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The Use of Frozen, Food-Grade Blood to Successfully Maintain Colonies of Four Species of Mosquitoes (Diptera: Culicidae)

Kara Tyler-Julian,[1] Constance Darrisaw, Aaron Lloyd, and David Hoel

Lee County Mosquito Control District, 15191 Homestead Road, Lehigh Acres, FL 33971, USA
[1]Corresponding author, e-mail: tyler-julian@lcmcd.org

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Abstract

An essential component of all mosquito-rearing activities is the act of blood-feeding the mosquitoes (Diptera: Culicidae). Many options exist for this purpose including live host animals and a diverse array of artificial-feeding methods. Most of the published artificial-feeding methods involve expensive materials, custom-built devices, or are labor-intensive. All of the previously published methods utilize blood sources, which are either expensive, or difficult to obtain. Additionally, much of the research into artificial blood-feeding methods for mosquitoes has focused on two species: *Aedes aegypti* (Linnaeus) and *Aedes albopictus* (Skuse). This article presents a modified artificial blood-feeding method that uses affordable and easily sourced materials, does not require any technical knowledge to assemble, and requires minimal time and effort. The combination of inexpensive aluminum plates, Parafilm and polytetrafluoroethylene tape membranes, an electric germination mat, and frozen, food-grade blood produces exceptional feeding rates and abundant egg production. The method has been used for 2 yr at the Lee County Mosquito Control District to successfully maintain laboratory colonies of four species of mosquito: *Ae. aegypti*, *Ae. albopictus*, *Aedes taeniorhynchus* (Wiedemann), and *Culex quinquefasciatus* (Say). Variations of this method are reported, which can be used for wild and laboratory colonies of multiple species. This modified method is highly accessible for any small-scale mosquito rearing facility with labor or budgetary constraints.

Key words: mosquito, rearing, blood, feeding, membrane

One of the activities vital to mosquito control operations is the validation of mosquito control efforts through the testing of insecticides and other products used in the management of mosquitoes (Diptera: Culicidae). To accomplish this testing, colonies of susceptible and field populations of mosquitoes must be reared and maintained to test at any time. The susceptible, or naive, colonies are important to have as a control to compare against the wild populations when conducting bioassays in the laboratory and cage trials in the field. At the Lee County Mosquito Control District (LCMCD), located in Lehigh Acres, Florida, we maintain year-round colonies of both wild and susceptible (laboratory) colonies of at least four species: *Aedes aegypti* (Linnaeus), *Ae. albopictus* (Skuse), *Ae. taeniorhynchus* (Wiedemann), and *Culex quinquefasciatus* (Say). During the summer season, we maintain up to 19 colonies at once, with the addition of many wild populations used for cage trials and bioassays.

Essential to the maintenance of a mosquito colony is the provision of blood to adult females for egg production (Clements 1992). Live chickens have been used as a blood source for at least 10 yr at LCMCD. Live chickens are already maintained at the district year-round as part of our virus surveillance program, and additionally, live hosts were considered by many to be the most effective and efficient blood feeding method (Rutledge et al. 1964, Richards et al. 2012). Although artificial blood feeding methods can result in a lower feeding rate, this lower rate is generally acceptable for colony maintenance (Bailey et al. 1978, Alto et al. 2003, Deng et al. 2012, Dias et al. 2018).

Live animal use as a blood source carries the added expense of housing and caring for the animals, as well as the added risk of potential disease introduction into an otherwise sterile insectary environment (Bailey et al. 1978). Additionally, capturing and restraining or anesthetizing the animals can be a time-consuming and stressful process, for the animals as well as for the employees. This method can also result in added contaminants in the insectary (excrement and fur/feathers). In the interest of sterility, efficiency, consistency, and perhaps more importantly, ethical considerations regarding the use of animals (Ferdowsian and Beck 2011), we developed an alternate membrane feeding method to maintain our colonies starting in January 2019.

Many successful artificial blood-feeding methods have already been established for mosquitoes (Bailey et al. 1978, Tseng 2003,

Deng et al. 2012, Costa-da-Silva et al. 2013, Carvahlo et al. 2014). Artificial feeding methods have been used successfully for *Anopheles albimanus* (Wiedemann) (Bailey et al. 1978); *Ae. aegypti* (Rutledge et al. 1964, Alto et al. 2003, Deng et al. 2012, Carvahlo et al. 2014); *Ae. albopictus* (Alto et al. 2003, Lyski et al. 2011, Deng et al. 2012); *Aedes triseriatus* (Novak et al. 1991); *Culex pipiens quinquefasciatus* (Novak et al. 1991, Richards et al. 2012); *Culex tarsalis* (Novak et al. 1991); and others (Rutledge et al. 1964, Siria et al. 2018). One common method used to accomplish blood-feeding involves the use of expensive commercial blood-feeding devices (Gunathilaka et al. 2017), which can cost up to $2,500–$3,000 (Hemotek 2021). Other, more affordable methods involve technical ingenuity to construct custom devices (Rutledge et al. 1964, Deng et al. 2012) and use various membrane materials. We found these various combinations of methods to be either too expensive, too cumbersome, or too technical for widespread use and found the methods to be potentially deterrent to many insectaries attempting to switch from live hosts to a more efficient, affordable, and user-friendly method.

The elements needed to successfully elicit blood feeding by mosquitoes using artificial methods include the following: blood source, heat source, membrane, and a vessel or substrate to contain the blood. Optional additions to increase blood-feeding success include: host cues (e.g., odors from human skin; Costa-da-Silva et al. 2013, Carvahlo et al. 2014, Gonzales et al. 2015); a source of carbon dioxide (McMeniman et al. 2014); and phagostimulants such as ATP (Rutledge et al. 1964, Bailey et al. 1978, Gonzales et al. 2015, Sri-In et al. 2020). Different combinations of each of these components may be more appropriate for certain species and strains of mosquitoes. For example, the presentation of the bloodmeal can affect the feeding success of *Ae. albopictus* which prefers vertical presentation of artificial membrane feeders, but will feed on large horizontal presentations as well (Lyski et al. 2011). *Culex* mosquitoes feed better on quail skin membranes than on mouse skin or latex membranes (Novak et al. 1991).

