Large scale artificial rearing of *Anastrepha* sp.1 *aff. fraterculus* (Diptera: Tephritidae) in Brazil

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ABSTRACT: Some species of the genus *Anastrepha* (Diptera: Tephritidae) are successfully managed by matching the sterile insect technique with parasitoid releases. Such strategies used in integrated pest management can be implemented only where insect mass-rearing programs are feasible. In this study, we show the process of domestication, rearing technology and quality control data obtained from 54 generations of *Anastrepha* sp.1 *aff. fraterculus* (Wiedemann, 1830) kept under fully artificial conditions. Eggs were collected by an artificial oviposition panel consisting of one side of the cage made of blue voile fabric externally covered with a thin layer of silicon rubber. They were then air-bubbled in water at 25 °C for 48 h before seeding. Larvae were reared on the regular laboratory artificial diet with 66 % of agar reduction turning over a semi-liquid diet, which reduced costs and improved insect quality. The adult and larval diets were composed of local ingredients including hydrolyzed yeast. When large-scale production of this fly is contemplated, the critical stage is larval development. This system of artificial rearing for *A. fraterculus* sp.1 developed in Brazil, allows for the production of a large number of insects of excellent quality using local ingredients and less agar in diet composition than the original medium used for this species. By reducing the interval of egg collection, the system might be optimized in terms of insect yield and, therefore, meet the demands of *A. fraterculus* sp.1 with regard to integrated pest management purposes.

Keywords: insect production, insect domestication, sterile insect technique, integrated pest management, fruit fly

Introduction

Integrated Pest Management (IPM) programs targeted at fruit pests encourage biological control techniques, reduction of chemical insecticide use and increases in natural enemy populations. Besides biological control, the Sterile Insect Technique (SIT) is another strategy that meets the food safety and quarantine requirements of the international fruit trade. It entails routine releases of sterile insects over a wide area where they will mate with females in the wild and consequently give rise to a gradual decrease in the pest population (Knipling, 1955). Fruit flies count amongst the major pests in agriculture worldwide because they directly affect yield by causing damage to fruit. Furthermore, quarantine restrictions are placed on the international trading of fruit from areas where fruit flies are present, which can have serious negative impacts on a country's economy. Two strategies used worldwide in IPM fruit fly programs are Augmentative Biological Control (ABC) with parasitoids and SIT, which are complementary and work together quite efficiently (Knipling, 1992). To improve SIT, mass rearing of both target fly and parasitoids are required. For insect mass-rearing, procedures are needed to foster high production of high quantity offspring at the lowest cost. In the genus *Anastrepha*, *A. obliqua* and *A. ludens* in Mexico (Montoya et al., 2000), *A. ludens* in the USA (USDA, 2010) and *A. suspensa* in the USA (Sivinski et al., 1996) have been successfully man-

aged with SIT or ABC. Some studies testing those strategies for *A. fraterculus* control have also been conducted in Argentina (Allinghi et al., 2007; Gomez-Cendra et al., 2007; Ovruski et al., 2011; Rull et al., 2012; Segura et al., 2009; Vera et al., 2007).

In Brazil, *A. fraterculus* is the most important species among *Anastrepha* spp. in several fruit crops (Malavasi et al., 2000). This study aimed at developing a method of large-scale rearing for *A. fraterculus* with the lowest cost, which is a major step for implementing of integrated management programs using ABC and SIT. The study also presents production and quality control data spanning 54 generations of mass-rearing.

Materials and Methods

Domestication of *A. fraterculus* sp.1

Parental flies were collected from infested indigenous fruits (*Eugenia pyriformis* Cambess.), from Piracicaba (22°42′ S; 47°47′ W; 503 m), in the state of São Paulo, Brazil, and were kept in mesh-covered containers on vermiculite until all larvae had hopped off of the fruit and entered the pupal stage. Pupae were sieved from vermiculite, put into a small screened cage for emergence, and kept in a climate-controlled room (26 ± 2 °C); relative humidity (RH) of 70-80 %, and 14 h of photophase at approximately 12,000 lux.

Adults (262 females and 250 males) were transferred to screened cages (30 × 30 × 30 cm) where they

were given water (moist cotton), food (hydrolyzed yeast *Saccharomyces cerevisiae* and caster sugar at v : v ratio of 1 : 3 respectively), and one papaya fruit (*Carica papaya*) weighing about 350 g for natural oviposition. To assure there had been no previous fruit flies infestation fresh mature green papaya fruits were obtained from the local market (Seo and Tang, 1982).

