A New Method for Feeding Laboratory-Reared *Aedes aegypti* (Diptera: Culicidae) Adult Females[1]

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Abstract The mosquito *Aedes aegypti* (L.) (Diptera: Culicidae) transmits arboviruses including the causative agent for yellow fever and threatens human health in tropical and subtropical areas. To reduce the incidence of infection, a variety of tactics is being developed to keep the population density of this vector mosquito as low as possible. Laboratory research directed to this end requires maintaining laboratory colonies of the insect, which, in this case, requires providing immobilized live animals to *A. aegypti* adult females for a source of blood for production of viable eggs. Using live animals for this procedure is costly, time consuming, and requires rearing mammalian species in the laboratoy. This study evaluated the impact of supplying the adult females with blood from a domestic pig (*Sus scrofa domestica* L.) mixed with an anticoagulant (7 ml of heparin per liter of blood) on the oviposition rate of *A. aegypti.* Feeding *A. aegypti* females with pig blood obtained from a slaughter facility and mixed with heparin produced more eggs (6,994 $\pm$ 168) than those allowed to feed on a living hamster as a source of blood (5,483 $\pm$ 171). Thus, this methodology is more efficient and economical than the current use of live animals to feed females of *A. aegypti*.

Key Words blood feeding, laboratory rearing, arboviruses

The anthropophilic mosquito *Aedes aegypti* L. (Diptera: Culicidae) is the most dangerous animal to humans because it is a vector of arboviruses, which cause severe diseases such as dengue, dengue hemorrhagic fever, yellow fever, Zika, chikungunya, and Mayaro virus disease (Fernandez et al. 2020, Laverdeur et al. 2024). Rearing and maintaining these mosquitoes under laboratory conditions is important in conducting basic and applied research for physiological studies as well as the development of management tactics (Gloria-Soria et al. 2019).

Female mosquitoes must ingest vertebrate blood to complete the development of their eggs. Providing a blood meal is, thus, fundamental in their rearing (League et al. 2021, Luo 2014, Pitts 2014). Although females can mobilize nutrients

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acquired during the larval stage to develop the initial egg laying (Gonzáles and Hansen 2016), the blood meal is an essential source of amino acids for egg formation. The quality of the blood meal on which females feed affects egg nutrition, fecundity, oviposition, and hatch (Yan et al. 2021).

Currently, the breeding of *A. aegypti* depends on the blood supply of vertebrates such as rabbits (*Oryctolagus cuniculus* L.), guinea pigs (*Cavia porcellus* L.), rats (*Rattus* spp.), mice (*Mus musculus* L.), golden hamsters (*Mesocricetus auratus* [Waterhouse]) (CENAPRECE 2015, FAO/IAEA 2017, Fikrig et al. 2022, Gonzáles and Hansen 2016, Ote and Kanuka 2018), and even human blood (Baughman et al. 2017, Imam et al. 2014, Lusiyana et al. 2015). The logistics of providing these blood sources are complicated because of the costs, time, and growing concern for animal welfare in the laboratory. Researchers have been exploring options to provide blood without using live animals (Bursali and Simsek 2023, Gonzáles and Hansen 2016, Luo 2014).

Some researchers have proposed a blood-free diet (Deng et al. 2012, Gonzáles et al. 2018, Gonzáles and Hansen 2016, Huang et al. 2015, Luo 2014, Pitts 2014). Artificial diets and innovative methods for feeding female mosquitoes solve some drawbacks of conventional methods but are costly (Bursali and Simsek 2023, Gonzáles and Hansen 2016). We propose a methodology that consists of feeding *A. aegypti* females with blood obtained as a byproduct of slaughtered pigs (*Sus scrofa domestica* L.) mixed with heparin, an anticoagulant. Consumption of pork is common worldwide. Although some blood is used to prepare food (Chapa 2012), most is wasted. Consequently, obtaining that blood at a low price and even at no cost is feasible. Thus, the search for practical, easy-use, and inexpensive blood-feeding methods for females of these mosquito disease vectors is of paramount importance.