Membranes

A wide variety of membrane types have been used to blood-feed mosquitoes with varying success. These membranes include: natural sheep intestine (Bailey et al. 1978), collagen membrane sausage (Lyski et al. 2011, Deng et al. 2012), lambskin membranes (Lyski et al. 2011), and a variety of other animal skins and intestines (Rutledge et al. 1964, Novak et al. 1991, Harrington et al. 2001). These membranes can be difficult to source and cumbersome to use, requiring various soaking and rinsing methods before they can be used (Bailey et al. 1978, Kasap et al. 2003, Deng et al. 2012). Parafilm M (American National Can, Chicago, IL) is a common alternative to animal-based membranes and is simply stretched thinly and wrapped around a blood-containment vessel. Parafilm M has been successfully used for blood-feeding *Ae. aegypti* and *Ae. albopictus* (Tseng 2003, Costa-da-Silva et al. 2013, Carvahlo et al. 2014, Gonzales et al. 2015, Gunathilaka et al. 2017, Sri-In et al. 2020). Another alternative to animal-based membranes is the use of polytetrafluoroethylene (PTFE) tape ('plumber's tape'), which resulted in 100% feeding success in *Ae. aegypti* (Siria et al. 2018). The benefits of both the Parafilm M and PTFE tape membranes are their widespread availability and ease of use, not requiring any added processing or preparation outside of simple stretching.

Blood

Numerous sources of blood, and blood components have been used with varying success to feed adult mosquitoes. An option, where available, is to collect fresh blood regularly from a local slaughterhouse and store for short periods of time in the refrigerator (Bailey et al. 1978, Gunathilaka et al. 2017). This method, however, is not an option for many facilities that are located great distances from animal agriculture. A common source used by many facilities is a variety of animal blood that can be purchased from various scientific supply companies (Bailey et al. 1978, Novak et al. 1991, Tseng 2003, Lyski et al. 2011, Richards et al. 2012, Sri-In et al. 2020). The disadvantage of this option is the high price, which can be cost-prohibitive for laboratories with limited budgets. Expired human blood has been used successfully and can be purchased from blood banks and hospitals where available (Kasap et al. 2003, Nasirian and Ladonni 2006, Pothikasikorn et al. 2010). Another option is using components of blood in place of whole blood, such as bovine serum albumin (Rutledge et al. 1964, Gonzales et al. 2015), and erythrocyte extract (Rutledge et al. 1964). These options may also be cost-prohibitive or require technical knowledge to prepare the components in-house. Some facilities that house and maintain live animals can collect fresh blood and treat it to prevent coagulation (Rutledge et al. 1964, Novak et al. 1991, Deng et al. 2012, Siria et al. 2018). This method is not accessible to facilities that do not house animals and/or do not have the training or veterinary professionals required to safely harvest blood without harming the animals. Methods used to prevent coagulation when blood is collected fresh in situ include using EDTA (Deng et al. 2012), mechanically defibrinating the blood (Novak et al. 1991), and adding heparin solution (Rutledge et al. 1964). This adds an additional level of complexity that may be deterrent to many facilities. Although defibrinated blood is often used, Dias et al. (2018) found that citrated blood resulted in higher feeding rates by the three mosquito species tested, compared to defibrinated blood. Citrated blood is widely available from scientific supply companies, and can also be found at certain grocery stores as a frozen food-grade product.

Heat Source

Once the blood, membrane and substrate are chosen, the blood must be heated at an adequate temperature to induce feeding. Several methods are used to heat artificial blood meals in order to emulate a living host and induce feeding from adult female mosquitoes. These heating methods may use expensive or complicated devices (Rutledge et al. 1964, Gunathilaka et al. 2017, Dias et al. 2018), devices that were created in-house requiring technical knowledge and skills (Rutledge et al. 1964, Benzon and Apperson 1987, Kasap et al. 2003, Deng et al. 2012), or materials that require manual observation and reheating throughout the duration of feeding (Carvahlo et al. 2014, Gunathilaka et al. 2017, Siria et al. 2018). Commercial aquarium heaters have also been used inside of blood-filled casings (Kasap et al. 2003), but may not be appropriate for other membrane feeders.

These studies have established successful artificial feeding methods using many different combinations of membrane types, blood sources, and heat. However, none of these methods are widely accessible to facilities with limited resources and budgetary constraints due to the cost, technical nature of assembling the device, or the amount of labor and oversight required. All of the previous studies have used blood sources, which were sourced from laboratory supply companies or hospitals or obtained fresh from slaughterhouses or in-house animals. This is the first published account, to our knowledge, of the use of frozen, food-grade blood to feed mosquitoes. These previous studies also suffer from a lack of diversity of species tested, with the majority of the studies only testing laboratory colonies of *Ae. aegypti* or *Ae. albopictus* with the selected

feeding method. We sought a method that was affordable, that did not use a live animal, and that did not require technical expertise to construct a new device. Additionally, we developed a combination of methods that will work for four species of mosquitoes: *Ae. aegypti*, *Ae. albopictus*, *Ae. taeniorhynchus*, and *Cx. quinquefasciatus* and for both wild and laboratory populations.

One successful and easily assembled blood-feeding method is the use of an aluminum diamond plate and Parafilm (Nasirian and Ladonni 2006, Carvahlo et al. 2014, Gunathilaka et al. 2017). This method resulted in superior feeding rates for *Ae. aegypti* compared to use of glass plates or the Hemotek membrane feeding method (Gunathilaka et al. 2017). Using an aluminum plate with a Parafilm membrane resulted in a feeding rate of 90.9% for a colony of *Anopheles stephensi* (Lis.) (Nasirian and Ladonni 2006). Carvahlo et al. 2014) provide a detailed instructional article and video explaining how the bloodmeal is assembled. The successes and the availability of these materials in our laboratory led to our decision to test modifications of this method with our colonies. This modification includes a different heat source from previous studies: commercially available germination pads with temperature control devices. Additionally, we used cost-effective food-grade blood purchased from a grocery store and added the use of the PTFE tape membrane (Siria et al. 2018), with the aluminum plate. Previous studies using these methods have not included *Cx. quinquefasciatus* or *Ae. taeniorhynchus* mosquitoes. These modifications are presented for four species of laboratory and field populations of mosquitoes.

Experimental Design

The four species of mosquitoes maintained at LCMCD and used in these tests are as follows: *Ae. aegypti*, *Ae. albopictus*, *Ae. taeniorhynchus*, *Cx. quinquefasciatus*. Wild and laboratory colonies were used, where possible. Laboratory colonies of *Ae. aegypti* are the USDA Orlando strain, originally obtained from the USDA, ARS, SAA in Gainesville, FL, in March 2016 and maintained at the LCMCD insectary since that time. The laboratory colonies of *Ae. albopictus* are a USDA strain obtained from the same facility in October of 2014 and maintained in the LCMCD insectary since that time. The laboratory colonies of *Cx. quinquefasciatus* and *Ae. taeniorhynchus* are naive colonies of unknown origin that have been maintained for more than 60 generations in the insectary. All wild colonies of *Aedes* mosquitoes tested in this experiment are the F0–F1 generations, obtained as eggs or larvae from the field in Lee County, FL. The wild *Cx. quinquefasciatus* colony tested in this experiment is the F3–F4 generation and was collected originally as eggs in the field in Polk county, FL.

