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Effects of different diets on *Aedes aegypti* adults: improving rearing techniques for sterile insect technique

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Abstract

The aim was to evaluate the effect of different energy diets available in adulthood on the longevity, dispersal capacity and sexual performance of *Aedes aegypti* produced under a mass-rearing system. To evaluate the effects of diets in relation to the survival of the adult male insects of *Ae. aegypti*, six treatments were used: sucrose at a concentration of 10%, as a positive control (sack10); starvation, as a negative control (starvation); sucrose at a concentration of 20% associated with 1 g/l of ascorbic acid (sac20vitC); wild honey in a concentration of 10% (honey10); demerara sugar in a 10% concentration (demerara10); and sucrose at a concentration of 20% associated with 1 g/l of ascorbic acid and 0.5 g/l of amino acid proline (sac20vitCPr). Each treatment had 16 cages containing 50 adult males. For the tests of flight ability and propensity to copulation, five treatments were used (saca10; sac20vitC; mel10; demerara10; and sac20vitCPr), with males each for flight ability and females copulated by a single male for copulation propensity. The diet composed of sucrose at a concentration of 20% associated with ascorbic acid, as an antioxidant, improved the survival, flight ability and propensity to copulate in *Ae. aegypti* males under mass-rearing conditions, and may be useful to enhance the performance of sterile males, thus improving the success of sterile insect technique programmes.

Introduction

Aedes aegypti is one of the most epidemiologically important mosquito species in the spread of viral diseases in urban environments, with emphasis on dengue, Chikungunya, and Zika, which are recognised as a global public health problem (Silva *et al*., 2020). This vector is found mainly in eastern South America (Brazil), southern North America (Mexico, Cuba, and southeastern coast of the United States), Central Africa (Central African Republic), southeastern Asia (India and southeastern coast of China), and eastern Oceania (Ding *et al*., 2018; Ducheyne *et al*., 2018; Shriram *et al*., 2018), due to favourable weather conditions (Rodrigues-Alves *et al*., 2020).

Vector-borne diseases account for about 17% of all infectious diseases, causing more than 700,000 deaths per year (Guo *et al*., 2022). In Brazil, the control of *Ae. aegypti* is a great challenge for public health, due to its increasing territorial dispersion and the need for increasingly complex prevention and control actions. For this reason, arboviruses are responsible for significant human morbidity and mortality in the country (Andrioli *et al*., 2020). Thus, the magnitude of financial and political problems that these epidemics can bring to developing countries is still ignored. Therefore, the main measure for the treatment or prevention of diseases transmitted by *Ae. aegypti* depends entirely on vector control or interruption of mosquito contact with humans, thus ending the arboviruses transmission cycle (Rodrigues-Alves *et al*., 2020; Tajudeen *et al*., 2022).

Faced with the problem of growing statistics on cases of dengue, Zika, and Chikungunya in Brazil (Pescarini *et al*., 2022), it is emphasised that the traditional control programmes used are not being sufficient to obtain consistent results in reducing the epidemiological indices of diseases caused by the virus transmitted by *Ae. aegypti* (Brito *et al*., 2021; Macêdo *et al*., 2021). Thus, it is essential to adopt strategies that seek to controlling mosquito population, and provide sustainability to the actions established by health surveillance networks (Borre *et al*., 2022). It is clear that complementary mosquito population control tools must be effective, sustainable, and environmentally safe (Silvério *et al*., 2020; Pitton *et al*., 2023).

One of the promising alternatives is the use of sterile insects. The sterile insect technique (SIT) is based on releasing a large number of sterile males of the target species in a pre-established area (Knipling, 1955). But for such a strategy to be effective, it is essential to release high-quality insects, so that they are able to disperse, survive, and compete with wild males to copulate with wild females (Balestrino *et al.*, 2022; Gómez *et al.*, 2022).

The first steps have already been taken in Brazil for the application of SIT (Gómez *et al.*, 2022; Silva *et al.*, 2023). Although there is already a protocol for mass rearing of *Ae. aegypti* (Moscamed Brasil Biofactory (BMB); Carvalho *et al.*, 2014a, 2014b), to the best of our knowledge, there are few studies that determine the influence of nutrition in the adult stage on the performance of *Ae. aegypti* produced males (van Schoor *et al.*, 2020) in tropical regions.