To start the colony off, the first generation was reared only on fruit, from which 100 pupae were obtained (81 % adult eclosion; 42 females, 39 males). For the second generation, besides papaya for oviposition, the cage had also one side made of blue voile fabric that was externally covered with a thin layer of silicon rubber (< 0.5-mm thick), which served as an oviposition panel. Papaya fruits were replaced every other day and set onto a thin layer of vermiculite on plastic trays where they remained for 13 days to facilitate larval development. Third instar larvae were removed from the rotten papayas and kept for eight days in vermiculite for pupation.

Pupae were sieved and put in a new cage to begin the next generation of the colony. The small number of eggs that were laid on the oviposition panel were carefully removed from the outer surface with a soft brush and placed on wet filter paper inside Petri dishes and kept at 25 $\pm$ 1 °C for 72 h. The newly-hatched larvae were transferred with a soft brush to the regular artificial diet (50 larvae per 10 mL of diet) where they remained for the whole larval development period (Salles, 1992). Adults originating from these eggs were maintained in cages without papaya to artificially select for females to oviposit through the oviposition panel.

By using papaya as an oviposition and a larval substrate, the first generations reared in the laboratory were guaranteed and no additional collections of infested fruit in the field were needed as in the case of high insect mortality or poor oviposition under artificial conditions. Thus, throughout the domestication period of five generations, two colonies of *A. fraterculus* sp.1 were kept; one was reared directly on papaya fruit, and the other included artificial oviposition and larval substrates.

Artificial rearing of *A. fraterculus* sp.1

From the sixth generation onwards, *A. fraterculus* sp.1 rearing was conducted using artificial conditions exclusively. Several specimens of adults, larvae and eggs were identified. Morphology of the aculeus and eggs as well as larva chromosomal analysis confirmed it to be a pure colony of *Anastrepha* sp.1 *aff. fraterculus* (Araújo and Zucchi, 2006; Selivon et al., 2005).

Adults were kept in cages (30 × 50 × 100 cm) with bottom and corners made of aluminum, and front and back of acrylic; top and one side of screen for ventilation, and the remaining side (50 × 100 cm) an oviposition panel made of blue voile fabric that was externally covered with a thin layer of silicon rubber (< 0.5-mm thick). At the top of the cage, there was a food station shaped in the form of a half-cut pipe (diameter of 10 cm). In generations 6-16 adults were fed the same diet as de-

scribed earlier for domestication. From the 17th generation onwards, raw wheat germ was added into the diet to improve egg hatch (Vera, M.T. personal communication) at the v:v ratio of one part of raw wheat germ : one part hydrolyzed yeast : three parts sugar.

In the 26th generation the imported yeast was replaced by a local one, Bionis YE MF® (Morelli et al., 2012; Silva Neto et al., 2012). Below the food station there was also a water supply (deionized water and sodium benzoate solution at 0.03 % to avoid fungal growth), which was closed but had a single longitudinal gap at the top through which a filter paper allowed access to water. Each cage held 70 mL of pupae, which corresponded to approximately 1,960 adults. Adults were held under controlled conditions (26 $\pm$ 2 °C; 70-80 % RH, 14 h of photophase at about 12,000 lux).

A moistened spongecloth was attached to a transparent PVC cover and placed outside in parallel position to the oviposition panel to prevent egg desiccation. Ovipositing females were attracted to the panel by fluorescent lamps placed behind it. They introduced the ovipositor through the fabric mesh leaving the eggs trapped on the outer surface of the voile fabric. Eggs were removed by applying a gentle spray of water onto the panel and gathering them with a tray placed under the cage every 24 h for 15-20 days (Vera et al., 2007) and, thereafter, the adults were discarded.

The eggs were washed with filtered water solution of sodium benzoate at 0.03 % for five minutes to reduce microbial contamination of the artificial diet. After measuring the volume of eggs collected (1 mL contains 11,700 eggs), they were transferred to an Erlenmeyer flask (500 mL) for air bubbling in filtered water (v:v ratio of 1 mL of eggs: 20 mL of water) at 25 °C for 48 h before seeding.