The mosquitoes are maintained in the insectary at 25–27°C and 70–80% humidity with a light:dark cycle of 14:10. Adult mosquitoes of all four species are housed in 46 × 46 × 46 cm screened cages (Bioquip Products, Rancho Dominquez, CA) at a density of 3,000–8,000 mosquitoes per cage for the laboratory colonies and a density of 30–300 mosquitoes per cage for the wild colonies (depending on species and larval hatch rate). Larvae are reared in trays at a rate of approximately 250 larvae per 1,000 ml of water. Various water sources can be used to rear the larvae including tap water aged 24 h, filtered tap water to remove the chlorine, and water that has been filtered through Reverse Osmosis (RO). All species of larvae in the insectary are reared on ground and powdered Mazuri Rat & Mouse Diet (Mazuri Exotic Animal Nutrition, St. Louis, MO) at a rate of 0.3–0.6 mg/larva per day, increasing with instar. Once the larvae are more than 50% pupated, or on the second day of pupation, the trays are drained and the pupae are placed into 16-ounce plastic deli cups containing 300 ml of filtered tap water. The pupal cups are then placed into the cages for emergence. A cotton pad soaked with 20% sucrose solution and 0.2% (w/v) methyl paraben is provided to each cage and replaced weekly. Methyl paraben is added to the sucrose solution to reduce fungal growth and increase longevity of the mosquitoes (Benedict et al. 2009).

Approximately 3–4 d after the last pupae have emerged in the cage, the sucrose source is removed from the cage. The bloodmeal is offered the following day, approximately 5 d postemergence and 16–24 h after the removal of the sucrose. The blood used in our insectary is purchased at a local Asian grocery store in 100-ounce quantities. Three blood types are available at this vendor including citrated bovine blood, citrated pork blood, and pork blood with water and salt. The blood is purchased frozen and thawed before use. The two main blood types used in our insectary are the citrated bovine blood and the pork blood with water and salt. We have stored the blood for up to 1 yr in a freezer at –18 to –20°C with no noticeable change in blood quality or mosquito response. Once thawed, the blood can be stored for 1–2 wk in the refrigerator at 1.7–4°C. The complete list of prices and materials used for the blood-feeding procedure can be seen in Table 1.

Two days after the blood feeding event eggs are collected from each *Aedes* species. Eggs are collected from *Ae. albopictus* and *Ae. aegypti* using 16 oz black polypropylene cups lined with paper towels and filled with 100 ml of filtered tap water. The cups are left in the cage for 2 d, at which point the paper towel (egg paper) is removed and replaced with a fresh egg paper and fresh water. The cup is then placed back in the cage for two more days before being removed. The egg papers are laid flat to dry for 2–3 d before storing in one-gallon plastic storage bags for up to 3 mo.

Eggs are collected from *Ae. taeniorhynchus* by way of a large roll of moist cotton. Nonsterile cotton rolls are cut into 121 × 30 cm sections and rolled tightly into a 30 cm × 10 cm log shape. This section of cotton is soaked with filtered tap water and placed on top of the cage where it remains for 2 wk. Every 3–4 d, the oldest section of the cotton containing freshly oviposited eggs is cut from the remaining roll and laid flat to dry for up to 6 d. The dry sections of cotton and eggs are folded carefully and stored in one-gallon plastic storage bags in the insectary for up to 3 mo.

Egg collection from the *Cx. quinquefasciatus* colonies takes place only one to two times per month/cage based on when larvae are needed for stocking cages or conducting experiments. A minimum of 5 d is allowed to pass between the blood feeding event and the first attempt to collect eggs. A 16-ounce deli cup containing 300 ml of filtered tap water is added to the cage and left for the necessary amount of time to obtain the desired number of egg rafts. Depending on the number of egg rafts needed this can require from 4 to 24 h.

Blood-Feeding Procedure

For each cage, two 10 × 10 cm aluminum plates are used. Thawed blood is measured out in 40-ml increments for each cage of adult mosquitoes. The blood is poured into a polystyrene cup, which is placed into a 400-ml glass beaker containing 200 ml of water and set onto a hot plate (Fig. 1). A digital thermometer is placed into the blood, and it is heated on the hot plate at a low heat until it reaches 36–38°C. Although the blood is heating, the membrane is prepared. The membrane and metal plate preparation is derived from the method presented by Carvahlo et al. 2014) and tested by Gunathilaka et al. (2017). Two membranes are used in our adaptation of this method, Parafilm and PTFE tape depending on the species and colony (wild or laboratory) of mosquito. Parafilm is cut into

Table 1. List of the materials used for this method, and the sources and prices of those materials

Item name	Product number	Source	Use	Cost
0.08" Aluminum Tread Plate, 4" × 4"	12509	https://www.onlinemetals.com/en/buy/aluminum/0-08-aluminum-tread-plate-3003-h22/pid/12509	Substrate	$2.60
Parafilm M wrapping film, 4"	S37440	https://www.fishersci.com/shop/products/parafilm-m-wrapping-film-3/p-2379782	Membrane	$34.50
PTFE thread tape roll, 2"	B008HPVP6S	https://www.amazon.com/Gasoila-Standard-Density-Performance-Temperature/dp/B008HPVP6S?ref_=ast_sto_dp&th=1	Membrane	$7.08
Germination mat	B07F821DKQ	https://www.amazon.com/gp/product/B07F821DKQ/ref=ppx_yo_dt_b_asin_title_o04_s00?ie=UTF8&psc=1	Heat source	$35.99
Pork blood with water and salt	N/A	Local Asian food grocer	Blood source	$3.99
Hot plate	N/A	Varies	Blood warming	Varies
Glass Beaker, 400 ml	N/A	Varies	Blood warming	Varies
Digital thermometer	B01LKRHW3E	https://www.amazon.com/Habor-Thermometer-Instant-Digital-Temperature/dp/B01LKRHW3E/ref=psdc_289810_t1_B001XMFM2A	Blood warming	$12.99
Plastic cup, 8 oz	N/A	Varies	Blood warming	Varies
Screened cage, 18 × 18 × 18"	1450C	https://www.bioquip.com/Search/DispProduct.asp?pid=1450A	Laboratory rearing	$249.47
Sterile cotton	2904X10	https://www.thomassci.com/Laboratory-Supplies/Cotton/_/COTTON-ROLLS?q=Sterile%20Cotton%20Roll	Laboratory rearing	$24.35
Sugar	N/A	Varies	Laboratory rearing	Varies
Mazuri rat and mouse diet	52166	https://www.mazuri.com/mazuri/small-animal/squirrel/rat-mouse-diet	Laboratory rearing	$12.99