Materials and Methods

This study was conducted from June 2020 to December 2021 in laboratory facilities housed at the Colegio de Postgraduados, Campus Montecillo, Texcoco, Estado de México, Mexico.

***Aedes aegypti* population.** We used the New Orleans population of *A. aegypti* mosquitoes provided by the University of Nuevo Leon, Mexico. This population was reared according to the methodology established by the World Health Organization (2005) in bioclimatic chambers (Thermo Scientific Model TFFU2065FWA, Waltham, MA) under controlled conditions of 27°C $\pm$ 2°C and a 12:12-h photoperiod. The larvae were provided laboratory rodent chow (Rodent Lab Chow® 5001, Purina, St. Louis, MO) as food. After pupation and adult emergence, *A. aegypti* mosquitoes were introduced into entomological cages (40 $\times$ 40 $\times$ 40 cm) covered with organza cloth. Adult mosquitoes were fed a 10% (w/w) sugar solution, and female mosquitoes were blood-fed according to the treatments subsequently described.

Live hamster feeding. Live golden hamsters, *Me. auratus*, were used as a source of blood for feeding female *A. aegypti* mosquitoes. Several hamsters were maintained in the laboratory so that hamster hosts could be rotated to avoid reuse for mosquito feeding within 1 week. The hamsters were fed daily ad libitum with

drinking water and ground croquettes (Rodent Lab Chow 5001, Purina). To facilitate mosquito feeding, the dorsal side of the hamsters was shaved.

The female mosquitoes were fed every 72 h by placing a hamster (without sex differentiation) into the mosquito rearing cage and immobilized inside a 5.1 × 12.7 cm metal box (Itasa Américas, S.A. de C.V., ITSA S.A. de C.V., El Marqués, Querétaro, Mexico) adapted with metal mesh to prevent the hamster's escape. The hamster was kept inside the rearing cage for 3 h while the female mosquitoes had free access to the animal to take blood meals.

Pig blood feeding. The pig blood was obtained as a byproduct of pig slaughtering for food in the region of Texcoco, state of Mexico. The blood was collected immediately after slaughter, when sodium heparin (Inhepar® 1,000 IU, Pisa, Guadalajara, state of Jalisco, Mexico) was added to the blood at 7 ml/l of blood. Once conditioned with this anticoagulant, it was used immediately or stored for up to 7 d at 4°C for later use.

Before use, the stored blood (50 ml) was removed from the refrigerator, placed in a plastic container, kept at room temperature for 15 min, and then dipped into a water bath at 80°C to raise the blood temperature to 40°C, which took approximately 10 min. Subsequently, 10 ml of warmed blood was placed in an 80-ml plastic container (Reyma, Nuevo León, Mexico), and the opening of the container was covered with Parafilm-M® (VWR, Visalia, CA). Human sweat was collected in 2-ml microcentrifuge tubes (Eppendorf®, Framingham, MA) for phage stimulation. This sweat preferably comes from a person who is highly susceptible to mosquito attacks. The collected sweat was stored at 4°C and used for 3 mo. The outer part of the Parafilm membrane that covered the container with the pig blood was impregnated with the human sweat. Subsequently, three blood supply containers were inverted on the mosquito-rearing cage so that females could pierce the Parafilm to obtain the pig blood meal.

Experiment. The two treatments—blood from live hamsters or pig blood mixed with heparin—were evaluated in a completely randomized design with four replicates and under laboratory conditions of 27°C ± 2°C, 75% ± 5% relative humidity (RH), and on a 12:12-h photoperiod. Twenty-five adult couples were placed inside an entomological cage (40 × 40 × 40 cm) as the experimental unit and reared as described above. One ovitrap per entomological cage was placed and replaced daily to collect eggs until oviposition ceased in both treatments. The ovitrap consisted of a container covered with Kraft paper (Kraft Paper Services, Addison, IL) and 200 ml of tap water.