It is known that in the winged phase, for *Ae. aegypti*, haematophagy is restricted to females, while both sexes can obtain energy from plant sources. Adult insects need to ingest sugar to satisfy the energy demands for basal metabolism and escape from predators and unfavourable environments (Barredo and Degennaro, 2020). Thus, the need to test formulations of energy diets is highlighted in order to improve the performance of insects, obtaining males that are more sexually competitive, with better dispersal and survival capacity, to be used in sterile insect control programmes.

In this sense, we hypothesise that adult males of *Ae. aegypti* produced under mass rearing and fed with energy diets show improvement in survival rates, propensity to copulate, and flight ability. Therefore, the objective of this study was to evaluate the effect of different energy diets available in adulthood on the longevity, dispersal capacity, and sexual performance of *Ae. aegypti* produced in a mass-rearing system.

Materials and methods

Experimental site and maintenance of the Ae. aegypti *colony*

The research was carried out in the entomological laboratory of BMB, located in Juazeiro-Bahia, Brazil (9°27′33″ S and 40° 29′14″ W). The *Ae. aegypti* insects used in the research were obtained from colonies maintained at the BMB Production and Laboratories Unit, maintained in air-conditioned rooms with a temperature of 27 ± 1°C, relative humidity 70 ± 10%, and a photoperiod of 11:13 (light:dark) hours.

The MBR001 strain was used in the bioassay. The multiplication of this lineage was conducted following the protocol described by Carvalho *et al.* (2014a, 2014b). During the larval stage, a commercial ground food (Sera Vipan Premium®; SeraGmbH, Heinsberg, Germany) was offered to the larvae, according to a predefined feeding regime (table 1).

Separation of pupae

Sexual separation was performed at the pupal stage, due to the size dimorphism between male and female pupae: females are generally larger (Focks, 1980; Carvalho *et al.*, 2015). A separator (Moscamed Brazil) was used, formed by two overlapping glass plates and four valves that, when closed, press the glass plates and they start to function as a funnel with a horizontal linear format, with the separation of the pupae: first phase (upper portion) female pupae, second phase (central portion) male pupae and, in the third phase (lower portion), the larvae.

Table 1. Larval feeding regimen used in mass rearing of *Ae. aegypti*

	Number of larvae/tray (g)		
Day	6750	8000	9000
1	0.4	0.5	0.5
2	0.5	0.6	0.7
3	1.1	1.3	1.4
4	2.1	2.5	2.8
5	4.3	5.1	5.8
6	2.2	2.6	2.9
7	1.4	1.6	1.8
8	0.7	0.9	1.0
9	0.5	0.6	0.7
10	0.2	0.3	0.3

Energy diets for adult insects and experimental design

The experimental design used was completely randomised. To evaluate the effects of the diets in relation to the survival of the adult male insects of *Ae. aegypti*, six treatments were used, namely: sucrose in a concentration of 10%, as a positive control (sack10); starvation, as a negative control (starvation); sucrose at a concentration of 20% associated with 1 g/l of ascorbic acid (sac20vitC); wild honey from *Apis mellifera* in a concentration of 10% (honey10); demerara sugar in a 10% concentration (demerara10); sucrose at a concentration of 20% associated with 1 g/l of ascorbic acid; and 0.5 g/l of amino acid proline (sac20vitCPr). Each treatment had 16 repetitions (cages) containing 50 adult males.

For the flight ability and copulation propensity tests, five treatments were used (saca10; sac20vitC; mel10; demerara10; and sac20vitCPr). However, for the flight ability tests, three replicates (flight ability device) containing 100 males each were used.

Adult survival

To evaluate insect survival, groups of 50 male pupae were placed in plastic pots (7.5 cm in height × 12 cm in diameter) fitted with voal pot lids. After 2 days, the pots were drained and cotton soaked with each diet was placed on top of the pot to feed the mosquitoes. Since their emergence, the insects were maintained under *ad libitum* feeding with their respective diets, receiving food for 4 days.

On the fifth day, the diets were withdrawn and the insects from each treatment were divided (eight replications) into two scenarios: (1) supply of water only and (2) total starvation, without access to any nutrients, to simulate the days in which that would receive diet in the BMB, and possible difficulties in locating natural sources of food, after release. The insects were kept in rooms under controlled conditions of temperature, relative humidity, and photoperiod for 30 days. The evaluations were measured daily during the morning, the dead adults were removed from the pots with the aid of a brush, being then counted and then discarded. Mortality was evaluated daily, in the morning, for 30 consecutive days, because despite the literature showing that adult insects live an average of 30–35 days,

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those from mass rearing have reduced longevity, reaching up to 20 days, according to Villiard and Gaugler (2015).