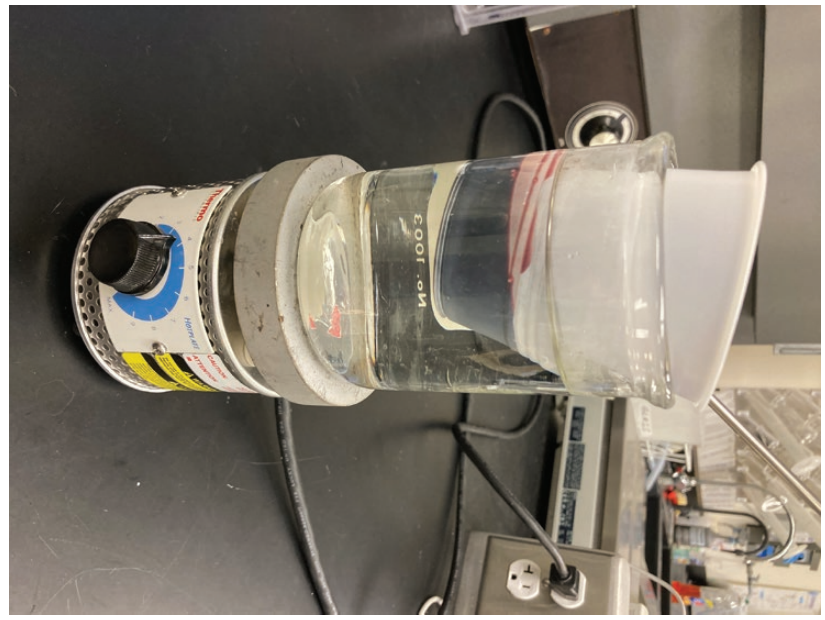

Fig. 1. Preparation for blood-feeding: Heating process. The blood is contained in a plastic cup placed in a beaker of water on top of the hot plate on medium-low heat. It is stirred and monitored until reaching 36–38°C.

10 × 10 cm sections, one section for each plate. PTFE tape is cut into 5 × 18 cm sections. Both membranes are then rubbed against clean human skin, free of any lotions or perfumes. The membranes are then stretched as thinly as possible and placed over the rough side of the aluminum plate. Three sides of the membrane are sealed onto the back of the plate, leaving the fourth side open for the addition of blood (Fig. 2).

Once the membrane has been stretched over the plate and the blood has reached the proper temperature, 10–20 ml of blood is poured into each aluminum plate depending on the mosquito density of the cage. The fourth side of the membrane is then pressed tightly against the back of the aluminum plate to form a water-tight seal. The blood meals are then placed membrane-side down onto the top of the cage. A germination mat is placed on top of the blood meals with the temperature probe placed against the back of one of the aluminum plates (Fig. 3). A paper towel is placed between the germination mat and the blood meals to protect from accidental leaks, and the temperature control of the germination mat is set to 37°C. A few breaths are exhaled into the cage to further stimulate interest, and the blood meals are left for 2–4 h.

The Parafilm membrane is used routinely for the following species and populations: Wild and laboratory *Cx. quinquefasciatus*, wild *Cx. nigripalpus*, and wild and laboratory *Ae. albopictus*. The PTFE tape membrane is used for wild and laboratory populations of *Ae. taeniorhynchus* and laboratory populations of *Ae. aegypti*. Wild colonies of mosquitoes often need to be deprived of sucrose for a longer duration before they will feed on the artificial membrane. For these populations the sucrose can be removed up to 4 d before

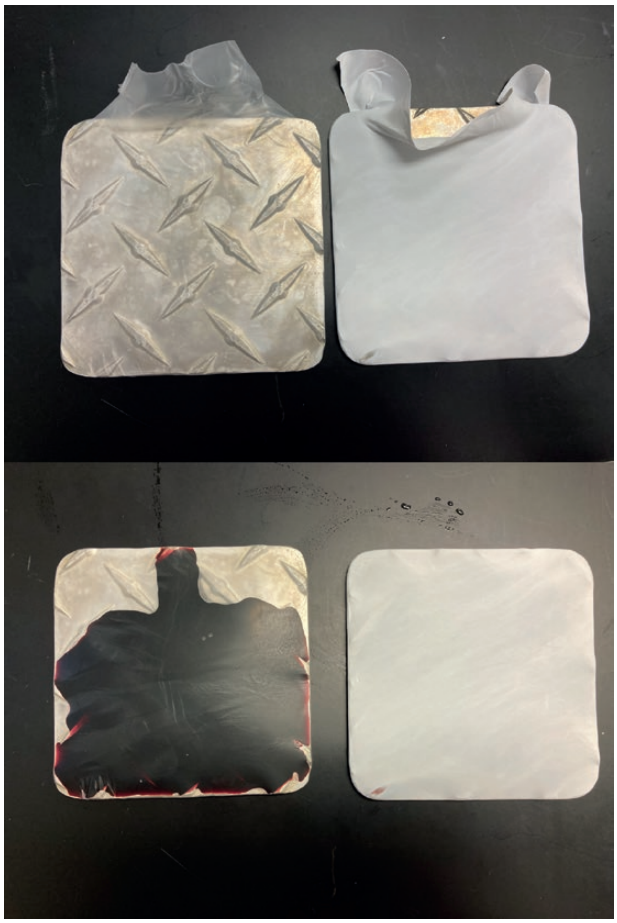

Fig. 2. Preparation of the plate. Parafilm (left) or PTFE tape (right) is stretched over the aluminum plate forming a pocket on the diamond side of the plate. Three sides are sealed on the opposite side of the plate. Blood is poured into the open panel, creating a pool on the diamond side. The open side of the Parafilm is then sealed tightly against the back of the plate.

feeding. When sucrose is removed, a ball of cotton saturated with filtered tap water is offered as a moisture source. Additionally, the natural feeding behavior of the wild mosquitoes should be taken into consideration. Wild *Culex* spp. are fed in the evening by setting the blood meals in place and leaving the germination mat set overnight. The germination mat can be adjusted to a lower temperature (35°C) in these cases.

Over the course of 2 yr, we have offered all three blood options and both membrane types to wild and laboratory colonies of all four species. Alternate substrates, such as petri dish lids, have also been used to determine the adaptability of the method. Feeding behavior is observed, and the percentage of females engorged in each condition is recorded. The preferred membrane and blood combination is used regularly for each colony. For routine colony maintenance of the *Aedes species* the mosquitoes are fed 5 d after adult emergence and then weekly thereafter. *Cx. quinquefasciatus* mosquitoes also receive an initial bloodmeal 5 d after adult emergence, but are only fed one time, unless more feedings are needed to produce additional eggs.