Larval emergence. Eggs (3–45 d old) from each treatment, placed on Kraft paper, were separated in groups of 100 to 224 (experimental unit) and placed in water to hatch. The hatching percentage was increased using the method of Quispe-Pretel et al. (2015), which involves heating tap water until reaching the boiling point, then removing from the heat source. When the boiled water reached 30°C, we added sugar (24 g/L) and used it at room temperature. Then, eggs were placed in containers with 150 ml of this water to hatch. The experimental units were maintained in bioclimatic chambers (Model TFFU2065FWA; Thermo Scientific) under controlled conditions of 26°C ± 2 °C, 70% ± 5% RH, and on a 12:12-h light:dark regime. After 48 h, we evaluated the number and percentage of emerged larvae. Each treatment was replicated 10 times.

Table 1. Basic statistics of number of eggs laid by *Aedes aegypti* fed two different blood sources.

Variable	Source of Blood for Feeding	
	Live Hamster	Pig Blood + Heparin
Days to the first egg laid	2	3
Days to the last egg laid	75	100
Average number of eggs*	5,483 ± 171	6,994 ± 168**

* Total number of eggs laid during the experiment by 25 females.
** Significantly better oviposition with pig blood feeding than with live hamster feeding (two-sample Student's *t* test, $P < 7.39 \times 10^{-4}$).

Statistical analysis. The number of eggs from each ovitrap was counted under a stereoscope, and the mean number of eggs per female was calculated. The data were grouped into 5-d periods, and a nonpaired Student's *t* test ($P < 0.005$) was performed every 5 d to determine the differences between the two methods of blood feeding. A two-sample *t* test ($P = 0.05$) was used to determine if there were statistical differences in the percentage of larval emergence between the two treatments. All analyses were conducted using the RStudio Statistical Computing Program, version 4.4.1 (https://www.r-project.org).

Results

The *A. aegypti* females that fed on live hamsters began laying eggs after 2 d, whereas those fed pig blood containing heparin started laying eggs after 3 d (Table 1). In the group that received pig blood with heparin, the last oviposition occurred on day 100, 25 d later than in the group fed live hamster blood. On average, 5,483 eggs were counted in the live-hamster-feeding treatment group, and 6,994 eggs were counted in the pig-blood-plus-heparin treatment group. On the basis of these results, oviposition was significantly greater with the pig blood feeding method than with the live hamster ($P < 7.39 \times 10^{-4}$) (Table 1).

In the first two periods (0–5 and 6–10 d), *A. aegypti* females oviposited more eggs when fed live hamster blood than when fed pig blood with heparin ($P < 0.0001$). During the fourth period (16–20 d), there were no significant differences ($P = 0.58$) in the number of eggs laid for either type of blood source (Table 2, Fig. 1). From the 5th to the 13th period (21–65 d), *A. aegypti* females oviposited more eggs when fed pig blood than when provided blood meals with live hamsters (Table 2). No significant differences in the number of eggs laid in the two treatments were seen between days 66 and 80 (Table 2, Fig. 1).

The mean percentage of larval emergence at 48 h was 87.9 and 89.9% from eggs oviposited by females fed a live hamster or pig's blood + heparin, respectively (Table 3). The two treatments were not statistically different ($P = 0.05$) in terms of larval emergence.

Table 2. Comparison of number of eggs laid by *Aedes aegypti* fed on two blood sources.

	Source of Blood for Feeding				
	Live Hamster		Pig Blood + Heparin		
Period (d)	Total*	Mean** ± SE	Total*	Mean** ± SE	Probability < *t*
0–5	2,599	26.0 ± 0.84	1,638	16.4 ± 0.47	<0.0001§
6–10	4,581	45.1 ± 1.39	3,996	40.0 ± 0.36	0.01§
11–15	2,342	23.4 ± 0.46	3,526	35.3 ± 0.87	<0.001§
16–20	2,863	28.6 ± 0.63	2,913	29.1 ± 1.15	0.58
21–25	2,711	27.1 ± 1.19	3,696	37.0 ± 0.74	4.18^{-4}§
26–30	1,998	20.0 ± 1.00	2,502	25.0 ± 1.05	0.013§
31–35	1,814	18.14 ± 0.85	2,143	21.4 ± 0.53	0.017§
36–40	989	9.89 ± 0.65	1,350	13.5 ± 1.34	0.05§
41–45	853	8.53 ± 0.27	1,552	15.5 ± 1.16	0.001§
46–50	158	1.58 ± 0.23	826	8.3 ± 0.49	<0.0001§
51–55	405	4.05 ± 0.55	755	7.5 ± 0.18	9.73
56–60	266	2.66 ± 0.36	832	8.3 ± 0.25	<0.0001§
61–65	96	0.96 ± 0.06	843	8.4 ± 0.18	<0.0001§
66–70	158	1.58 ± 0.17	490	4.9 ± 0.43	0.14
71–75	86	0.86 ± 0.11	243	2.4 ± 0.19	4.88
76–80	20	0.20 ± 0.2	309	3.01 ± 0.40	6.66
81–85	0	0	188	1.9 ± 1.32	
86–90	0	0	85	0.85 ± 0.51	
91–95	0	0	64	0.64 ± 0.64	
96–100	0	0	25	0.25 ± 6.25	

