

CHAPTER 7

Methods to Assess Blood and Nectar Meals in *Aedes aegypti* Mosquitoes

Krithika Venkataraman,^{1,3} Veronica Jové,^{1,3} and Laura B. Duvall^{2,4}

¹The Rockefeller University, New York, New York 10065, USA; ²Department of Biological Sciences, Columbia University in the City of New York, New York, New York 10027, USA

Male and female *Aedes aegypti* mosquitoes survive by feeding on floral nectar for metabolic energy, but females require blood protein, obtained from biting a host, for egg development. Although males exclusively derive energy from nectar sugars, females must select the meal that best matches their present metabolic and reproductive needs. In females, blood and nectar promote independent feeding behaviors with distinct sensory appendages, meal sizes, digestive tract targets, and metabolic fates. Understanding how male and female mosquitoes recognize, locate, and metabolize nutrients is essential for characterizing the survival and reproductive capabilities of this mosquito. Here, we provide an introduction to blood versus nectar feeding and methods to quantify nectar and blood meal sizes in individual *Ae. aegypti* mosquitoes. Precise quantification of meal size is crucial for ensuring consistency in assays that record events downstream of feeding behavior, including host attraction or fecundity.

BLOOD AND NECTAR MEALS

Although male and female *Aedes aegypti* mosquitoes can sustain energy metabolism with carbohydrates from nectar sugars, females require protein from vertebrate host blood to develop eggs and reproduce (Fig. 1A; Briegel 2003; Duvall et al. 2019). Nectar feeding and blood feeding are linked to behaviorally discrete feeding programs, which are defined by the use of different feeding appendages, meal sizes, and digestive tract targets (Fig. 1B; Gordon and Lumsden 1939; Trembley 1952). Nectar is detected by the labium, an external feeding appendage that ensheathes the stylet (Fig. 1C; Pappas and Larsen 1978; Sanford et al. 2013). Blood is detected by the stylet, which comes into direct contact with blood as a female pierces through her host's skin and retracts the labium (Fig. 1D; Gordon and Lumsden 1939; Trembley 1952; Jové et al. 2020). Females typically take small, frequent nectar meals with an average meal size of 0.87 μL (Jové et al. 2020). In contrast, blood meals approximately double the body weight of a female and have an average size of 3.20 μL (Liesch et al. 2013; Sissoko et al. 2019; Jové et al. 2020). The large blood meal contains enough protein to produce 100–150 eggs and suppress the drive to blood feed for multiple days (Brown et al. 1994; Klowden and Lea 1979; Duvall et al. 2019). The nectar meal is typically routed first to a temporary food storage organ called the crop, leaving the midgut free in case the female encounters an opportunity to feed on blood (Fig. 1B; Gordon and Lumsden 1939; Trembley 1952). Female *Ae. aegypti* mosquitoes thus have mutually exclusive

³These authors contributed equally to this work.

⁴Correspondence: lbd2126@columbia.edu

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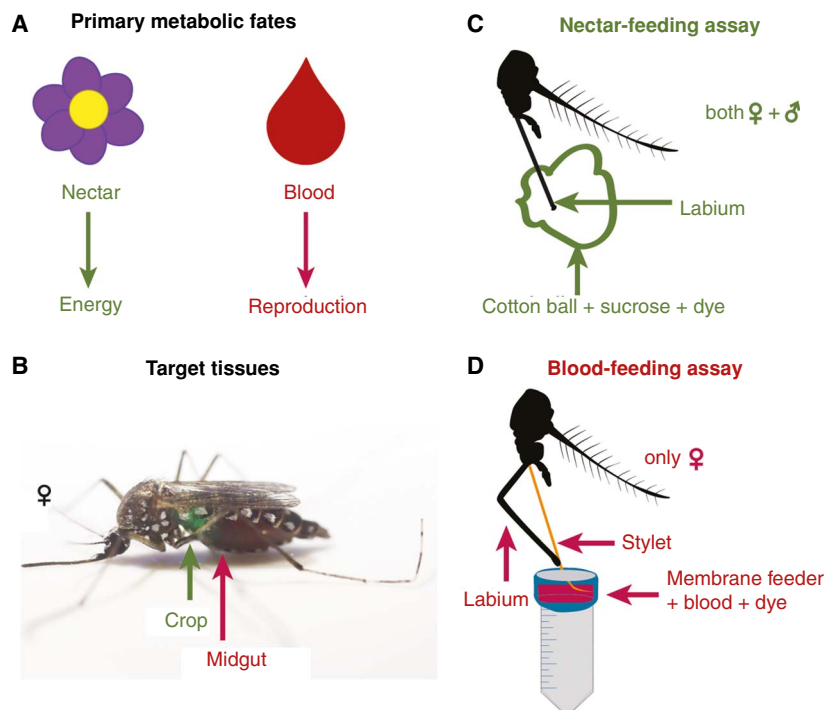


