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ARTIFICIAL BLOODFEEDER GLYTUBE: EVALUATING DIFFERENT TYPES OF MEMBRANES AND BLOOD SOURCES FOR FEEDING *AEDES AEGYPTI* AND *AEDES ALBOPICTUS*

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ABSTRACT. Mosquito colony maintenance in the laboratory is essential for research but presents logistical and ethical problems with the use of live animals for bloodfeeding. The Glytube is an artificial bloodfeeding system for mosquitoes that uses Parafilm-M® membrane and human blood to feed *Aedes aegypti*. This study evaluated the efficiency of Glytube with different types of membranes and chicken blood to feed *Ae. aegypti* and *Ae. albopictus*. We evaluated 2 artificial (thread seal tape [TST], Parafilm-M) and 2 natural membranes (pork, sheep intestine). The results for *Ae. aegypti* suggest that TST was the best membrane because it presented a high percentage of fed females (63%), a high average number of eggs per female (54.65), and an egg viability rate significantly similar to control (mouse). For *Ae. albopictus*, there was no significant difference between the membranes and the control; however, the use of TST is suggested due to the low cost and easy manipulation. The treatments that used chicken blood did not present significant differences in the egg viability when compared with the control. The Glytube functionality can be increased by replacing the Parafilm-M membrane by TST and human to chicken blood.

KEY WORDS *Aedes aegypti*, *Aedes albopictus*, artificial bloodfeeder, Culicidae, maintenance of mosquito colonies

INTRODUCTION

The mosquitoes *Aedes aegypti* (L.) and *Ae. albopictus* (Skuse) are vectors of several global arboviruses, including dengue virus (Simmons et al. 2012), yellow fever virus (Jenntes et al. 2010), chikungunya virus (Leparc-Goffart et al. 2014), and Zika virus (Zara et al. 2016). *Aedes albopictus*, beyond these arboviruses, is associated with the transmission of eastern equine encephalitis (Gomes et al. 1999) and Japanese encephalitis (Consoli and Oliveira 1994).

Experimental studies on these vectors, as well as their interaction with pathogens, require the maintenance of mosquito colonies in the laboratory (Deng et al. 2012, Costa-da-Silva et al. 2013). Several different blood sources were used for artificial feeding: animals, such as rabbits (Novak et al. 1991), bovines (Davis et al. 1983), hens (Bishop and Gilchrist 1946), sheep (Rodhain et al. 1912, Phasomkusolsil et al. 2013), and horses (Costanzo et al. 2015), in addition to human and mice blood (Pina and Fonseca 1999). Currently, one of the most used methods is adult sedated mice (Kogan 1991, Novak et al. 1991).

Therefore, maintenance of mosquito colonies in the laboratory implies the need for parallel maintenance of animal facilities for the supply of animals to be used as a food source, and their financial costs (Pina and Fonseca 1999, Deng et al. 2012). In addition, there are all the ethical issues related to the use of other species for the benefit of human health (Rivera 2001). All these difficulties when bloodfeeding mosquito females in the laboratory led to several studies attempting to replace this procedure with artificial bloodfeeding systems through membranes (Deng et al. 2012).

Artificial feeders are an interesting solution found to address this problem. They are devices that propose the facilitation of feeding colonies, either by reducing the time, through the precision in the quality of the blood offered or by high mosquito acceptance. These devices range from a simple glass with a membrane at the bottom sheltering the blood, to more elaborate models involving water baths and incubators. However, certain elements tend to be common to all feeders: cages, blood—covered by a membrane, and temperature control (Rutledge et al. 1964, Gunathilaka et al. 2017).

Artificial feeders have great flexibility of use. They have already been used to feed *Culicoides mississippiensis* Hoffman (Diptera: Ceratopogonidae) (Davis et al. 1983), *Culex quinquefasciatus* (Say), and *Anopheles aquasalis* Curry (Costa-da-Silva et al. 2013), demonstrating the plasticity of use within the order Diptera. In addition to colony maintenance to help provide individuals with physiological, behavioral, and parasite–vector relation-

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ships (Novak et al. 1991), they can be used in experiments that involve vector infections (Bishop and Gilchrist 1946, Reeves and Miller 2013).