* Total number of eggs laid by period.
** Mean number of eggs laid by a female.
§ Significantly better oviposition with pig blood feeding than with live hamster feeding (two-sample Student's *t* test, $P < 0.05$).

Discussion

Aedes aegypti females fed pig blood obtained from slaughter facilities and treated with heparin produced more eggs (6,994 ± 168) than those females allowed to take blood meals from hamsters (5,483 ± 171). Larval emergence from those eggs was not statistically different between the two treatments ($P = 0.05$). Thus, it is possible to avoid using live animals as a source of blood to feed *A. aegypti* females in laboratory-reared colonies. In fact, we have successfully used pig's blood + heparin instead of live

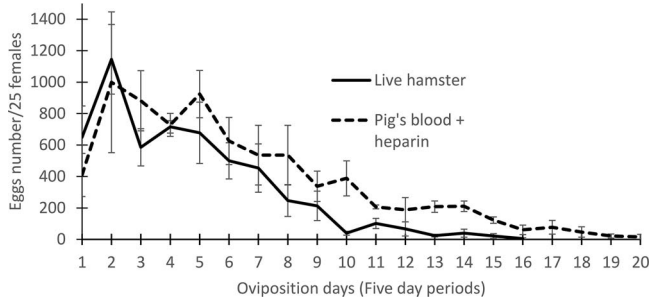

Fig. 1. Oviposition rates of *Aedes aegypti* fed live hamster or pig blood + heparin.

hamsters as a source of blood meals to feed *A. aegyti* females since 2022. Our intention of replacing live hamsters as a source of blood for feeding the females was not to obtain a higher oviposition rate but to obtain similar results and mitigate the use of live animals to maintain our colonies of *A. aegypti*. However, using pig blood + heparin led to improved oviposition rates.

The more common blood meal sources for laboratory rearing of mosquitoes are human arms, guinea pigs, mice, rats, hamsters, monkeys, and chickens (Imam et al. 2014). Although direct human blood feeding is possible, live animals have been used as a food source for female mosquitoes because of the risk of disease transmission to human subjects. However, there has been increasing concern about the well-being of laboratory animals and the economic, time, and logistical inconveniences of raising these animals for blood meal sources (Deng et al. 2012, Gonzáles and Hansen 2016, Luo 2014). The golden hamster, *Me. auratus,* is commonly used as a blood meal source for female *A. aegypti*; however, according to Blaurock et al. (2022) and Handley et al. (2023), this hamster species is a host for acute respiratory syndrome coronavirus type 2, thus raising concerns of the risk of zoonotic spillover of the virus to humans.

These circumstances have forced researchers to search for alternative blood meal sources for mosquitoes (Baughman et al. 2017, Gonzáles and Hansen 2016). Pitts (2014) proposed a blood-free diet consisting mainly of bovine serum albumin dissolved in a phosphate-based buffer provided to mosquitoes through membranes. Huang et al. (2015) suggested feeding female mosquitoes a mixture of 0.8 g of NaCl, 0.1 g of $NaHCO_3$, 1 g of dextrose, and 1 g of albumin in 100 ml of water, to which adenosine triphosphate (ATP) was added. Baughman et al. (2017) proposed a stable diet on the basis of human blood plasma supplemented with ATP for the *Aedes* and *Anopheles* spp. Gonzáles et al. (2018) developed an artificial diet (SkitoSnack) for feeding female *Ae. aegypti* that consisted of bovine serum albumin (200 mg/ml), bovine hemoglobin (5 mg/ml), chicken egg yolk (5 mg/ml), glucose (50 mM), ATP (3 mM), sodium chloride (150 mM), sodium bicarbonate (23 mM), potassium chloride (4 mM), calcium chloride (2.5 mM), and magnesium chloride (0.8 mM).