Up to the 10th generation, larvae were reared on the diet promulgated by Salles (1992) as follows: sugar (6.0 %), wheat germ (6.0 %), brewer's yeast (6.0 %), sodium benzoate (0.1 %), methylparaben (0.8 %), hydrochloric acid (0.6 %), agar (1.0 %) and water (q.s.). From this generation onwards, the diet was modified by reducing the agar concentration. The 11th generation was selected for defining the appropriate agar concentration for mass-rearing diets with lower concentrations of agar (0.30, 0.32, 0.34, 0.36, 0.40 and 0.70 %) which were then compared with that of Salles (1992) with regard to quality parameters to be applied to the flies. Each treatment was replicated ten times. The eggs were bubbled in water as described above, and samples of 0.1 mL were seeded on 200 mL of diet placed in plastic pots (500 mL).

The pots were covered with perforated lids and kept in a climate-controlled room (24 $\pm$ 2 °C, 70 % RH) for 10-12 days until larvae reached late third instar. Larvae were collected by dissolving and washing the diet under water on a sieve. They were then placed on thin and moist vermiculite (v:v ratio of 1:2) in plastic trays for pupation. These trays were kept in dark rooms (24 $\pm$ 2 °C, 65-75 % RH) for eight days. Pupae were then softly sieved and counted. To estimate the quality parameters,

two samples of 100 pupae (eight days old) were taken from each replicate: one sample was used to assess pupal weight and flight ability according to standard protocols (FAO, 2003), and the other sample was used to determine emergence rate and sex ratio.

Egg-adult period was considered to be the number of days between egg collection and the date of emergence; emergence rate was estimated as (number of emerged flies) 100^{-1}; the sex ratio as (number of females) (number of females + males)$^{-1}$; flight ability as (number of flying adults) (number of adults emerged)$^{-1}$ and the egg-pupae recovery as (number of eggs seeded) (number of pupae recovered)$^{-1}$. Larval viability was assessed by inoculating 200 newly hatched larvae in each diet pot. Late third instar larvae were separated from the diets and placed on vermiculite for pupation as described above. Recovered pupae were counted and larval viability was estimated as (the number of larvae inoculated) (number of pupae recovered)$^{-1}$.

With regard to the rearing procedures, one to three kilograms of larval diet (0.34 % of agar) were placed in plastic trays (35 × 25 × 7 cm) and the bubbled eggs were seeded on larval diet at 1:100 volume ratio of eggs: diet. The trays were covered with crimp fabric and kept in a climate-controlled room (24 ± 2 °C, 70 % RH) for about 10-12 days for larvae to achieve the late third instar, which was characterized by larval orientation and location on the diet. When the majority of larvae (about 80-90 %) were moving horizontally on the diet, it indicated that they had reached the end of the third instar, had stopped feeding, and were crawling around to find suitable sites for pupation.

Late third instar larvae were separated by sieving the diet under water. The volume collected was recorded, and the larvae were placed on a thin layer of moist vermiculite (at 1:2 volume ratio of larvae:vermiculite) in plastic trays for pupation, which were kept in dark rooms (24 ± 2 °C, 65-75 % RH) for eight days. Pupae were then softly sieved and kept under the same conditions for at least 24 hours before placement in the adult holding cages.

Quality control parameters

Several parameters related to rearing and quality of *A. fraterculus* sp.1 were assessed for each egg collection, such as eggs collected (mL), eggs hatched, pupae collected (mL), larval viability, pupal weight (mg), egg-pupae recovery, emergence, sex ratio, egg-adult period (days). For egg hatch, a sample of 100 eggs was collected after the bubbling period and placed on a black filter moist paper inside a Petri dish for incubation. After two days, the number of hatchings was counted, and the egg hatch rate was estimated based on (number of eggs hatched) (total eggs in the sample)$^{-1}$.

Data analysis

A total of 54 insect generations were reared to maturity under laboratory conditions in the period between November 2005 and August 2012, 53 of which were under entirely artificial conditions. Means and standard errors were estimated using the statistical package R version 2.15.1. The optimal agar concentration in larval diet was determined using generalized linear models (GLMs). Emergence and flight ability, egg-pupae recovery and larval viability data were submitted to logistic regression models adjusted for over-dispersion (Crawley, 2007). Afterwards, rank means were pair-wised compared by non-parametric t-test and α adjustment by Bonferroni's correction. The weight of the 100 pupae and egg-adult period data were analyzed by linear regression and means compared by Tukey test (α = 0.01). All statistical analyses were run using the R program version 2.10.1.