FIGURE 1. Nectar and blood feeding in mosquitoes involve distinct sensory appendages, target tissues, and metabolic fates. (A) Nectar and blood meals have distinct metabolic fates in energy and reproduction, respectively. (B) Photograph of an *Aedes aegypti* female with a 10% sucrose meal containing food dye in the crop (green), and a sheep blood meal in the midgut (red), illustrating the different target tissues for different meal types. Schematics of example nectar-feeding (C) and blood-feeding (D) assays that can be used to deliver meals to mosquitoes in the laboratory. (Adapted from Jové et al. 2020.)

feeding programs for blood and nectar meals—from the sensory periphery, to visceral organs, and to metabolic fate (Fig. 1).

Features of each behavioral program can be precisely quantified in the laboratory using nectar- and blood-feeding assays (Costa-da-Silva et al. 2013; Liesch et al. 2013). Room-temperature sucrose meals are presented to females on a cotton ball so that the labium directly contacts the meal upon landing, similar to nectar feeding from flowers (Fig. 1C). In blood-feeding assays, warmed blood meals are offered to females in the presence of attractive human cues like CO₂ and heat to promote landing on an artificial feeder (Fig. 1D; McMeniman et al. 2014; Liu and Vosshall 2019). An artificial membrane prevents the female from directly contacting the blood meal, similar to the way that skin prevents direct blood contact when the female lands on a host. Like with skin, the female must pierce the membrane with the stylet to directly contact the blood meal.

Nectar Feeding

In the wild, female and male *Ae. aegypti* mosquitoes obtain sugars from floral nectar, which is primarily composed of sucrose, fructose, and glucose (Van Handel 1972). In the laboratory, mosquitoes are routinely reared on 10%–20% sucrose solutions as a substitute for floral nectar. When mosquitoes feed on nectar and sucrose solutions, the nectar-feeding appendage, the labium, directly contacts the meal. Sugars are detected by gustatory receptors, and exogenous activation of neurons that express a conserved sweet taste receptor, *Aedes aegypti Gr4*, is sufficient to promote nectar-feeding behavior in the absence of a real nectar meal in these mosquitoes (Jové et al. 2020). It is important to quantify nectar-feeding behavior, because energy derived from sugar metabolism can impact longevity, fertility, and flight time (Harrington et al. 2001; Briegel 2003; Barredo and DeGennaro 2020). In females, nutrients from sugars can also synergize with those from blood to control fecundity. For

example, isoleucine from human blood enhances egg production only in females that have fed on sugar (Harrington et al. 2001).

Blood Feeding

Female *Ae. aegypti* must ingest vertebrate blood protein to reproduce, and domestic strains of this species preferentially bite human hosts to obtain the required nutrients for egg production (McBride et al. 2014). As a female bites her victim, the needle-like stylet pierces the skin and injects saliva. Compounds in the female's saliva trigger the host's immune response and prevent the blood from clotting while she feeds. As she pumps blood through the stylet, the female's meal is directed to the midgut for digestion (Gordon and Lumsden 1939; Trembley 1952). When allowed to feed to repletion, the volume of blood ingested reliably doubles the female's body weight and takes several days to fully digest (Klowden 1990). Upon ingesting the blood meal, the protein obtained from blood suppresses a female's attraction to human hosts and triggers egg development (Judson 1967; Klowden and Lea 1979; Davis 1984a,b; Klowden 1990; Duvall et al. 2019).

During blood feeding, *Ae. aegypti* are capable of transmitting viral and parasitic pathogens that cause diseases including dengue, lymphatic filariasis, yellow fever, chikungunya, and Zika (World Health Organization 2020). When a mosquito consumes a blood meal from an infected host, she ingests blood-borne pathogens into her midgut, which can then enter the mosquito's circulatory system and migrate to her salivary glands where they are poised to be passed to the next animal that she bites (Rückert and Ebel 2018). After the female lays her clutch of eggs, she is once again motivated to blood feed from her next host, initiating another cycle of host seeking and egg laying (Judson 1967; Brown et al. 1994; Liesch et al. 2013; Duvall et al. 2019). When they feed on subsequent hosts, infected female mosquitoes spread disease by injecting pathogens along with their saliva. Thus, blood-feeding behavior is a crucial step for both reproduction and disease transmission.