These alternative methods have the disadvantage of requiring a variety of laboratory reagents that may not be available in developing countries that are under

Table 3. Emergence of *Aedes aegypti* larvae from eggs laid by females fed with two different sources of blood.

	Source of Blood to Feed Females					
	Live Hamster			Pig′s Blood + Heparin		
		Larval Emergence (48 h)			Larval Emergence (48 h)	
Replication	*n*	Amount	Percent	*n*	Amount	Percent
1	127	116	91.3	127	117	92.1
2	167	154	92.2	124	123	99.2
3	155	127	81.9	127	106	83.5
4	200	168	84.0	128	110	85.9
5	179	163	91.1	224	205	91.5
6	180	167	92.8	100	90	90.0
7	191	152	79.6	103	82	79.6
8	177	150	84.7	106	97	91.5
9	150	133	88.6	190	164	86.3
10	165	156	94.5	175	165	94.3
	1691	**1486**	$\bar{x} = \mathbf{87.9}$	**1404**	**1259**	$\bar{x} = \mathbf{89.6}$

Equal variance assumed, probability $> |t| = 0.59233$.
Equal variance not assumed (Welch correction), probability $> |t| = 0.59239$.

threat of diseases transmitted by *Ae. aegypti*. Deng et al. (2012) evaluated a feeding method with membranes and blood from mini pigs, *C. porcellus* L. They reported a fecundity of 71.17 ± 1.68 eggs per female per week. Costa-da-Silva et al. (2013) proposed a system in which they used Falcon-type conical tubes with a screw cap. These tubes were fitted with sealing film and parafilm. The latter was covered with a hole in the tube cap. Blood (1.5 ml) at 37°C was placed between the sealing film and the parafilm; the remainder of the tube was filled with 40 ml of glycerol at 50°C to maintain warm temperature. Luo (2014) proposed a membrane system for feeding mosquitoes consisting of four tubes containing pig blood inserted into a larger tube and kept in a water bath at 38.5°C. To use them, the lower end of each tube containing blood was closed with a bovine collagen membrane from which the female mosquitoes could feed directly. Balestrino et al. (2014) proposed a mass-rearing cage for *Aedes albopictus* (Skuse) that is thermostatically controlled to maintain blood at 37°C and is offered to mosquitoes through a porcine intestine. Ote and Kanuka (2018) proposed a methodology to feed mosquitoes with mouse blood treated with an anticoagulant (heparin 1 mg/ml).

Anticoagulants are necessary to feed female mosquitoes with blood from any vertebrate animal. Lusiyana et al. (2015) evaluated various types of anticoagulants

in human blood in the diet of *Ae. aegypti* and found heparin the best anticoagulant for breeding this vector species. It is also crucial to include phagostimulants in addition to anticoagulants. We used human sweat as a phagostimulant. The outer part of the parafilm covering the container with pig blood and heparin mixture was impregnated with human sweat and placed in an inverted position on the entomological cage containing the adults of *Ae. aegypti*.

In conclusion, we determined and demonstrated that rearing *Ae. aegypti* in the laboratory can be effectively and efficiently accomplished by providing females with pig blood obtained from local slaughter facilities and mixed with heparin (7 ml/l of blood). We further designed and developed the methodology for providing the blood meal through a parafilm membrane treated with human sweat as a phagostimulant. Egg production by these females was significantly higher than the production by females fed by the more conventional method of providing the blood meal via living animals. Further, we saw no impact on the quality of the eggs produced, as larval emergence from the eggs fed with this method did not differ from that of eggs produced by females fed on living animals. We have thus successfully instituted this method of blood meal provision in our laboratory rearing protocols.

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