Results

Among the agar concentrations assessed, the 0.34 % level resulted in better values over almost all the parameters evaluated (Table 1). The decrease from 1 to 0.34 % of agar improved pupal weight by 5 % ($F_{(7, 72)}$ = 8.96; $p < 0.001$), flight ability by 25 % ($F_{(7, 72)}$ = 18.09; $p < 0.001$) and egg-pupae recovery by 31 % ($F_{(7, 72)}$ = 18.82, $p < 0.001$). Larval viability (81 %) and emergence (81 %) rates did not vary with agar concentrations in the diet ($F_{(7, 40)}$ = 0.86, p = 0.55 and $F_{(7, 72)}$ = 1.66, p = 0.13 respectively). Egg-adult period was influenced by the agar concentration ($F_{(7, 72)}$ = 3.89; p = 0.0012); however, the values obtained at 1 and 0.34 % agar did not differ (27 days).

The volume of eggs collected varied greatly over the 54 generations of *A. fraterculus* sp.1 reared under artificial conditions (Figure 1) with an average of 102 ± 13.03 mL (n = 53) per generation and 5.03 ± 0.10 mL per day (Table 2). The volume of pupae produced reflected the volume of eggs inoculated, which varied according to the demand for insects. Maximum volumes were 591.15 mL of viable pupae per day and 18.10 L of eggs per generation, and means values were 296.52 ± 6.46 mL (n = 1,155) and 5.54 ± 0.69 L (n = 53) respectively. Egg hatch rate increased in two intervals: the first between the 13th and the 18th generations and the second between the 25th and the 30th generations (Table 2). Values above 75 % were achieved only after the 43rd generation.

Larval viability had a drastic decrease between the 25th and the 30th generations but over the next six generations (31st to 36th) a slight improvement in larval viability was detected, and from the 37th generation onwards, larval viability again reached values close to the highest peak over the period. However, the average value of 0.29 was too low and must be increased. Pupal weight and emergence rate had averages of 14.26 mg pupa^{-1} and 85 % respectively (Table 2). Sex ratio averaged 0.53 female biased. Egg-pupae recovery was low, only 0.17 of the embryos reached the pupal stage. The egg-adult period lasted from 28 to 31 days, averaging 30 days.

Table 1 – Quality parameters of *Anastrepha* sp.1 *aff. fraterculus* (mean ± SE) when larvae were reared on artificial diets with concentrations of agar.

Agar concentration	Pupal weight	Larval viability	Emergence	Flight ability	Egg-adult period	Egg-pupae recovery
%	mg	rate[1]	rate	rate	days	rate
0.30	15.3 ± 0.2 b	0.77 ± 0.04 a	0.79 ± 0.02 a	0.52 ± 0.03 de	27.6 ± 0.16 a	0.14 ± 0.02 f
0.32	15.5 ± 0.1 ab	0.80 ± 0.03 a	0.79 ± 0.02 a	0.64 ± 0.01 bc	27.4 ± 0.22 a	0.39 ± 0.04 cde
0.34	16.1 ± 0.1 a	0.83 ± 0.02 a	0.85 ± 0.01 a	0.74 ± 0.02 a	27.2 ± 0.33 a	0.63 ± 0.02 a
0.36	16.1 ± 0.1 a	0.82 ± 0.05 a	0.82 ± 0.02 a	0.69 ± 0.02 ab	27.1 ± 0.38 a	0.53 ± 0.02 b
0.38	15.8 ± 0.1 ab	0.84 ± 0.03 a	0.80 ± 0.02 a	0.58 ± 0.02 cd	27.5 ± 0.17 a	0.28 ± 0.05 ef
0.40	15.2 ± 0.2 b	0.79 ± 0.04 a	0.79 ± 0.02 a	0.57 ± 0.03 cd	27.3 ± 0.30 a	0.32 ± 0.05 de
0.70	14.7 ± 0.2 b	0.85 ± 0.03 a	0.80 ± 0.02 a	0.46 ± 0.02 e	25.9 ± 0.23 b	0.43 ± 0.03 bcd
1.0	15.3 ± 0.1 b	0.81 ± 0.01 a	0.84 ± 0.01 a	0.55 ± 0.02 de	27.3 ± 0.30 a	0.48 ± 0.04 bc

Means followed by the same letter in columns do not differ ($p < 0.01$); [1]n = 6 replicates; the remaining parameters had n = 10.