Flying ability of adult insects

The mosquitoes' ability to fly was assessed using a device developed by the International Atomic Energy Agency. The evaluation was carried out through the ability of mosquitoes to escape from a series of flight tubes in a period of 2 h (Culbert *et al.*, 2018).

For this evaluation, a sample of 100 adult males per device was used, with each treatment being replicated three times. Insects were aspirated and placed inside the flight tubes through a small 1 cm diameter hole located at the bottom. To encourage mosquitoes to escape from the base of the device, a mosquito attractant (BG-Lure Biogents®; 003-DS-RAC007, France) was placed in the upper outer part of the containment cage, directly below a 12 V fan with a speed of 6000 rotations per minute, capable of generating an air flow of 11.9 m^3/h.

After 2 h, the fan was switched off and the experiment was considered as completed. Mosquitoes that successfully escaped into the containment cage and those that remained in or under the tubes were aspirated and cold immobilised for later counting. The escape rate was obtained by dividing the number of mosquitoes that escaped (contained in the containment tube) by the total number of males inserted in the device.

Propensity to copulation

The propensity to copulate was developed according to Balestrino *et al.* (2017). This analysis was determined through the number of virgin females that a single male after 4 days post-treatment, with the respective energy diets, could successfully inseminate during a period of 15 h (two twilight periods). For this, a single virgin male (about 96 h after emergence) from each treatment was transferred to a plastic cage ($30 \times 30 \times 30$ cm^3) with ten virgin females of the same age. Six replications of ten observational units (female) were used for each treatment.

Subsequently, each female was dissected using a stereomicroscope and the three spermathecae were removed to verify the presence or absence of spermatozoa.

Statistical analyses

The results obtained were analysed using Statistical Analysis System software, previously checking the normality of the residues and the homogeneity of the variances using the Shapiro–Wilk test using the PROC UNIVARIATE procedure. The data were subjected to analyses of variance and when a significant difference was detected ($P < 0.05$), the means of the treatments were compared using Tukey's test. The statistical model of the applied design is as follows:

$$y_{ij} = m + t_i + e_{ij} \quad (1)$$

where y_{ij} is the observed value of the characteristic studied, in treatment i ($i = 1, 2,\ldots, I$) and in repetition j ($j = 1, 2,\ldots, J$); m is the overall mean (of all observations) of the experiment; t_i is the effect of treatment i; e_{ij} is the error associated with the y_{ij} observation or the effect of uncontrolled factors on the y_{ij} observation.

Results

Survival of adult insects

Survival rates for 30 days of insects subjected to experimental diets are shown in figs 1 and 2. Figure 1 represents scenario 1, in which insects, from the fifth day onwards, were provided