Accurate and precise quantification of the blood meal is important for many experimental approaches, because protein content ultimately controls oogenesis and host-seeking suppression behavior (Briegel 1990). If a female fills to repletion on blood, she can develop an entire clutch of more than 100 eggs and will fully suppress her host-seeking drive (Liesch et al. 2013; Duvall et al. 2019). However, partial meals may lead to no or reduced egg production (Briegel 1990) and incomplete host-seeking suppression (Klowden and Lea 1978). Consequently, females will continue to host seek to find an additional source of blood and thereby refeed on multiple hosts. Refeeding on multiple hosts facilitates disease transmission and can also occur if the female experienced poor larval nutrition (Farjana and Tuno 2013).

QUANTIFYING MEAL VOLUME

Selecting a Method

Meal size can be quantified as either an end-point assay by measuring fluorescence (Duvall et al. 2019; Jové et al. 2020) after mosquitoes have consumed meals containing known amounts of fluorescent dye or, dynamically, using video recording and live tracking (Hol et al. 2020). When choosing a measurement method, it is important to consider whether an end-point assay will provide sufficient temporal resolution of the feeding event, and whether live animals will be needed for subsequent experimentation (Fig. 2A). If live mosquitoes are required for downstream experiments, meal size consumed can also be measured by weighing small groups of anesthetized mosquitoes (Fig. 2B). Weights can be benchmarked to known volumes determined by the end-point homogenization assay in which fluorescent dyes are added to the meal (Fig. 2C). To quantify features of blood-feeding behavior prior to engorgement, video recording can be combined with either quantification assay (Fig. 2D; Hol et al. 2020).

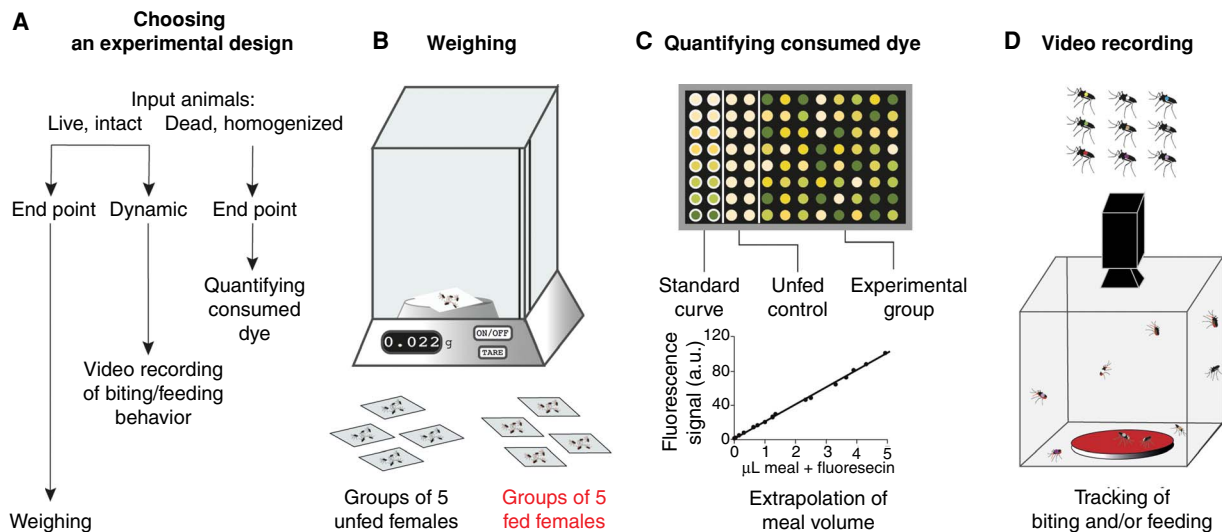


FIGURE 2. Multiple methods can be used to quantify meal volumes consumed by mosquitoes. (A) A meal size quantification method should be chosen depending on the ultimate experimental goals and the final state of animals required during/after meal size quantification. (B) Mosquitoes can be weighed in small groups if live animals are no longer required after meal size quantification, a more precise end-point method can be used. Meals should contain a quantifiable dye that is released upon homogenization of animals after feeding. Note that the standard curve in C depicts a schematic, not real data. (D) For dynamic monitoring of biting or feeding behavior, animals can be tracked using video recordings, with or without being individually marked.