Membrane Preference Test Methods

Membrane preference tests were conducted to determine the preference of each species and colony for each membrane, and the details of these methods and the results are shown below.

Fig. 3. The feeding plate is set on top of the cage with the blood facing down. The temperature probe is placed against the back of one feeding plate and the germination mat is set on top. A paper towel can be placed between the probe and the mat to absorb leaks. A metal plate is tested with Parafilm membrane in this photograph.

To determine the membrane preference of each species and population (wild or laboratory), we conducted a series of choice and no-choice membrane feeding tests. All feeding tests were conducted between 08:00 and 14: 00 h with the exception of the wild *Cx. quinquefasciatus*, which were tested between 16:00 and 08:00 h. The intent of the choice feeding test was to determine initial attractiveness of the membranes to the mosquitoes. The no-choice tests were then conducted to determine the feeding success of each membrane. In the choice feeding tests, we presented two metal plates at the same time to each cage of mosquitoes. Each plate was wrapped in the normal manner with one of the membranes and filled with 10 ml of warmed blood. Each cage was presented with one PTFE-wrapped plate and one Parafilm-wrapped plate. Citrated bovine blood was used in the preference tests for all species except for *Ae. albopictus*, for which we used pork blood with water and salt. Upon presentation of the plates and addition of the germination mat, a timer was set for 2 min of observation for the laboratory colonies. Two observers were present and each observer watched one of the plates and used a handheld tally counter to record the number of female mosquitoes to land on and probe the membrane in the 2-min observation period. Wild colonies were observed in the same manner with a longer time limit of five minutes due to lower cage populations and a reduced interest in the membranes compared to laboratory colonies. In the course of this study, we were only able to actively observe wild populations of *Ae. albopictus* and *Ae. taeniorhynchus* as we did not have any wild *Ae. aegypti* in the laboratory, and the wild *Cx. quinquefasciatus* would only feed during the night. Only one to two repetitions were conducted for each population. The ages of the mosquitoes in the choice test ranged from 5 to 25 d old.

In the no-choice feeding tests, mosquitoes were blood fed according to the normal routine (once per week for *Aedes* spp., once per month/cage for *Cx. quinquefasciatus*) using the method presented above. Data were collected in such a way as to depict the expected routine feeding success of each membrane. Each cage was presented with two metal plates wrapped in the same membrane, each containing 10 ml of warmed blood and heated with the germination mat. Four hours after the bloodmeal was presented (16 h for the wild *Cx. quinquefasciatus*), four haphazardly chosen 10 × 10 cm quadrants of the cage were observed with a flashlight. The number

of engorged and unfed females was counted using handheld tally counters, and the percentage of blood fed females was calculated by dividing the number of engorged females by the total number of females counted and multiplying by 100. Mosquitoes are considered to be engorged if the abdomen presents with clearly visible red coloration in stage 3 through stage 5 using the coarse grading scale of Pilitt and Jones (1972). No attempt was made to quantify the volumes of the blood meals. The no-choice tests were repeated three to seven times for each membrane type and each population, with the exception of the wild *Cx. quinquefasciatus* and the PTFE tape, which stopped after one repetition due to the physical nature of the PTFE tape causing blood to dry before these mosquitoes could feed. These trials were conducted on the colonies over the period of 3 mo with the mosquito ages ranging from 5 to 38 d old. The data presented represent laboratory colonies on their 12–16th generation of feeding on artificial blood meals, and wild colonies are the F_0–F_4 generation.

Fecundity and Fertility Measurements

Once the preferred membrane type was determined, fecundity was measured. The fecundity of females was determined as the total amount of eggs produced from each cage per month. Fecundity was quantified over the course of 3 mo/cages worth of egg production for each species. For *Ae. aegypti*, *Ae. albopictus*, and *Ae. taeniorhynchus*, fecundity was measured by weighing out the total amount of eggs produced in 1 mo. Eggs were first brushed from the oviposition papers or cottons after drying and aging for a minimum of 2 wk. Next, the total quantity of eggs for that month was weighed on an analytical balance. The approximate number of eggs per gram was then quantified by taking three subsamples of eggs from each batch, weighing the subsamples, and counting the total number of eggs in each subsample under a microscope. This approximate eggs/gram value was then multiplied by the weight of eggs for that month and used to estimate the approximate total number of eggs produced in 1 mo.

To quantify the number of eggs produced by *Cx. quinquefasciatus*, the number of egg rafts produced by a cage in 1 mo was counted for 3 mo. Eggs were collected from each cage twice, approximately 1 wk apart by placing a 16 oz deli cup full of 300 ml of filtered tap water into the cage for 4 h at the first collection and 24 h at the second collection. A subsample of 20 egg rafts was collected from each cage, photographed under 40× magnification with the images transferred to a computer. The total number of eggs in each raft was counted using the computer images of the rafts. This count was averaged over the subsample to produce an average number of eggs per raft. This number was multiplied by the total number of egg rafts produced by each cage in each month to obtain a monthly total. The 3 mo of data were averaged to obtain an average expected egg count per month per cage.

Fertility was calculated as the percentage of eggs to successfully hatch in a 48-h period. This was measured for all species by taking three subsamples of a known quantity of eggs (1,300–2,600 eggs per subsample) and allowing the eggs to hatch over 48 h. Four- to 6-wk-old eggs of *Ae. aegypti*, *Ae. albopictus*, and *Ae. taeniorhynchus* were flooded in 16 oz deli cups containing 300 ml of filtered tap water with 300 mg of food. Egg rafts of *Cx. quinquefasciatus* were carefully placed into a 16 oz deli cup containing 300 ml of filtered tap water within 36 h of oviposition. Upon hatching, 300 mg of food was added to the deli cup and the larvae were allowed to grow for 48 h. When the 48 h hatching period was reached, larvae were drained from the water and placed into 70% isopropyl alcohol to allow for larval counting under 40× magnification. Due to low population sizes and a limited timeframe, we did not measure fertility or fecundity for any of the wild mosquito populations.

Results

Membrane Preference

The results of the membrane choice test for each species are shown in Fig. 4. Both the laboratory and wild populations of *Ae. taeniorhynchus* show an initial preference for the PTFE tape over the Parafilm membrane. The laboratory population of *Ae. aegypti* also shows a clear initial preference for PTFE tape. Laboratory populations of *Ae. albopictus* and *Cx. quinquefasciatus* both show an initial preference for the Parafilm membrane, whereas the wild *Ae. albopictus* population showed an initial preference for the PTFE tape. The number of wild *Ae. albopictus* observed was very low (n = 21) and is not likely to be a good representation of the preference of this species. This choice test only indicated initial attraction and did not measure feeding success.