The use of artificial feeders has advantages over natural feeding for infection studies, as it allows researchers to specify the infecting concentration of a microorganism in the blood that will be offered to the vector. It facilitates the transmission of different types of pathogens and potentiates the chances of infection besides being more secure in carrying out the process (Pina and Fonseca 1999).

In an attempt to construct an efficient artificial feeding system, various types of materials have been used, such as natural membranes from animal skins, including hens (Bishop and Gilchrist 1946), mice (Rodhain et al. 1912), pork intestine (Costanzo et al. 2015), sheep intestine (Novak et al. 1991), and bovine collagen (Deng et al. 2012), being used the most as they usually present better results. However, artificial membranes, such as Parafilm and latex membranes, also provide promising results (Deng et al. 2012). Latex condoms and paraffin films (Novak et al. 1991, Phasomkusolsil et al. 2013), as well as silicone membranes (Pina and Fonseca 1999), are also used.

The Glytube system of artificial bloodfeeding for mosquitoes (Costa-da-Silva et al. 2013) used Parafilm and human blood to feed *Ae. aegypti*. It is effective, easy to assemble, and a cost-effective artificial feeder.

Our study aimed to evaluate different types of membranes using Glytube for feeding *Ae. aegypti* and *Ae. albopictus* females. Additionally, the study also evaluated the effect replacing human blood with chicken blood on bloodfeeding and egg development in both species.

MATERIALS AND METHODS

Aedes aegypti and *Ae. albopictus* rearing

All the experiments were carried out in the insectary of the Parasitology Laboratory (Institute of Biological Sciences, Federal University of Pará), using female *Ae. albopictus* and *Ae. aegypti* from the 3rd- to the 5th-generation colonies maintained according to Eiras and Jepson (1994). The colonies were established with mosquitoes captured by ovitraps in the urban area of Belém (Pará, Brazil). The insectary was maintained at a temperature between 27°C and 29°C, RH above 70%, with a photoperiod of 12 h light and 12 h dark. Adults were fed ad libitum with a water and sugar solution, and the larvae were fed with fish food (TetraMin®, Melle, Germany).

Artificial feeder

The Glytube feeder was produced according to Costa-da-Silva et al. (2013). It consists of a 50-ml conical-bottom polypropylene tube with a modified screw lid. Thirty milliliters of glycerin was placed inside the tube and sealed with a heat-resistant film, commercially used for food cooking, attached with adhesive tape.

The glycerin tube was heated in a water bath at 60°C. The screw lid was cut in the center, creating an opening of 2.4 cm in diam, to allow the insects access to the blood. The membranes tested were fastened externally to the cap, covering this circle.

Four types of membranes were tested: 2 artificial (thread seal tape [TST], also known as plumber's tape or polytetrafluoroethylene, and Parafilm-M®) and 2 natural membranes (pork and sheep intestine). The artificial membranes were simply stretched and fixed to the lid. The natural membranes were bought, clean, and salted. The piece of tripe used was washed with running water to remove the salt, cut vertically, stretched over the lid, and tied with a string. After fixing the membrane, 1 ml of heparinized chicken (*Gallus gallus domesticus* (L.)) blood was added inside the lid, and then was screwed onto the tube. A heat-resistant film separated the blood from the glycerin, allowing heat transfer from the latter to the blood without contamination. Glycerin was chosen for having low thermal conductivity, thus taking time to lose the acquired heat and, consequently, keeping the blood warm for a longer period.

Experimental design

All the tests were performed at the end of the afternoon when the 2 target species show their highest activity. For the experiments, small feeding cages (15.0 cm × 10.0 cm × 10.0 cm), containing 10 adult mated female mosquitoes, within 5–10 days old were used. The feeder, using one of the membrane types, was placed over the cage for 1 h. As a control, anesthetized adult mice (*Mus musculus* (L.)) of the Swiss Albino strain were used. The animals were laid over the test cages, with the abdomen exposed to the females' bites. Between the cage and the mice, a paper barrier was added so that the surface of the mice available to the mosquitoes was a circular area of 2.4 cm in diam, similar to that made available in the feeder experiments.

During the experiment the following variables were observed: 1) pre–blood meal time (min)—the time required for the 1st female to look through the feeder; and 2) percentage of females that were fed engorged, showing dilated abdomen (Pina and Fonseca 1999).