In our associated protocol (Protocol 1: Size Quantification of Blood and Sugar Meals in *Aedes aegypti* Mosquitoes [Venkataraman et al. 2022]), we detail a high-throughput, end-point assay to quantify meal volumes consumed by individual mosquitoes.

Applications

Meal size quantification methods are useful for understanding how variables such as the contents of a meal or the genetic background of the mosquito can influence feeding behavior. For example, genetic modification of sweet taste receptors has been shown to modify nectar-feeding behavior in females (Jové et al. 2020), but the analogous receptors for blood-feeding behavior remain unknown. These feeding and quantification assays can also measure changes in feeding vigor observed upon addition of pharmacological compounds like feeding deterrents. Furthermore, dyes of distinct spectra can be added to compare feeding vigor across multiple meal compositions or feeding instances. Because the amount of blood protein consumed determines physiology downstream of feeding—like oogenesis and host-seeking suppression behavior—meal size quantification is important to standardize results in subsequent assays that measure fecundity and host attraction. Finally, these quantification assays can be extended in a time course series to track the meal as it is digested and excreted (Duvall et al. 2019).

CONCLUSIONS

The nectar- and blood-feeding programs in *Ae. aegypti* are linked to nutritional value and reproductive needs. Carbohydrates acquired from floral nectar are sufficient to sustain energy metabolism in females and males, but protein obtained from vertebrate blood is needed for females to produce viable eggs (Briegleb 2003; Duvall et al. 2019). If a female ingests blood from an infected host, she can transmit pathogens that cause diseases including Zika, dengue, and chikungunya to her next victim (Rückert and Ebel 2018). Thus, nectar- and blood-feeding behaviors are essential for the survival and repro-

ductive success of *Ae. aegypti* and underlie their capacity to transmit pathogenic viruses (Rückert and Ebel 2018). Precise measurements of meal size are important to understand how meal content, source, and availability can impact subsequent physiology and behavior, including metabolism, food search motivation, and egg development. These experimental manipulations of meals can be broadly applied to comparisons across mosquito sex, species, and genetic background.

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Protocol 1

Size Quantification of Blood and Sugar Meals in *Aedes aegypti* Mosquitoes

Krithika Venkataraman,^{1,3} Veronica Jové,^{1,3} and Laura B. Duvall^{2,4}

¹The Rockefeller University, New York, New York 10065, USA; ²Department of Biological Sciences, Columbia University in the City of New York, New York, New York 10027, USA

Both male and female mosquitoes consume sugar-rich nectar meals required for metabolic energy, but only females consume protein-rich blood meals, which are required for egg development. The size of each meal consumed has subsequent effects on behavior and reproduction; therefore, precise quantification is an important aspect of mosquito feeding behavior studies. This protocol describes a high-throughput, end-point assay to quantify meal volumes ingested by individual mosquitoes. The addition of a fluorescent dye to the meal allows for meal size quantification. Individual mosquitoes that have been fed this meal are homogenized in 96-well plates, and the fluorescence levels are measured with a plate reader. This protocol can also be adapted to determine if alteration of meal composition affects the ingested meal volume, if mosquito strain or genotype dictates consumption, or if meals are derived from multiple sources.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

Reagents

Adult *Aedes aegypti* mosquitoes

Females should be >3 d old to ensure competence to blood feed, although this can vary by strain. *Ae. aegypti* are generally maintained and reared at 25°C–28°C with 70%–80% relative humidity and a 14 h light:10 h dark photoperiod, and animals are allowed to mate freely. Adult mosquitoes are provided constant access to 10% sucrose, which is replaced with a water-soaked cotton ball (to prevent desiccation) ~24 h before performing feeding experiments to standardize feeding rates and meal sizes. Detailed rearing methods are described in DeGennaro et al. (2013) and can be modified to suit experimental needs.

Feeding deterrents (optional; see Step 1)

Feeding deterrents can be mixed with blood or with other meals delivered through a Glytube (Costa-da-Silva et al. 2013). Known feeding deterrents used with *Ae. aegypti* include 1% DEET (N, N-diethyl-meta-toluamide) or bitters like 10 mM denatonium, 1 mM lobeline, and 5 mM quinine, as previously described (Dennis et al. 2019).