Feeding success was measured in the no-choice tests, the results of which can be seen in Fig. 5. These results only include blood-feeding using the preferred blood types of pork blood with water

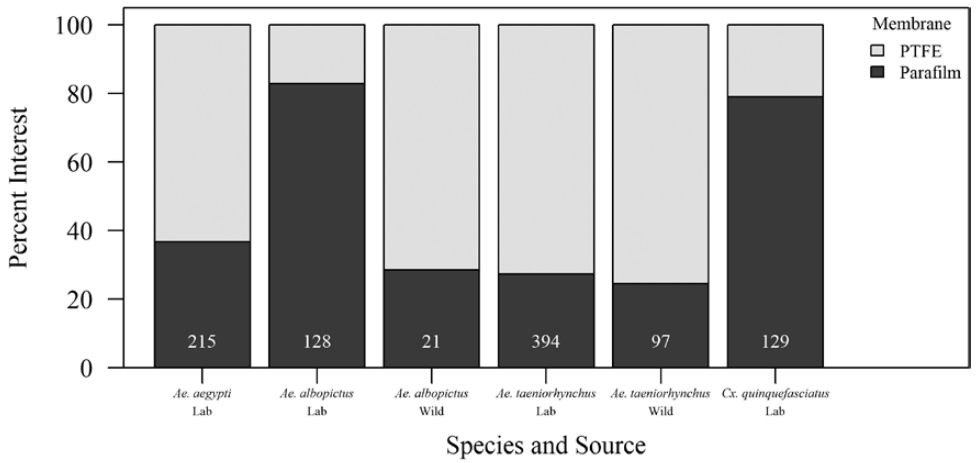

Fig. 4. The results of the choice test for each species and colony of mosquito. The bars represent the percent of mosquitoes that landed on each membrane long enough to probe the membrane. The number inside each bar is the total number of mosquitoes counted in the observation period.

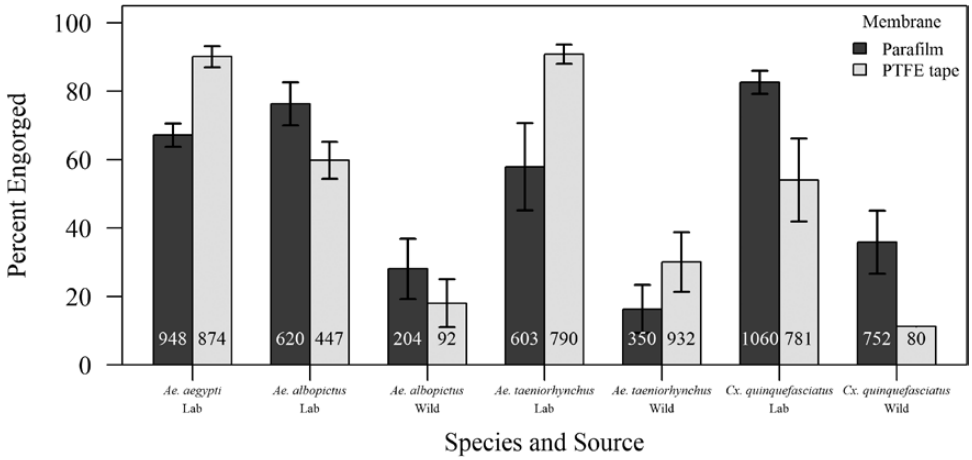

Fig. 5. The results of the no-choice feeding tests. Bars represent mean percent of engorged females (±SE) for each species and colony. The numbers inside the bars represent the total number of females counted over all replications.

and salt for *Ae. albopictus* and *Cx. quinquefasciatus* and citrated bovine blood for *Ae. aegypti* and *Ae. taeniorhynchus*. Initial feeding trials showed poor feeding by *Ae. albopictus* (7.61%) and *Cx. quinquefasciatus* (42.64%) when citrated bovine blood and Parafilm membranes were used, necessitating the switch to pork blood with water and salt for routine feeding and the membrane tests. Both blood types produce satisfactory feeding rates (over 80%) in our laboratory populations of *Ae. aegypti* and *Ae. taeniorhynchus* and the two types are used interchangeably for these two species based upon availability.

Regardless of membrane used, feeding rates are much higher in the long-term colonized (generation > F30) laboratory populations (Fig. 5). Despite the lower feeding rate exhibited by the wild populations, their feeding patterns in the no-choice tests were consistent with those observed for the laboratory populations of the same species. Sample sizes are included on the bars in Fig. 5. Laboratory populations of *Ae. aegypti* fed at higher rates on the PTFE tape membrane (mean ± SE, 90.12% ± 3.10) than on the Parafilm membrane (63.83% ± 0.92), although both membranes result in a feeding rate greater than 60%. Both laboratory and wild populations of *Ae. albopictus* exhibited higher rates of feeding on the Parafilm membrane (laboratory, wild; 76.30% ± 6.26, 28.09% ± 8.82) than on the PTFE tape membrane (laboratory, wild; 65.11% ± 2.07, 14.28% ± 6.23). A higher proportion of laboratory and wild populations of *Ae. taeniorhynchus* fed on the PTFE tape membrane (laboratory, wild; 90.82% ± 2.82, 30.09% ± 8.73) than on the Parafilm membrane (laboratory, wild; 57.95% ± 12.74, 16.37% ± 7.06). Both populations of *Cx. quinquefasciatus* fed at higher rates on the Parafilm membrane (laboratory, wild; 82.61% ± 3.39, 36.15% ± 11.90) than on the PTFE tape membrane (laboratory, wild; 54.11% ± 12.13, 11.25%).

Fertility and Fecundity

Results of the fecundity and fertility evaluations, as well as cage density and the feeding rate for the preferred membrane/blood combinations, can be seen in Table 2. When using the preferred membrane and blood type for each species, all laboratory colonies reach an average feeding rate of at least 76%. Average fertility for all species was greater than 60%, with *Ae. taeniorhynchus* exhibiting the lowest fertility rate of 61.44% and *Ae. aegypti* reaching the highest fertility rate of 93.06%. Average per cage monthly fecundity of *Cx. quinquefasciatus* was the lowest of the four species tested at 28,587.77 eggs. The highest monthly fecundity reached was 137,516.95 eggs by *Ae. aegypti*. These numbers align with the different cage densities.

Overall, this feeding method works acceptably well for all of the species and populations tested in our insectary. Two different membranes and blood types are used according to the observed response of each species and population. We have successfully maintained all of our laboratory and wild colonies of mosquitoes for 2 yr using this artificial feeding method. Egg production has remained adequate to rear enough mosquito larvae to successfully maintain a colony of predatory *Toxorhynchites rutilus rutilus* (Coquillett) and provide ample larvae for experiments and educational displays for 2 yr.