Table 2 – Rearing and quality control parameters (mean ± SE) of *Anastrepha* sp.1 *aff. fraterculus* produced under artificial conditions.

Generations	Daily volume of eggs	Egg hatch	Larval viability	Daily volume of pupae	Pupal weight	Emergence	Sex ratio	Egg-pupae recovery	Egg-adult period
	mL	rate	rate	mL	mg	rate	rate	rate	days
2nd to 6th[a]	0.04 ± 0.00	0.28 ± 0.01	0.45 ± 0.04	10.04 ± 2.72	-	0.51 ± 0.04	0.48 ± 0.02	0.14 ± 0.01	28.45 ± 0.54
7th to 12th	0.42 ± 0.05	0.15 ± 0.01	0.41 ± 0.04	62.19 ± 7.95	-	0.70 ± 0.03	0.48 ± 0.02	0.06 ± 0.01	29.23 ± 0.40
13th to 18th	4.58 ± 0.61	0.35 ± 0.02	0.39 ± 0.03	216.22 ± 39.95	-	0.87 ± 0.02	0.51 ± 0.01	0.12 ± 0.01	28.65 ± 0.34
19th to 24th	5.62 ± 0.23	0.49 ± 0.01	0.34 ± 0.02	291.71 ± 16.36	13.91 ± 0.15	0.85 ± 0.01	0.52 ± 0.01	0.15 ± 0.01	30.75 ± 0.18
25th to 30th	5.61 ± 0.22	0.72 ± 0.01	0.15 ± 0.01	208.32 ± 11.14	14.63 ± 0.12	0.82 ± 0.01	0.55 ± 0.00	0.11 ± 0.01	30.65 ± 0.15
31st to 36th	6.82 ± 0.23	0.73 ± 0.00	0.22 ± 0.01	397.11 ± 11.37	14.42 ± 0.09	0.90 ± 0.00	0.52 ± 0.00	0.16 ± 0.00	30.52 ± 0.08
37th to 42nd	3.84 ± 0.12	0.72 ± 0.01	0.38 ± 0.01	405.68 ± 14.18	13.53 ± 0.05	0.86 ± 0.00	0.54 ± 0.00	0.30 ± 0.01	28.43 ± 0.22
43rd to 48th	4.04 ± 0.15	0.76 ± 0.01	0.35 ± 0.01	377.26 ± 12.87	14.49 ± 0.06	0.87 ± 0.01	0.56 ± 0.00	0.27 ± 0.01	29.66 ± 0.16
49th to 54th	5.43 ± 0.20	0.80 ± 0.01	0.29 ± 0.01	454.60 ± 26.24	14.30 ± 0.08	0.89 ± 0.01	0.55 ± 0.01	0.23 ± 0.01	30.61 ± 0.17
Mean[b]	5.03 ± 0.1	0.64 ± 0.01	0.29 ± 0.01	317.55 ± 6.48	14.26 ± 0.04	0.85 ± 0.003	0.53 ± 0.002	0.17 ± 0.003	30.04 ± 0.07

-Data not available; [a]Domestication period (criteria used to group the generations in intervals); [b]Excludes domestication period, N = 1076 egg collections.
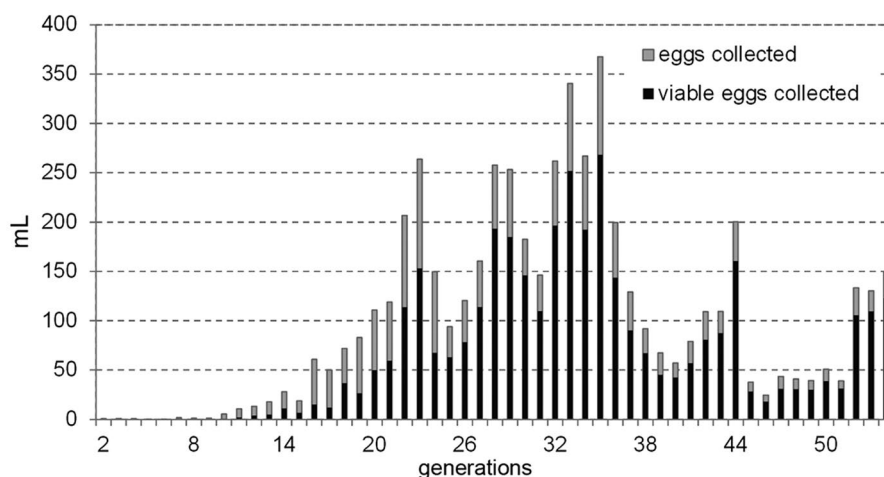
Figure 1 – Total volume of eggs per generation of *Anastrepha* sp.1 aff. *fraterculus* collected under artificial conditions.