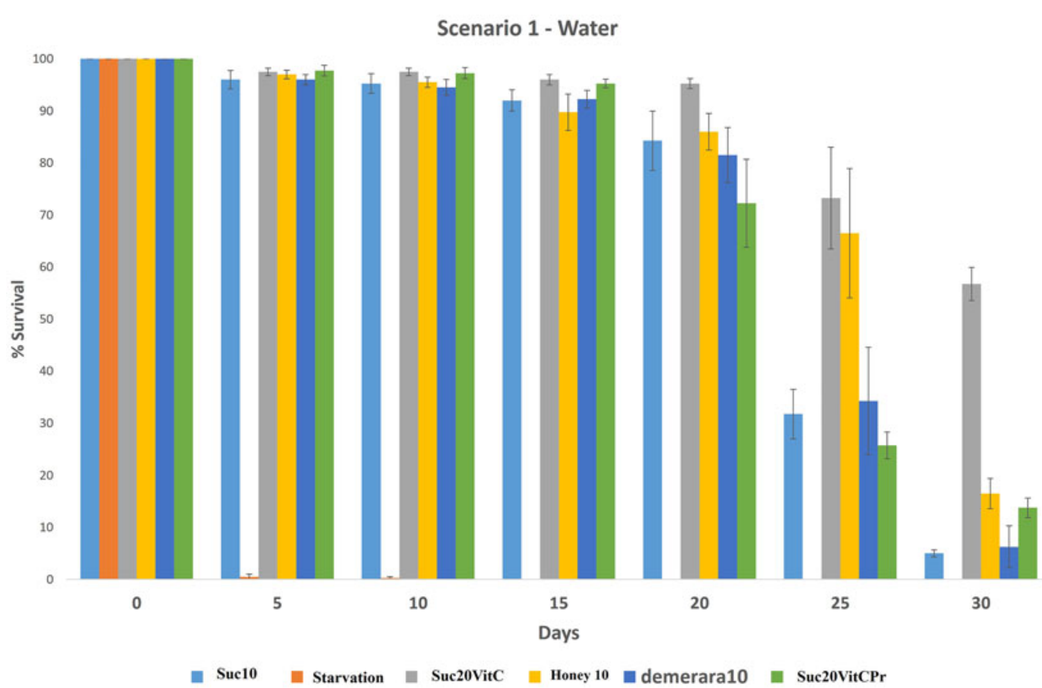

Figure 1. Average survival rate of *Ae. aegypti* adults subjected to different feeding regimes and from day 5, fed with water (scenario 1). Different letters represent statistically significant differences in means at the $P < 0.05$ level. Uppercase letter compares treatment within the day and lowercase letter compares treatments across days.

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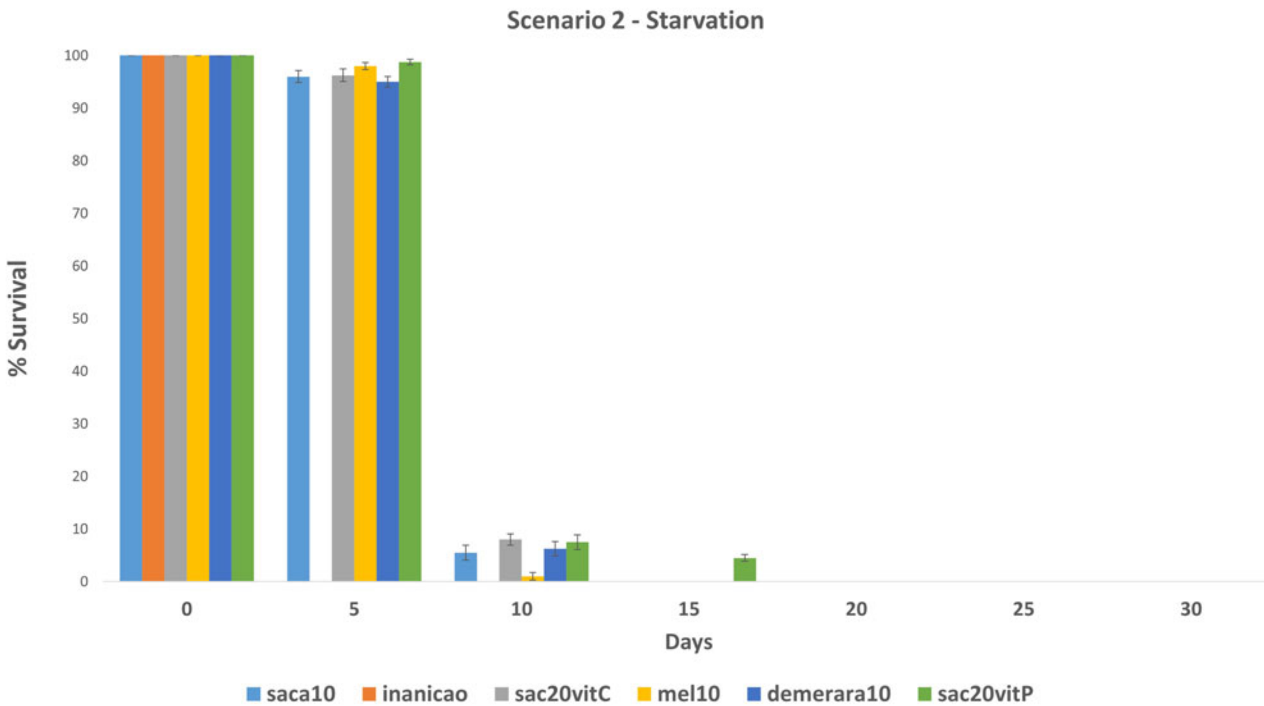

Figure 2. Average survival rate of *Ae. aegypti* adults subjected to different feeding regimes and from day 5, starvation (scenario 2). Different letters represent statistically significant differences in means at the $P < 0.05$ level. Uppercase letter compares treatment within the day and lowercase letter compares treatments across days.

with water *ad libitum*. Figure 2 represents scenario 2, in which the insects are subjected to total starvation from the fifth day until the end of the tests. Regardless of the experimental diet or scenario evaluated, no immediate mortality was observed. From the fifth day on, the starvation treatment showed almost total mortality (fig. 1), with only 0.5% of live insects remaining, and showed total mortality in scenario 2 (fig. 2).