Discussion

This is the first publication, to our knowledge, reporting successful blood-feeding of mosquitoes using frozen, food-grade pork and bovine blood. This blood is purchased for a very low price at a local Asian food market and is likely readily available in many communities. The blood is already treated with citrate or water and salt, negating the need for the user to add any preservatives. The blood is easily stored in the freezer for many months while still eliciting acceptable feeding rates once it is thawed and heated. The ease of obtaining and using these affordable blood sources to successfully feed multiple species of mosquito is a benefit to any rearing facility.

Additionally, this study tested the artificial membrane preferences of four species of mosquitoes using a combination of choice and no-choice tests and metal plates. Contrary to the results obtained by others (Rutledge et al. 1964, Kasap et al. 2003), we found Parafilm to be very successful as a feeding membrane, with high feeding rates in laboratory colonies of *Ae. albopictus* and *Cx. quinquefasciatus*, with lower rates of feeding in wild *Ae. albopictus* and *Ae. aegypti*. Our results are in agreement with the success of the aluminum plate and Parafilm membrane method seen in previous studies (Nasirian and Ladonni 2006, Carvahlo et al. 2014, Gunathilaka et al. 2017). The feeding rates observed in our colonies on Parafilm are much higher than those found by Tseng (2003) using Parafilm membranes with sheep blood and no heat source. The feeding rates observed in our laboratory are also higher than those obtained with *Ae. aegypti* (51.30%) feeding on the affordable Glytube device (Costa-da-Silva et al. 2013), but are similar to the high rates seen in other studies testing *Ae. aegypti* feeding rates

Table 2. Mean percentage of engorged females, mean monthly egg production (over three months), mean egg hatch rate (three replicates), and approximate number of adult mosquitoes per cage

Species	Membrane (blood)	Number of females counted (reps)	Blood feeding rate (%)	Number of eggs produced per cage/month	Egg hatch rate (%)	Total number of adults per cage (range)
Aedes aegypti	PTFE (Bovine)	874 (6)	90.12 ± 3.1 (77.86–99.43)	137,516. 95 ± 13,794.52 (119,200.00–164,542.52)	93. 06 ± 0.40 (92.28–93.53)	6,000–8,000
Aedes albopictus	Parafilm (Pork)	620 (5)	76.3 ± 6.26 (62.02–92.68)	68,877.41 ± 18,071.09 (45,646.36–104,470.67)	76. 30 ± 1.47 (73.66–78.73)	3,000–6,000
Aedes taeniorhnchus	PTFE (Bovine)	790 (5)	90.82 ± 2.82 (83.54–98.54)	113,548.56 ± 26,607.63 (80,324.78–166,160.91)	61. 44 ± 4.08 (56.59–69.54)	6,000–8,000
Culex quinquefasciatus	Parafilm (Pork)	1,060 (7)	82.61 ± 3.39 (71.81–95.83)	28,587.77 ± 510.38 (27820.00–29554.20)	89. 59 ± 2.41 (84.95–93.03)	3,000–5,000

Values presented represent the mean ± SE, followed by the range in parenthesis, where appropriate. The values only represent the preferred blood type and membrane combination for each species and only include laboratory colonies.

Fig. 6. Feeding rate response of the germination mat, parafilm, and pork blood combination as shown by engorged *Culex quinquefasciatus* females of a laboratory colony. Also shown is the feeding response of *Aedes aegypti* to a combination of a petri dish, pork blood, and PTFE tape.

on Parafilm (Dias et al. 2018) and PTFE tape (Siria et al. 2018). Our modifications of using commercially available, food-grade frozen pork and bovine blood and a germination mat appear to be a comparable method. The aluminum plate may also have contributed to the higher success observed, as found by Gunathilaka et al. (2017). Similar to Siria et al. (2018), we found the PTFE tape resulted in a higher feeding rate in certain species and populations of mosquitoes than the Parafilm M membrane and have adjusted our use of the two membranes accordingly. Additionally, at facilities where aluminum plates are not readily available, other substrates can be used with either of these artificial membranes. We have also used petri dish lids with these two membranes on occasion (Fig. 6) and have had similar success to that seen with the cups used by Siria et al. (2018). The higher rate of successful feeding reported here may also be due to adaptation over the generations to the artificial feeding method, similar to that reported by Deng et al. (2012). We did not record the rates of feeding when we first began transitioning from a live host to the artificial method and cannot make any conclusions in this regard.

Multiple studies describe *Ae. albopictus* as being hesitant to blood feed in general compared with other species, whether on a live host or artificial membrane (Alto et al. 2003, Tseng 2003, Lyski et al. 2011). Additionally, a higher proportion of *Ae. albopictus* feed on live hosts than on artificial membranes (Alto et al. 2003). We found the same pattern in the wild, F_0 *Ae. albopictus* that we tested, however, the

current feeding rate of our laboratory *Ae. albopictus* colony on artificial membranes is remarkably high compared to other studies. Using Parafilm membranes and pork blood achieved a much higher feeding rate (76.3%) than the 31% feeding rate obtained using citrated sheep blood and Parafilm by Tseng (2003). This could be due to using a different method and blood source from the previous studies, but it could also be due to adaptation (Deng et al. 2012) considering we are reporting on the 12th generation of artificially fed *Ae. albopictus*. Indeed, the F_0 wild *Ae. albopictus* population tested in our laboratory displays a feeding rate (28%), which is closer to that found by Tseng (2003). However, early on in our attempts to transition to artificial feeding from live hosts, we found the laboratory colonies of *Ae. albopictus* to be hesitant towards feeding on any artificial membranes when citrated bovine blood was used. Only when we began to try pork blood with water and salt did we finally see feeding rates higher than 10% in our laboratory colonies of *Ae. albopictus*. These results demonstrate that even recalcitrant mosquitoes can adapt to an artificial feeding method when the appropriate combination of membranes and blood types are used.

Although defibrinated blood is often used in artificial feeding, Dias et al. (2018) found that citrated blood resulted in higher feeding rates by three mosquito species tested [*Ae. aegypti*, *Cx. quinquefasciatus*, and *Anopheles aquasalis* (Curry)], compared with defibrinated blood. We have also found high acceptance of citrated blood in *Ae. aegypti* and *Ae. taeniorhynchus*, but higher acceptance of pork blood with water and salt than citrated bovine blood was found in our *Ae. albopictus* and *Cx. quinquefasciatus*. The isoleucine content of blood may be an important factor driving egg production and mosquito preference in certain species of mosquitoes, further supporting our decision to use pork blood as the main blood source for *Ae. albopictus* and *Cx. quinquefasciatus* mosquitoes (Lea et al. 1956, Harrington et al. 2001). The drawback to the frozen pork blood with water and salt is that it does not contain an anticlotting agent and thus contains a very large amount of clotted blood. This is easily sieved out of the blood once it is thawed, but it is the reason we still prefer to use citrated bovine blood for our other species that do not exhibit strong blood preferences (*Ae. taeniorhynchus* and *Cx. quinquefasciatus*). Ongoing studies are being conducted in our laboratory to compare the different food-grade blood types in regards to their effects on feeding rate, fecundity, and fertility for the four species in our insectary. Other facilities should conduct their own comparisons between the blood types before choosing one to use as different strains and species of mosquitoes may respond differently.