Discussion

The volume of insects produced is an important parameter in this study, since it provides a measurement of the *A. fraterculus* sp.1 production in the present facilities. However, it is important to consider both production and the quality parameters for the insects. Egg hatch, egg-pupae recovery, larval viability, pupal weight, emergence, and sex ratio reflect the quality of the insects produced and production efficiency at different stages of insect development.

Egg hatch is a parameter that requires improvement. The addition of raw wheat germ (*Triticum aestivum* L.) in adult diet and the change in the protein source from imported to local hydrolyzed yeast (Morelli et al., 2012), as well as the bubbling of eggs before seeding, increased egg hatch in the present methodology that can be improved further by more effectively preventing egg desiccation. Vera et al. (2007) reported egg hatch rates above 80 % when the relative humidity in the room containing adult cages was controlled with a humidifier. Alternatives that retain moisture on the oviposition panel are also promising, such as Fuceleron™ gel which has been used for *Anastrepha* spp. mass-rearing in Mexico (Flores et al., 2012).

The drastic decrease in larval viability between the 25th and 30th generations was due to a batch of wheat germ that killed the larvae, which was replaced once the problem was detected. The use of dietary ingredients from different batches or product suppliers may interfere in larval performance as, for example, in the case of contamination with pesticide residue, which likely occurred. Nevertheless, larval viability rate was also very low, and improvements should be made in diet consistency as well as in handling.

Vera et al. (2007) achieved a value of 67 %, and the main difference between that study and the current one was in the method used to collect larvae. Vera et al. (2007) placed the diet trays on sterilized sand, and once larvae had completed the third instar, they stopped feeding and moved out of the trays by themselves. Thus, multiple larval harvestings from a single diet tray were done on different days. In this study, larvae were removed by washing the diet on a sieve when most larvae had stopped feeding, thus harvesting larvae only once per tray. Since not all larvae had reached late third instar at the time of harvesting (10-20 %), the amount of pupae recovered was lower than with multiple harvestings (Vera et al., 2007), which directly affected larval viability and egg-pupae recovery.

Pupal weight and emergence were considered satisfactory according to standard protocols (FAO, 2003) and above the values obtained by Vera et al. (2007). FAO (2003) does not specify standards for *A. fraterculus*, however, both values are very close to those recommended for *A. obliqua* and *A. suspensa*. The sex ratio also met FAO (2003) standards. So, despite the low larval viability, both the parameters of pupal weight and high adult emergence demonstrate that the diet is nutritionally adequate, because it produces high-quality larvae and pupae, but the physical structure of the diet and handling of larvae can be improved. The current method for collecting the larvae favored the homogenization of insect development in the rearing and seemed to select more vigorous insects, once the pupae quality was higher.

Egg-pupae recovery was low, even though flies had been selected for artificial diet over several generations. Values above 0.4 can be achieved (Jaldo et al., 2001; Vera et al., 2007). The process of larval collection resulted in recovery rates of second and early third-instar larvae around 10-20 %, discarding deformed puparium, and therefore, low pupae recovery. Vera et al. (2007) used a different system for larval collection and achieved values greater than 50 %. Despite the lower yield compared to the system of Vera et al. (2007), our system provides higher-quality insects. Collecting eggs at shorter intervals as suggested here would be an easy alternative to implement for improving the current system of larval collection because it would homogenize larval development and thus increase egg-pupae recovery. Eight-hour intervals between egg collections would be logistically feasible as they coincide with the employees' shifts at fruit fly biofactories, which operate 24 h.

This system of artificial rearing for *A. fraterculus* sp.1, developed in Brazil, allows for the production of a large amount of insects of excellent quality using local ingredients and little agar in the diet composition. By reducing the interval of egg collection, the system might be optimized in terms of insect yield and, therefore, supply the demands of *A. fraterculus* sp.1 for IPM purposes. However, studies that address sterilization dosage and mating competitiveness of the sterile males, as well as mating compatibility between the reared strain and target populations, are recommended to improve further SIT.

Acknowledgements

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