After the blood meal, females were transferred to a larger cage (23.0 cm^3) for feeding on sugar solution and egg laying. The mean number of eggs laid per female was quantified (total number of eggs laid/number of live females), and the viability of the eggs was determined (number of eggs hatched/number of eggs laid). All experiments were repeated 10 times, totaling 100 females per experiment of each membrane for both species.

Table 1. Mean (± SD) of the variables analyzed for the 4 membrane types and the control treatment for *Aedes aegypti*: pre–blood meal time (PbMT), percentage of fed females, number of eggs per female, and number of viable eggs (viability).[1]

Treatment[2]	PbMT	% fed females	Eggs/female	Viability
Mice	2.01 ± 1.42 a	27.00 ± 1.70 b	56.89 ± 30.15 a	72.00 ± 0.12 a
TST	1.48 ± 1.29 a	63.00 ± 1.83 a	54.65 ± 37.14 a	64.00 ± 0.24 a
Parafilm-M®	1.34 ± 0.99 a	45.00 ± 1.35 b	66.05 ± 15.11 a	66.00 ± 0.18 a
Pig intestine	0.90 ± 0.51 a	57.00 ± 1.70 a	43.45 ± 18.48 a	71.00 ± 0.08 a
Sheep intestine	2.18 ± 1.92 a	38.00 ± 2.44 b	37.06 ± 21.41 a	81.00 ± 0.15 a
ANOVA				
F	1.55	6.17	1.97	0.14
P	0.202	0.000*	0.114	0.965

[1] Lowercase letters present the results of the averages comparisons between treatments by Tukey test. Different letters represent significantly different means ($P < 0.05$), identical letters represent absence of significant difference.
[2] TST, thread seal tape; ANOVA, analysis of variance.
* Means significantly different between treatments ($P < 0.05$).

Statistical analysis

Comparison of the variables (pre–blood meal time, percentage of fed females, number of eggs/females, and percentage of viable eggs) between the treatments (different types of membrane) were performed by analysis of variance, followed by Tukey test. *t*-test was used to compare the egg viability of the Glytube treatments with the control. Data were organized in Excel® (Microsoft, Redmond, WA) spreadsheets, and analyses were performed using the R program (R Core Team 2015).

All procedures using mice were approved by the Ethics Committee (CEUA/UFPA), of the Federal University of Para under protocol number 4237211117.

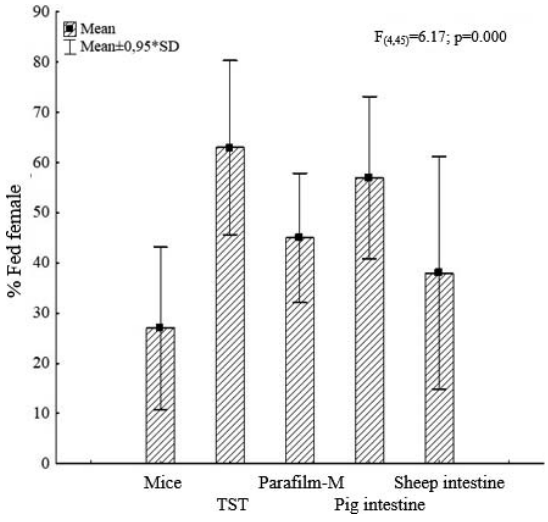

Fig. 1. Comparison of the mean (± SD) percentage of females fed per treatment for *Aedes aegypti*. Different letters on the bars indicate significantly different mean values, identical letters indicate mean values with no significant difference. TST, thread seal tape.

RESULTS

For *Ae. aegypti*, the pre–blood meal time ranged from 0.90 min for pork gut to 2.18 min for sheep intestine. However, there was no significant difference observed between treatments (Table 1). Comparing the percentage of fed females between treatments, it was observed that the averages surveyed for TST (63%) and pig intestine (57%), although statistically nonsignificant, were significantly superior to the other treatments (Fig. 1). The control treatment (mice) showed the lowest percentage of fed females. The number of eggs laid per female ranged from 37.06 for sheep intestine and 66.05 for Parafilm, although the differences between the treatments were not significant. When the viability of these eggs was evaluated, a high percentage of egg hatching was noted, ranging from 66% (Parafilm) to 81% (sheep intestine), with no significant difference observed between treatments.