Alto et al. (2003) reported that the blood-feeding rate on artificial membranes increased with age for *Ae. albopictus*, and followed a 2-d cycle in *Ae. aegypti*. A similar pattern of feeding behavior occurs in our colonies. Blood meals are offered 5 d after emergence, and then once per week after that. We notice an increase in the feeding rate at the second and third feedings by *Ae. albopictus*. By attending to the specific gonotrophic cycle of each species and population, blood-feeding success can further be increased to improve the efficiency of the operation. For the purposes of maintaining our colonies, weekly blood-feedings are more than adequate.

Consistent with previous reports, we have found that *Culex* feed less readily on artificial methods than *Aedes* species (Novak et al. 1991, Dias et al. 2018); however, our feeding method results in a higher feeding rate than what has been found in prior studies. The average feeding rate reported here of 82.61% by our laboratory colony of *Cx. quinquefasciatus* is much higher than the 48.00% found by Richards et al. (2012) using citrated bovine blood, and is on parity with the 81.00% feeding rate on live chickens reported in that study. It is also higher than the feeding rates obtained by Dias et al. (2018) using live guinea pigs (67.00%), and fresh citrated or defibrinated rabbit and sheep blood (37.00–48.00%). The fertility and fecundity rates obtained in our laboratory for *Cx. quinquefasciatus* are also higher than those obtained even with live chickens (89.59% fertility vs 83.00% fertility) in previous studies (Richards et al. 2012). The previous studies did not use the aluminum plate method or pork blood, both of which may have contributed to the success seen with our method. Additionally, *Cx. quinquefasciatus* mosquitoes used in previous studies may have been less adapted to artificial bloodmeals or another aspect of their rearing could have resulted in lower feeding rates, such as different heat treatments, less time allotted for feeding, or the time at which the bloodmeal was offered. Future studies should compare the different blood options and membrane devices with other *Cx. quinquefasciatus* colonies to elucidate whether our success is due to this particular combination of methods or an attribute of our specific colony. It bears mentioning that we are currently maintaining a wild colony of *Cx. quinquefasciatus* using our artificial feeding method and are on the F_4 generation with acceptable blood-feeding rates. The success obtained with this wild colony may be due to the different blood-feeding preparation. Sucrose is removed 48–72 h before the bloodmeal is offered, and the bloodmeal is placed on the cage at 1600 h and left overnight on a low heat setting (34°C).

This is the first report to our knowledge to assess the feeding rate of *Ae. taeniorhynchus* mosquitoes using artificial feeding methods. As such, we cannot compare our results to any previous studies on this species. We have achieved high rates of feeding with our laboratory colony using both membranes and both blood types with the metal plate method and a germination mat as the heat source. Additionally, we are able to successfully blood-feed and obtain eggs from wild populations of *Ae. taeniorhynchus* using this method, although the sucrose is removed for a longer period of time (48 h minimum) prior to blood-feeding to increase interest in feeding. Based on our success with this method, we can recommend the combination of frozen citrated bovine blood, PTFE tape membranes and metal plates as a promising method for other insectaries rearing this species.

Although our new artificial feeding method has been highly successful for all of our laboratory colonies for 2 yr (Fig. 7), there are a couple of peculiarities to mention. When using the PTFE tape membrane the blood begins to dry and congeal at a quick rate forming a dry crust and a viscous liquid within 2 h of presentation, and is the reason we could not continue with further replications using this membrane overnight with wild *Cx. quinquefasciatus*. This is

Fig. 7. Routine egg production using aluminum plates and pork blood. Left: Egg rafts produced by laboratory colony of *Culex quinquefasciatus*. Right: Eggs laid by laboratory colony of *Aedes aegypti*.

similar to what has been found with other membranes (Rutledge et al. 1964), but was not mentioned in the initial study of PTFE membrane feeding which inspired our method (Siria et al. 2018). This crust is difficult to clean from the aluminum plates, but does not seem to impede immediate feeding as most mosquitoes have finished feeding before congealing occurs. No such crust or viscosity was observed to form on the Parafilm membranes, even when left overnight. This attribute should be considered by any laboratory wishing to evaluate these membranes on other populations. The PTFE tape membrane works well for mosquitoes that will feed quickly upon presentation of the membrane, but is likely to be unsuitable for species or populations which require overnight or longer-duration feedings. In those cases, Parafilm is more likely to produce a successful result. The Parafilm also has disadvantages, however, the main one being the difficulty and subjectivity of stretching the membrane enough to allow the mosquitoes to pierce the membrane without causing tears and leaks. Leaks are a frequent occurrence when using the Parafilm, even by experienced technicians. The PTFE tape can be stretched extremely thinly without tearing or leaking as easily as the Parafilm. One other irregularity with this method is the nature of the pork blood with water and salt, which does not prevent the formation of clots, resulting in the presence of a large singular blood clot in the container when the blood is thawed. The blood is easily strained and the clot discarded, leaving a reduced volume of blood in the container. This is not a substantial problem, as the remaining blood is still consumed readily by the mosquitoes and the blood is comparably inexpensive making this still the most affordable option (in most areas).

Many smaller mosquito control and research facilities may not have the resources to purchase and source the various components needed to utilize the blood feeding methods that are currently published and widely known. The method presented here is highly adaptable and affordable for any district or educational facility to use with different species of mosquitoes. The ease of sourcing the frozen blood and other materials and conducting the blood-feeding is such that this method could easily be adapted for use in\science classrooms to maintain small colonies of mosquitoes for students to readily observe the life history of mosquitoes and conduct biological experiments with minimal resources. By modifying existing artificial feeding methods (Carvahlo et al. 2014, Gunathilaka et al. 2017, Siria et al. 2018) through the addition of commercially available, frozen food-grade blood, different membranes for different species, and commercially available germination mats with automatic temperature control, we have developed a highly successful artificial feeding method that can be used for multiple species of mosquitoes at almost any facility.

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Author Contributions

Conceptualization, data curation, formal analysis, methodology, visualization and writing, K.T.J.; Investigation, K.T.J and C.D.; Project administration, resources, and supervision, A.L. and D.H.; Writing-review and editing, K.T.J., C.D., A.L., and D.H.

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