In scenario 1, on the fifth and tenth days, the average insect survival among the experimental diets was between 96.8 and 96.0%, respectively, with the exception of the starvation treatment. On the 15th day, mean survival was 93.9%, with the exception of the honey diet10 and starvation, which were 89.8 and 0%, respectively. On day 20, the highest insect survival was possible with the sac20vitC diet with 95.2%, followed by sac10, honey10, and demerara10 diets, which showed an average survival rate of 79.3%. The lowest insect survival rate was obtained with the sac20VitCPr diet with 72.2% of live insects. Between days 25 and 30, the best survival rate was observed for the group of insects that received the sac20vitC diet, with values of 73.2 and 56.7%, respectively (fig. 1).

Comparing the treatments during the observation period (fig. 1), it can be noted that the survival rate of the treatment composed of 20% sucrose associated with ascorbic acid, reduced significantly from day 20, gradually reducing its values until day 30. The other treatments showed a reduction on day 5 (starvation), on day 10 (honey10 and demerara 10), and on day 15 (sac10 and sac20vitCPr).

In scenario 2, the average survival among the experimental diets was 96.8% on day 5, except for the starvation treatment, which no longer contained any live insects. On the tenth day of evaluation, the diet with 20% sucrose associated with ascorbic acid or also with proline had higher average survival rates (7.8%), followed by diets with 10% sucrose and 10% demerara (5.9%). The diet that showed the lowest insect survival rate within day 10 was composed of honey at a concentration of 10%, with only 1% of insects surviving. On day 15, only insects previously fed with the diet containing 20% sucrose with ascorbic acid and proline had a survival rate (4.5%) (fig. 2).

Comparing the diets between days in scenario 2 (fig. 2), it can be noted that the survival rate of insects that received the diet containing 10% honey reduced significantly from the fifth day of evaluation, while for the other diets, insect survival rates were reduced from day 10, except for the starvation treatment.

Flying ability of adult insects

The escape rates of males fed with different diets differed between treatments and varied between $69.8 \pm 0.58\%$ (sac10) and $80.3 \pm 0.78\%$ (sac20vitC) ($P < 0.001$; fig. 3). Insects fed after emergence with a diet composed of sucrose at a concentration of 20% associated with ascorbic acid showed better flight ability, achieving an average value of an escape rate of $80.3 \pm 0.78\%$, with an escape rate like the treatment that used 20% sucrose associated with ascorbic acid and incremented with the amino acid proline, which showed an escape rate of $76.5 \pm 0.57\%$ ($P < 0.001$). A 10% sucrose-based diet provided the insects with the lowest flight ability, with an average escape rate of $69.8 \pm 0.58\%$ ($P < 0.001$; fig. 3).

Propensity to copulation

The mean rates of insemination of females that copulated with males fed with different diets showed differences between the tested diets ($P < 0.001$; fig. 4). The diet composed of sucrose at a concentration of 20% associated with ascorbic acid and proline showed the best propensity for copulation, with an average insemination rate of $63.33 \pm 2.11\%$ (fig. 4). However, its insemination rate was similar to the diet based on 20% sucrose associated

