: We thank the reviewers for catching this – we have increased the quality of the exported figures.

Figure 4/5 and legends (line 640 and 652)
"Percent of females fed scored using fluorescence readings. Each point represents a measurement from an individual female. Data is shown as median with range, n = 12."
I am assuming that the % of fed females (nº of fed females over total nº of *100), if so individual measurements are not compatible. Could you please clarify.
Response: Thanks for catching this mistake - we have rewritten the figure legend to show that the data in Figure 4A represents trials [n = trial (12 - 16 females/trial)] and Figure 4B represents individual females from a single trial.

REPRESENTATIVE RESULTS: I believe it is a journal policy to avoid the use of personal pronouns.
Response: We have rewritten the text to avoid the use of personal pronouns in this section.

Reviewer #3:
Manuscript Summary:
In this manuscript, the authors present a method for artificial blood feeding in Aedes mosquitoes.

Major Concerns:
While the manuscript is well written and experiments are conducted in a rational way, this work does not add any knowledge to the existing literature. There have been many papers published on artificial blood and membrane feeding for mosquitoes. The authors' claim that most mosquito labs use animals for blood-feeding is not correct. Most labs working on mosquitoes, use simple membrane feeding protocols by using Petri dishes, culture vials, and even just cotton soaked in blood. Several labs (Luckhart lab for instance) have already published artificial blood composition to avoid any growth factors etc. Sugar meal with ATP or saline with ATP is a standard procedure to trick mosquitoes in taking up the saling in midgut instead of the crop. I do not see any labs that could benefit from this work.
Response: While we agree that many protocols for artificial feeding and formulations for artificial meals exist, this video protocol will be useful as a visual guide to researchers who are using pharmacological and genetic manipulations to study mosquito blood-feeding and post-blood-feeding behavior. This protocol provides:

- Innovative application of existing tools to deliver meals of varying composition and pharmacological compounds to mosquitoes in a physiologically relevant, non-invasive, and efficient manner.
- Precise quantification of meals and additives delivered to mosquitoes.
- Controlled stimuli to allow for reproducible application and complete experimental transparency of artificial feeding protocols with a focus on making them broadly accessible to many labs.

This work complements the stated mission of this journal: "JoVE is a scientific methods journal providing rapid and efficient publication of methodology in biological, medical, chemical and physical research. JoVE articles are video based (we call them video methods articles) which ensures a more effective transfer of information and experimental detail than with traditional text-based articles. JoVE publishes novel methods, innovative application of existing techniques, and gold standard protocols that enable a greater level of experimental transparency."

Author Bios:

Veronica Jové (vjove@rockefeller.edu)
Veronica received her B.A. degree from Columbia University in 2014 in Biological Science and Hispanic Studies. At Columbia, she worked in the laboratory of James Manley on MecP2 gene expression and splicing as affected by ALS mutations. Her research uses behavior, genomics, and calcium imaging to map the circuitry that detects blood and initiates blood-feeding behavior in *Aedes aegypti* mosquitoes.

Krithika Venkataraman (kvenkatara@rockefeller.edu)
Krithika Venkataraman earned a B.A. from Smith College in 2015 where she studied gene regulation in filarial parasites in the laboratory of Steven A. Williams. Krithika was a summer intern in the laboratory of Dr. Utpal Tatu at the Indian Institute of Science, Bangalore, India, where she worked on methods to control infections caused by protozoan parasites. Currently, Krithika studies how female *Aedes aegypti* mosquitoes regulate their attraction to humans depending on their reproductive physiology. Specifically, she is interested in how mosquito-specific genes and endocrine signaling modulate mosquito attraction. The broader goal of her work is to elucidate novel points of intervention to break the deadly biting cycles of disease vector mosquitoes.

Thomas M. Gabel (tg2738@columbia.edu)
Tom has worked with a variety of invertebrate communities, in both field and laboratory settings. He earned his Bachelor's degree in Ecology & Evolutionary Biology at the University of Colorado, Boulder, where he studied the impacts of climate change on the diversity and developmental stability of grasshopper assemblages in the Colorado Front Range. At California State University, East Bay, he investigated threats to native ant biodiversity in the San Francisco Bay Area, including the invasive Argentine ant, Linepithema humile. He then spent 3 years with the California Department of Fish & Wildlife on long-term monitoring projects studying the zooplankton and endangered planktivorous fish communities of the San Francisco Bay Estuary, including the endemic Delta Smelt, Hypomesus transpacificus. He joined the Duvall lab in the fall of 2019 as a Staff Associate, rearing and maintaining research colonies of Aedes aegypti for research into the neural mechanisms of blood-feeding behavior.

Laura B. Duvall (lbd2126@columbia.edu)
Laura received her B.A. in Biochemistry and Biological Basis of Behavior from the University of Pennsylvania in 2007. She then went on to complete a PhD with Paul Taghert at Washington University in St. Louis studying the neuropeptide regulation of circadian behavior in *Drosophila*. She conducted postdoctoral research with Leslie Vosshall at the Rockefeller University where she switched her studies to *Aedes aegypti* and focused her research efforts on understanding the regulation of feeding and mating behaviors in the mosquito. She started her own lab at Columbia University in 2019 where she is a member of the Department of Biological Sciences and an affiliate of the Zuckerman Institute. The Duvall lab focuses on using pharmacological and genetic approaches to understand how mosquitoes regulate their innate behaviors.