For *Ae. albopictus*, we observed the lowest pre–blood meal time in sheep intestine (1.89 ± 1.48 min), as compared with a higher time (2.59 ± 2.04 min) in the control (Table 2). The mean percentage of females fed in the control treatment (29%) was lower than that observed in all the membranes; however, the number of eggs per female was the highest. Evaluating the viability of these eggs, it was observed that the control treatment presented a lower viability than most treatments. There was no significant difference between treatments for any of the variables evaluated (Table 2).

Comparing the results presented by the 2 species, it can be observed that the pre–blood meal time for *Ae. aegypti* was lower than that of *Ae. albopictus* in all the treatments, except for the sheep intestine (Fig. 2), which was significantly different from the pig intestine. The percentage of fed *Ae. aegypti* females was also higher than *Ae. albopictus* for all treatments, except for the control where they were practically the same (Fig. 2). This difference was significant for TST and pig

Table 2. Mean (± SD)[1] of the variables analyzed for the 4 membrane types and the control treatment for *Aedes albopictus*: pre–blood meal time (PbMT), percentage of fed females, number of eggs per female, and number of viable eggs (viability).

Treatment[2]	PbMT	% fed females	Eggs/female	Viability
Mice	2.59 ± 2.04	29.00 ± 1.45	57.58 ± 17.32	76.00 ± 0.13
TST	2.52 ± 1.70	35.00 ± 0.97	46.12 ± 16.88	72.00 ± 0.18
Parafilm-M®	2.63 ± 1.84	36.00 ± 1.07	55.44 ± 15.06	93.00 ± 0.07
Pig intestine	2.48 ± 2.30	36.00 ± 1.84	42.84 ± 17.62	77.00 ± 0.11
Sheep intestine	1.89 ± 1.48	32.00 ± 1.48	45.72 ± 23.83	90.00 ± 0.05
ANOVA				
F	0.25	0.47	1.26	0.42
P	0.906	0.752	0.298	0.786

[1] Mean of 10 replicates with 10 female mosquitoes per replicate.
[2] TST, thread seal tape; ANOVA, analysis of variance.