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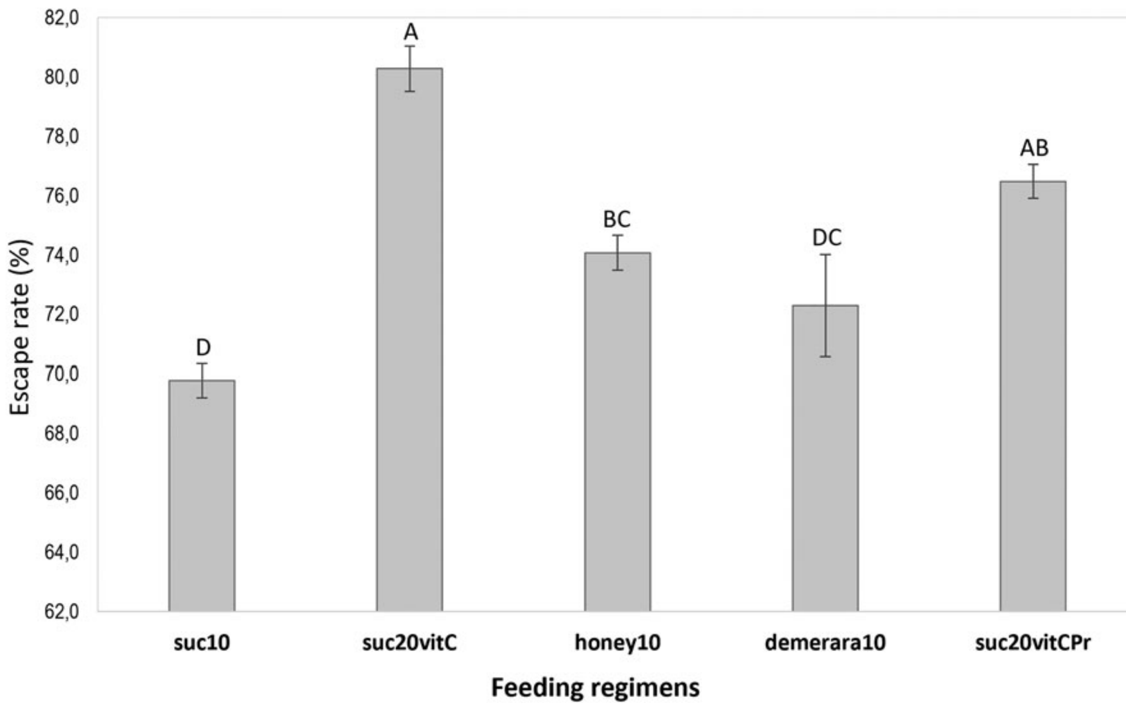

Figure 3. Mean escape rate of *Ae. aegypti* adults subjected to different feeding regimes: sac10 (sucrose 10%); sac20vitC (20% sucrose + ascorbic acid); honey10 (honey 10%); demerara10 (demerara sugar 10%); and sac20vitCPr (20% sucrose + ascorbic acid + proline). Different letters represent statistically significant differences in means at the $P < 0.05$ level.

with ascorbic acid and the diet composed of 10% demerara sugar, with values of 55 ± 3.42 and 53.33 ± 2.10%, respectively ($P > 0.05$; fig. 4).

Insects fed with honey at a concentration of 10% showed the worst propensity to copulate, exhibiting an average insemination rate of 41.67 ± 3.07%, so that its rate was equal to the composite control diet by 10% sucrose (44.98 ± 1.30%) ($P > 0.05$; fig. 4).

Discussion

Few studies that contemplate the use of different sources of sugars for adult males of *Ae. aegypti* reared under mass conditions are found in the literature. However, research reports the importance of using, shortly after emergence and before release into the environment, an energy diet for artificially reared males, since, during this period, processes that require a significant amount of energy are initiated, such as dispersing, compete with wild males, copulate, and seek shelter (Balestrino *et al.*, 2017; Barredo and Degennaro, 2020; van Schoor *et al.*, 2020).

Among all the parameters evaluated in this study (survival, flight ability, and propensity to copulate), the diet composed only of sucrose was inferior to the other sugary foods tested, including honey at a concentration of 10%. Contrary to our results, Bellini *et al.* (2014) verified that the supply of sucrose