³These authors contributed equally to this work.

⁴Correspondence: lbd2126@columbia.edu

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Fluorescent dye(s) (palatable, nontoxic)

For illustrative purposes, this protocol uses fluorescein as the fluorescent dye. Fluorescein sodium salt (Sigma-Aldrich F6377) must be prepared as 2% aqueous stock. It is light-sensitive and must be kept dark and/or always covered in aluminum foil both as a stock solution and when added to a meal.

If the volume of multiple different meals consumed must be measured, dyes with distinct excitation and emission spectra must be added to each of the meals (see Step 1). For example, fluorescein could be added to one meal, whereas rhodamine B or other water-soluble dye could be added to another meal (Kumar et al. 2021).

Meal(s) appropriate for the experiment

Mammalian blood

Any commercially available defibrinated mammalian blood (i.e., whole blood from which fibrin has been removed to prevent clotting) can be used for laboratory feeding, including blood from sheep, guinea pigs, or humans (Ross et al. 2017). Although optional, feeding rates can be boosted by the addition of 1–2 mM adenosine 5'-triphosphate (ATP) disodium salt hydrate buffered in an aqueous solution of sodium bicarbonate (Galun 1963).

Sucrose (10%)

10% sucrose in aqueous solution is an adequate substitute for nectar in the laboratory and can be delivered to mosquitoes for rearing and meeting metabolic energy needs.

Phosphate-buffered saline (PBS) (1×)

We purchase 10× PBS (Biotium 22020) and dilute 1/10 in DI water.

Equipment

Bead mill homogenizer for use with 96-well plates (e.g., TissueLyser II) (optional; see Step 11)

Another option is to use a pestle grinder (e.g., Kontes Pestle Pellet Grinder) and microcentrifuge tubes to process the samples individually (see Step 11).

Black polystyrene plate (96-well)

Use a plate with a solid bottom if the fluorescence reading is taken from above the plate or a clear bottom if the reading is taken from below.

Borosilicate solid glass beads (3-mm-diameter)

Centrifuge for 96-well plate

Feeder appropriate for the experiment (see Step 4)

Artificial membrane feeder (for blood feeding)

For blood and other meals that trigger engorgement, female mosquitoes must pierce a membrane to consume the meal. Examples of artificial membrane feeders include the commercially available Hemotek feeder or the Glytube (Costa-da-Silva et al. 2013), which can be assembled with standard laboratory equipment.

Cotton balls (for nectar feeding)

Meals ingested via the labium can be delivered easily using cotton balls soaked in the meal solution with added dye.

Fluorescence plate reader

Fluorescence plate readers that can work with a range of excitation and emission wavelengths, as well as those that can take optical density measurements are ideal.

Forceps

Microcentrifuge tubes

Multichannel pipette (optional; see Steps 8 and 14)

PCR plate (96-well)

Sealing film for 96-well plates

Vortex mixer

METHOD

This protocol has been adapted from Liesch et al. (2013), Duvall et al. (2019), and Jové et al. (2020a,b). In these examples, 7- to 21-d-old female mosquitoes are anesthetized at 4°C, sorted into groups of 15 to 20 females, and placed into a 32-oz high-density polyethylene (HDPE) plastic cup (VWR 89009-668) and provided with a water-soaked cotton ball (to prevent desiccation) ~24 h before performing feeding experiments to standardize feeding rates and meal sizes. After at least 15 min of reacclimating to assay room conditions, 1.5–2 mL of fluorescent meal is offered to each cohort of females using an artificial feeder like the Glytube (Costa-da-Silva et al. 2013). Quantification of meal volume is then performed as described below.

Organization is critical for this protocol. Be sure to set aside enough of the meal containing fluorescein at the start of the experiment. This will be used to generate the reference curve and is critical for reliable results. Label all tubes and organize the work area in advance to speed up sample handling. In this protocol, meal volume quantification is performed in a high-throughput manner by using 96-well plates, but it can be scaled up or down as needed. See Figure 1 for recommended plate organization. Carry out all quantification steps at room temperature.

1. For each meal, add fluorescein to a final concentration of 0.002% (e.g., 2 μ L of 2% fluorescein stock in a total meal volume of 2 mL).

If desired, add deterrents to some of the meals at this step. If alternative dyes are to be used, they should also be added at this step.

2. Set aside at least 1 mL of the final meal that has 0.002% fluorescein. To ensure that this sample is comparable to the one delivered to mosquitoes, expose it to the same heating and lighting conditions while preparing the meal for the mosquitoes.