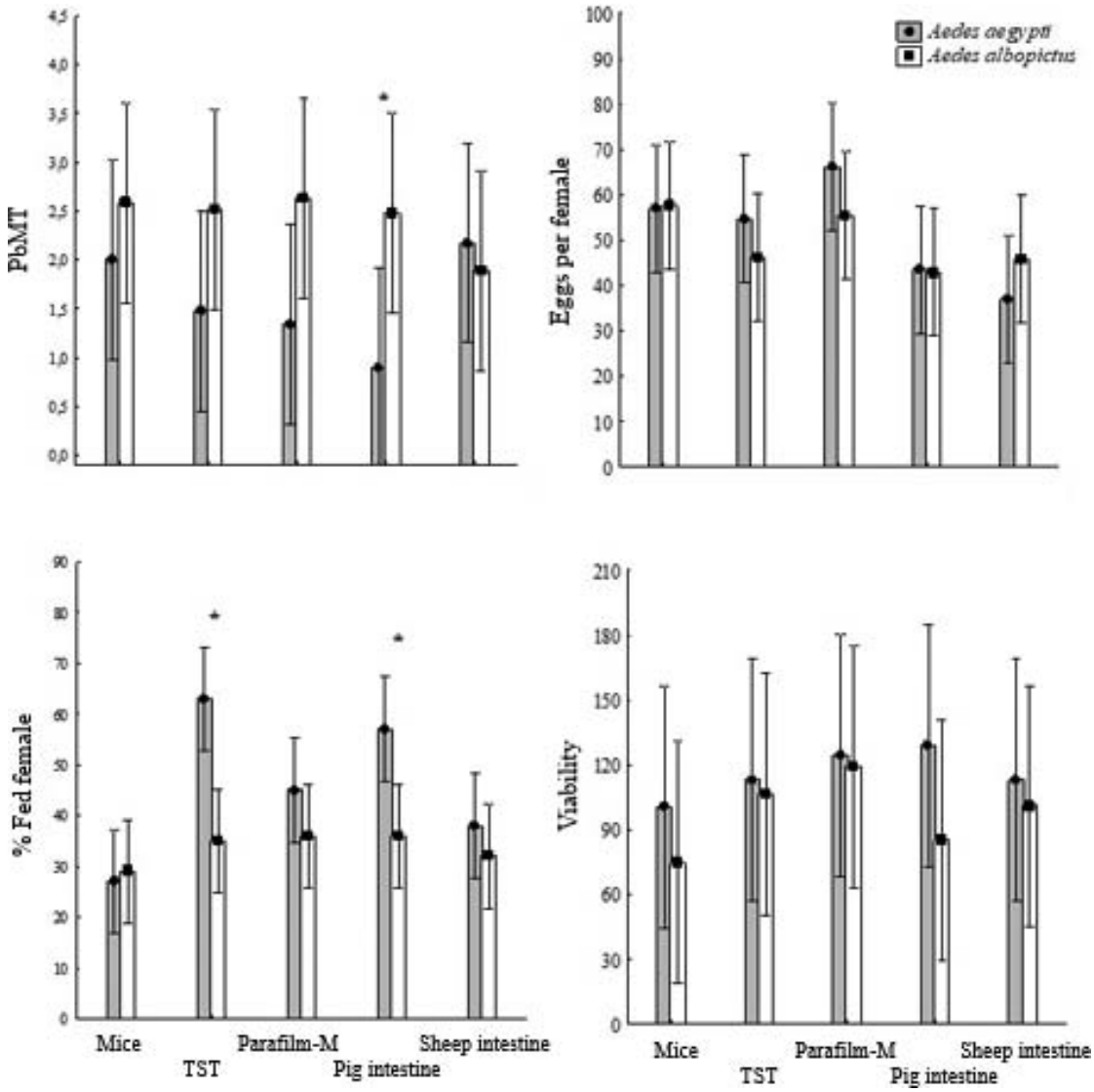

Fig. 2. Comparison between the results presented by *Aedes aegypti* and *Ae. albopictus*. Asterisks on the bars indicate significantly different means. TST, thread seal tape.

intestine. The number of eggs and the viability of eggs laid by *Ae. aegypti* was higher than those of *Ae. albopictus* (Fig. 2). However, no significant differences were observed.

DISCUSSION

Experimental studies with mosquito vectors require a larger number of replicating and, for this, a larger number of individuals. The logistical difficulties and high cost of bloodfeeding mosquito females using animals stimulated the development of several models of artificial feeders (Rutledge et al. 1964, Pina and Fonseca 1999, Costa-da-Silva et al. 2013, Luo 2014).

In studies evaluating artificial feeders, the time of exposure of the mosquitoes to the feeder varies widely. It can range from 30 min (Pina and Fonseca 1999, Tseng 2003, Phasomkusolsil et al. 2013) to 1 h (Rutledge et al. 1964, Deng et al. 2012). In our study, the artificial feeder was available to the mosquitoes for 1 h in order to evaluate the feeder's ability to keep the blood warm. It was found that Glytube was able to maintain the blood temperature even after 1 h. Additionally, at the end of the experiment, the blood showed no signs of coagulation. It is important to emphasize that keeping the feeder available for 1 h was completely unnecessary since females able to feed did it in a very short time. Similar behavior has been observed in other studies (Novak et al. 1991, Luo 2014). In our study, the mean pre–blood meal time for both *Ae. aegypti* and *Ae. albopictus* ranged from 0.9 min to 2.63 min, and no statistically significant difference was found between the membranes and the mice (control), suggesting similar effectiveness between the 2 systems.

The percentage of mosquitoes fed is a crucial factor in evaluating the efficiency of an artificial feeder that is usually quantified in the studies (Novak et al. 1991, Costa-da-Silva et al. 2013, Phasomkusolsil et al. 2013, Luo 2014). In our study, the average percentage of mosquitoes fed by Glytube was higher than that of the mice for all the membranes tested, demonstrating the acceptance of the feeder by both *Aedes* species. It should be emphasized that, for *Ae. aegypti*, the average percentage of fed females using TST (63%) and pork intestine (57%) was significantly higher than that observed for the mice (27%). Costa-da-Silva et al. (2013) obtained 51.3% of *Ae. aegypti* engorged females using the Glytube with a Parafilm membrane. This value was similar to that found in our study for the same membrane (45%), but lower than that observed for the TST (63%), which suggests that this artificial membrane is more efficient in terms of the percentage of fed females, being significantly superior to mice.