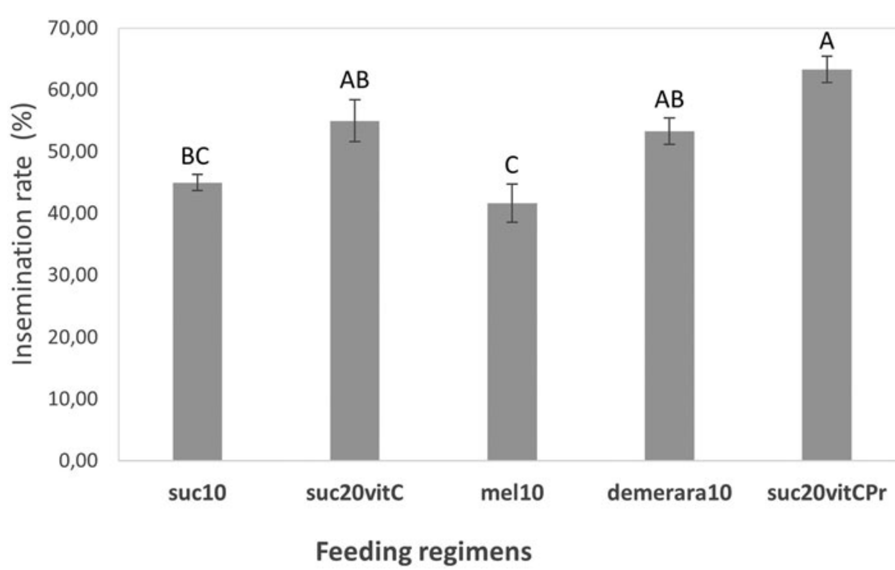

Figure 4. Average insemination rate of *Ae. aegypti* adults subjected to different feeding regimens: sac10 (sucrose 10%); sac20vitC (20% sucrose + ascorbic acid); honey10 (honey 10%); demerara10 (demerara sugar 10%); and sac20vitCPr (20% sucrose + ascorbic acid + proline). Different letters represent statistically significant differences in means at the $P < 0.05$ level.

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increases the probability of survival and sexual performance of *Aedes albopictus* insects, defining that sugar is the fuel for the effectiveness of survival, dispersion, and copulation of insects used in SIT.

Thus, after the release of sterile males, the duration of adult survival will likely influence the number of mating opportunities and the area over which sterile males disperse (Bond *et al.*, 2019). Therefore, it is extremely important that the male is offered a diet that favours these actions after release into the environment (Conway *et al.*, 2023), since in this habitat, survival will probably be lower than that under laboratory conditions, as in this environment insects find favourable conditions for their survival: controlled temperature and humidity conditions (Moura *et al.*, 2021), protection against predators, and *ad libitum* food supply (Bond *et al.*, 2019).

In this sense, it was observed in scenario 1 (supply of water *ad libitum*) that the best survival rates, in relation to the diets and between the evaluation days, was the diet composed of 20% sucrose associated with ascorbic acid, which, surprisingly, had a survival rate greater than 50% at 30 days of evaluation. However, the treatment with the lowest survival rates, as expected, was starvation (negative control). Regarding scenario 2, the diets that provided better survival rates were composed of 20% sucrose associated with ascorbic acid and the treatment in which proline was used, comparing the diets within days, until the tenth day of evaluation. However, the diet to which proline was added remained with a small percentage of live insects (4.5%) until day 15. Therefore, the findings of the present study refine our understanding of the survival of male adults of *Ae. aegypti*.

The results demonstrate the importance of using an energy diet in the adult phase, soon after its emergence, as well as doubling the sucrose concentration used as a standard in the mass rearing of this insect and associating it with ascorbic acid, which possibly acted as an antioxidant, proving to be an effective and low-cost alternative. Thus, on the 30th day of evaluation in scenario 1, the control treatment (diet containing 10% sucrose) represented only 8.8% of the survival rate of the diet containing 20% sucrose associated with ascorbic acid, that is, this treatment increased the survival of adult insects by 51.7% during this period.

In this context, it is important to note that reactive oxygen species (ROS) are present in the intestinal epithelium of sugar-fed mosquitoes (Bombaça *et al.*, 2021). However, in females after the blood meal, ROS decrease drastically, due to the mechanisms involved in the degradation of haemoglobin which, within the intestine of these insects, releases large amounts of the haem group, which has potential pro-oxidant and cytotoxic effects when not bound to proteins (Oliveira *et al.*, 2011; Bottino-Rojas *et al.*, 2019). Thus, to control these deleterious effects, some haematophagous organisms present in the intestine of these insects, decrease the generation of ROS by changing the energy metabolism to a system based on glycolysis in an anaerobic way to avoid oxidative stress (Ferreira *et al.*, 2018; Chen *et al.*, 2021). However, this effect seems not only to affect the gut, but also may constitute a systemic trend, as respiration and H_2O_2 generation in the flight muscle mitochondria of female *Aedes* are also reduced after a blood meal (Noce *et al.*, 2019).