This sample will be used to generate the reference standard curve for volume calculations.

3. Set aside 32 mosquitoes to use as unfed controls.

These animals will never be offered a meal and are used to account for tissue autofluorescence. They can be frozen at –20°C until quantification of experimental groups.

4. Deliver the meal to the experimental group of mosquitoes via the appropriate feeding method.
 - Use a saturated cotton ball for nectar feeding.
 - Use an artificial membrane feeder (e.g., Hemotek or Glytube) for blood feeding.

Detailed methods for feeding labeled or artificial blood meals are included in Costa-da-Silva et al. (2013) and Jové et al. (2020b).

5. Collect the animals after feeding is complete.

See Troubleshooting.

To measure meal size immediately after feeding, proceed to Step 6. Alternatively, freeze the animals at –20°C until quantification.

6. Prepare a serial dilution of the reference meal (containing 0.002% fluorescein) that was set aside in Step 2 as follows.
 - i. Prepare the first solution by adding 50 μ L of meal containing 0.002% fluorescein to 950 μ L of 1 \times PBS. Vortex it so that the fluorescein is uniformly mixed.

In Step 7, 100 μ L of each dilution from the series will be used to generate a standard curve in which fluorescence levels are plotted against the known meal volume (Fig. 1). For this first standard curve solution, the final 100 μ L volume contains 5 μ L of meal containing 0.002% fluorescein and 95 μ L of 1 \times PBS.

- ii. Prepare the remainder of the standard curve solutions (eight in total) by performing a twofold dilution each time. For each step in the series, take 500 μ L from the previous tube and add it to a new tube containing 500 μ L of 1 \times PBS. Vortex the tube well before preparing the next twofold dilution.

In the final 100- μ L volume for each of these eight tubes, the amount of meal containing 0.002% fluorescein will be 5, 2.5, 1.25, 0.625, 0.3125, 0.15625, 0.078125, and 0 μ L, respectively.