For *Ae. albopictus*, the lack of significant difference in the percentage of fed females between the membranes and mice observed in our study demonstrated the acceptance of the artificial feeder by this species. The percentage of fed females on the membranes was similar to that found by Tseng (2003), using Parafilm (31%).

Some studies suggest that natural membranes are more effective than artificial membranes. Novak et al. (1991) found that the most efficient membranes for feeding *Ae. aegypti* were quail and mice skin. Sheep intestine also showed promising results, reaching on average 59% of *Ae. aegypti* fed females. These values are higher than those found in our study for the same membrane (38%), but similar to those presented by pig intestine (57%). In the current study, the percentage of *Ae. aegypti* fed on pig intestine was significantly higher than the control treatment.

However, natural membranes require some treatment and labor before being used (Luo 2014), such as salt removal, as was done in our study. They may be used fresh or frozen (Rutledge et al. 1946), although it is usually difficult to obtain them fresh daily, and they are challenging to handle when frozen. Artificial membranes, on the other hand, do not require such care and are therefore more practical.

An efficient blood meal implies good egg laying (Phasomkusolsil et al. 2013, Luo 2014, Gunathilaka et al. 2017). The mean number of eggs per fed female observed in our study was relevant when compared with other values in the literature. For *Ae. aegypti*, an average of 12.7 eggs per female were recorded using human blood (Pothikasikorn et al. 2010) and 49.9 eggs per female with pig blood (Luo 2014), while we recorded 66.05 $\pm$ 15.11 eggs per female with chicken blood. For *Ae. albopictus*, the mean number of 54.0 eggs per female reported with pig blood (Luo 2014) was similar to that observed in our study using chicken blood (57.58 $\pm$ 17.32). In our study, there was no statistically significant difference in the mean number of eggs per fed female between membranes and the mice, suggesting a similar pattern of blood intake between the 2 feeding processes.

Evaluating the viability of the eggs in this study, for both species, the average viability of eggs using feeding by mice showed no significant difference in relation to the membranes using chicken blood. These data demonstrate a successful use of chicken blood in the artificial feeding process for *Ae. aegypti* and *Ae. albopictus*.

The type of blood influences the acceptance of the artificial feeding by the female, measured here by the percentage of fed females, as well as the posture rate and the viability of the eggs. In their study on mosquito feeding patterns, Dos Santos et al. (2012) showed that the genus *Aedes* presented a wide range of hosts for feeding, with a greater emphasis on birds. Nevertheless, another study (Forattini et al. 1987) verified the preference of the genus *Aedes* was toward mammals.

Pina and Fonseca (1999) reported a high rate of *Ae. aegypti* females feeding on human blood through a silicone membrane (89%), showing the species as anthropophilic. However, when bovine or mouse blood was used, the rate of engorgement decreased to

25.8% and 22.5%, respectively, being lower than that found in our work with artificial membranes TST and Parafilm (63% and 45%, respectively) and chicken blood.

It is important to point out that there are considerable differences in the experimental conditions between the works, which makes a more efficient and comparative study difficult. Among the differences, we can mention the age and quantity of the females, the type of animal used as a control, the size of the container, and the exposure surface of the membrane, besides the time of exposure of the feeder (Rutledge et al. 1964, Novak et al. 1991, Pina and Fonseca 1999, Costa-da-Silva et al. 2013).

The Glytube is a feeder that is easily produced and handled. It consists of low-cost materials commonly found in laboratories. They also have an easy maintenance and high reuse rate, except for membranes, which are used only once, although these are also inexpensive. The experiments using them showed important results. For *Ae. aegypti*, the TST membrane was the most indicated, because it presented a high percentage of fed females, and a large number of eggs laid per female with a high egg viability. For *Ae. albopictus* there was no significant difference between the membranes; however, we also suggest TST due to low cost and easy manipulation. The use of chicken blood in the process was also effective as it did not present significant differences when compared with the mice and it was easily available in slaughterhouses, with zero cost. These data thus demonstrate the efficiency of the artificial feeder Glytube, especially if combined with artificial membranes and chicken blood.

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