Insect flight is accomplished by the contractile activity of the flight muscle, which has an extremely high-energy demand and represents one of the most metabolically active tissues found in nature (Fu *et al.*, 2022). Mitochondria present in the flight muscle of *Ae. aegypti* play a central role in energy metabolism, interconnecting nutrient oxidation to adenosine triphosphate (ATP) synthesis, but also represent an important site of cellular superoxide production, through respiration mediated by the oxidation of the amino acid proline (Soares *et al.*, 2015). Therefore, in the diet containing this important amino acid to produce ATP, as well as in the diet in which the sucrose concentration was doubled, ascorbic acid was added, as a possible antioxidant.

The insect's flight ability is known to be a direct and reliable marker of insect quality, since it is sensitive to the effects of different treatments that may interfere with the quality of the mass-produced male (Culbert *et al.*, 2018). The male flight ability methodology used in the present experiment, according to the creators of the device used, provides a valuable method to detect and correct inadequate procedures, contributing to maintain the optimal quality and field performance of released mosquitoes (Balestrino *et al.*, 2017). In this sense, the diet that showed the least effectiveness for the flight capacity of insects was the control diet, composed of sucrose at a concentration of 10%. The diet composed of sucrose at a concentration of 20% associated with ascorbic acid showed a higher percentage of males that managed to escape the tubes of the device after 2 h of evaluation, triggering an improvement of 10.5% in the ability of insects to fly, compared with the control diet. This increase in flight capacity is of great relevance, as it demonstrates the improvement in the quality of the male produced, as a biotechnological product to act in the control of the species' population.

Another parameter evaluated in the current study, and which plays a major role in the evaluation of the male produced is the propensity to copulate. Although male mosquitoes are not directly involved in the transmission of arboviruses, understanding their mating behaviour is of great importance for control methods involving the release of sterile insects. Our findings differ from the responses obtained by Culbert *et al.* (2018) when evaluating the flight ability of *Ae. aegypti* and *Ae. albopictus* insects for 15 days, using the same devices used in this experiment. These authors fed the insects with 10% sucrose and subjected them to copulation conditions for a period of 5 days, obtaining insemination rates above (80%) the rate observed in this study. However, in this experiment the mating period was only 15 h, comprising two twilight periods. This fact may explain the lower insemination rate compared to the study by Culbert *et al.* (2018), as males had a shorter period to mate with females. However, the diet based on 20% sucrose associated with ascorbic acid and proline showed better insemination rates, with rates above 50%. This rate is similar to the values found with diets based on 20% sucrose associated with ascorbic acid and 10% demerara sugar. Thus, the least effective diet for the male propensity to copulate was the diet composed of 10% honey.

The insemination rate assesses male performance; however, the specific behavioural differences that underlie the variation in success are poorly defined. Therefore, the role of female choice and the degree to which it influences mating outcomes are not yet fully elucidated (Aldersley and Cator, 2019). Another preponderant factor to be observed is that male insects are polyandrous, and their reproductive success depends on the number of females they can inseminate before semen depletion (Oliva *et al.*, 2014; Sears *et al.*, 2020). Their accessory glands secrete proteins that are transferred to females during mating and influence their blood-feeding and oviposition-seeking behaviour. Thus, the male's nutritional status may have an indirect effect on the frequency of female blood meals, since the male's diet influences the functioning of his accessory glands (Oliva *et al.*, 2014; Barredo and Degennaro, 2020).

In this context, studies recommend that when implementing SIT, retain adult males for 3 days after emergence and before release, feeding them with sources of sugar, to improve their mating success and consequently reduce the population of mosquitoes that transmit dengue, Zika, and Chikungunya arboviruses (Bellini *et al.*, 2014; Fikrig *et al.*, 2020; van Schoor *et al.*, 2020; Swan *et al.*, 2021). Therefore, assessing the quality of the sterile males produced and the impact they may have on immediate survival, sexual performance, and dispersal, play a crucial role in the application of SIT, and the provision of an adequate energy diet before release can trigger an advantage to sterile males in these parameters.

Therefore, the current study demonstrates the importance of evaluating new diets for adult males that can provide better survival, flight ability, and copulation propensity. The tested diets, especially the diet in which the concentration of sucrose associated with ascorbic acid was doubled, were able to significantly improve these parameters.

Under experimental conditions, the diet composed of sucrose at a concentration of 20% associated with ascorbic acid improves survival, flight ability, and propensity to copulate in *Ae. aegypti* males reared under mass conditions and may be useful to enhance performance of sterile males, thus improving the success of SIT programmes.

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Competing interest. None.

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