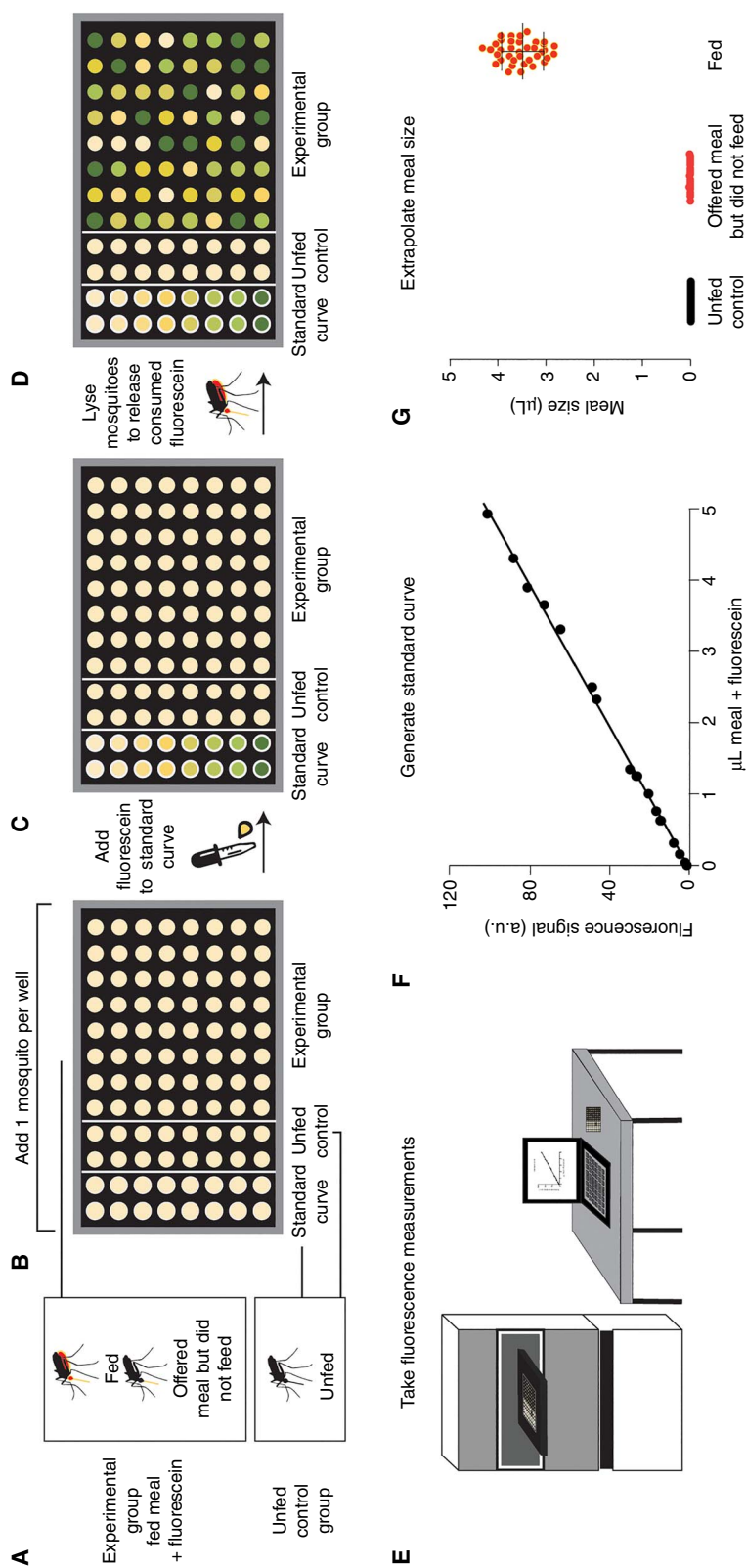


FIGURE 1. Schematic of steps to quantify meal size following nectar- or blood-feeding procedures. (A) Mosquitoes must be divided into two groups: an experimental group offered a meal containing a dye, such as fluorescein (*top*) and an unified negative control group given no meal (*bottom*). (B) Mosquitoes are individually transferred to the respective wells in a 96-well plate once feeding is completed. (C) Reference standard curve solutions with known amounts of meal containing 0.002% fluorescein are added in replicate to the 96-well plate. (D) Mosquitoes are lysed to extract consumed fluorescein. (E) A plate reader is used to quantify fluorescence levels in each well of the 96-well plate. (F) A reference standard curve is generated using the fluorescence readings for the known standard curve volumes of meal containing 0.002% fluorescein. (G) Meal volumes consumed by the experimental group are extrapolated from the standard curve generated, correcting for the baseline autofluorescence of unfed mosquitoes. The graphs in F and G depict schematics, not real data. This fluorescence quantification method is modified from Liesch et al. (2013). Portions of this figure are adapted from Jové et al. (2020b).

7. Pipette 100 μL of each solution from Step 6 into the first column of a 96-well plate to make the reference curve. Then, use forceps to place one unfed mosquito into each well in the first column of the plate to account for any tissue autofluorescence.

As shown in Figure 1C, we recommend adding a second column of unfed mosquitoes to generate a replicate curve and account for any edge effects of the plate reader.

If experimental groups were offered different meals (e.g., blood \pm drug), prepare a separate reference standard curve for each meal. Small differences in fluorescein addition can generate big differences in fluorescence levels and thus calculated volume, so each experimental group must be matched to its own reference curve. Calculate R^2 values to assess goodness-of-fit for linear regression to determine the reliability of the standard curve.

8. Add 100 μL of 1 \times PBS to each of the remaining wells using a multichannel pipette if available. In addition, add a borosilicate solid glass bead to each well.

A multichannel pipette is not an absolute requirement but improves accuracy and speed.

9. Prepare negative control columns by adding one unfed mosquito using forceps to each well in the next two columns of the plate.

The volume of fluorescein measured for these mosquitoes sets a baseline fluorescence measurement cutoff, to account for tissue autofluorescence. Use this cutoff to determine whether or not a mosquito in the experimental group is scored as fed.

10. Use forceps to place one mosquito from the experimental groups that were offered a meal into each of the remaining wells. Seal the plate tightly to avoid any leaks.

11. Use one of the following methods to thoroughly homogenize the abdomen and release the meal.

- Use a TissueLyser II with a borosilicate solid glass bead in each well at 30 Hz for 30 sec at room temperature.
- Use a vortex mixer with a borosilicate solid glass bead in each well. Vortex using a high setting for 15–30 sec at room temperature or until the tissue and blood meal are well-homogenized with no large pieces visible.

As an alternative, the tissue in each sample can be manually homogenized in a microcentrifuge tube using a disposable pestle grinder (without beads) until the consistency is uniform and no large pieces are visible. The samples must then be returned to the 96-well plate.

12. Centrifuge the plate for 1–2 min at 2000 rpm and collect the cleared lysate for use in subsequent steps.

13. Add 180 μL of 1 \times PBS to each well of a new black 96-well plate.

14. Transfer 20 μL of each lysate to the appropriate well of the black 96-well plate (which contains 180 μL of 1 \times PBS) for a final total volume of 200 μL . Mix using a multichannel pipette if available.

15. Using a plate reader, measure the fluorescence intensity of each well.

For fluorescein, the 485/520 excitation/emission channel is optimal, but this will vary depending on the dye used.

16. Plot the known meal volumes of the standard curve solutions against their corresponding fluorescence intensity measurements to establish a reference curve.

If applicable, generate the reference curve by averaging the known concentrations in the two reference rows.

17. Calculate the meal volume consumed by each experimental mosquito. To correct for tissue autofluorescence, average the fluorescence intensity reading among unfed mosquitoes, and subtract this average from the fluorescence intensity reading of each experimental mosquito.

Because a full blood meal approximately doubles the weight of the female, the volume varies depending on the size of the female; in previous studies (Duvall et al. 2019; Jové et al. 2020a) blood meal volumes from 2–5 μL were typically observed. Nectar meals are generally $<2 \mu\text{L}$.

See Troubleshooting.

TROUBLESHOOTING

Problem (Step 5): Females do not consume any of the meals offered.

Solution: Confirm that the dye does not render the meal unpalatable. This can be done by offering blood to a positive control cohort alongside the dye-fed cohorts and ensuring that the dye-fed cohorts feed at similar rates and consume similar volumes to the blood-fed cohort. Additionally, consider using a lower concentration of the dye in the meal. Addition of fluorescein has no effect on meal size, but this may not be true for all additives.

Problem (Step 17): The meal volumes calculated are wildly variable or unreliable.

Solution: Small differences in fluorescein addition can generate big differences in fluorescence levels and thus calculated volume. It is critical to confirm that each experimental group is matched to its own reference curve. If meal volumes are likely to be larger or smaller than the bounds of the standard curve suggested, then adjust the standard curve volumes prepared in Step 6 to the range of possible meal volumes. R^2 values can be calculated to determine goodness-of-fit for linear regression and to assess the reliability of the standard curve.

DISCUSSION

The size of each meal consumed by a mosquito has effects on its subsequent behavior and reproduction (Klowden and Lea 1978; Briegel 1990), so methods for the quantification of meal volume in mosquitoes are important. This protocol describes a way to precisely quantify the volume of a meal ingested by a single mosquito by adding a fluorescent dye to the meal.

The protocol can be adapted for nectar or blood meals, depending on the goals of the experiment. Both male and female mosquitoes consume sugar-rich nectar meals required for metabolic energy, but only females consume protein-rich blood meals required for egg development (Briegel 2003). This protocol can also be used to measure changes in feeding vigor in response to variables such as meal composition or genetic background (Jové et al. 2020a). For example, pharmacological compounds and/or feeding deterrents like DEET (*N, N*-diethyl-*meta*-toluamide) or bitters (such as denatonium, lobeline, and quinine) can be added to the meal to determine the impact on meal size (Duvall et al. 2019). This assay can also be modified to track the kinetics of meal digestion and clearance in individual mosquitoes. If dyes of distinct fluorescent spectra are added to each meal, researchers can compare the volume consumed across different meals (e.g., nectar and blood) or across multiple feeding instances (e.g., refeeding after a partial blood meal) (Klowden and Briegel 1994; Ponlawat and Harrington 2005).

Finally, it is important to note that this protocol describes a sensitive end-point assay to precisely measure the volume of meals consumed by individual mosquitoes. However, if mosquitoes will be used for subsequent behavioral testing or experimentation, then, instead, calculate blood meal size by weighing anesthetized mosquitoes in small groups and comparing prefed versus postfed weights as previously described (Duvall et al. 2019; Jové et al. 2020b). Weights can be benchmarked to the volumes calculated in this assay to determine volume consumed, although small nectar meals would be difficult to detect using weight measurements alone. Meal size measurements allow researchers to appropriately select animals for downstream *in vivo* assays that measure the effect of the meal on processes including fecundity or host attraction. This protocol can also be coupled to versatile feeding assays, such as those to measure feeding dynamics using real-time video recording (Hol et al. 2020), or to examine the effects of interrupted feeding.

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