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Research article

Lyophilized artificial diet for rearing the Neotropical *Euschistus heros* (Hemiptera: Pentatomidae)

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Abstract

An artificial diet to mass-rear *Euschistus heros* (F. 1798) (Hemiptera: Pentatomidae) was developed in the laboratory. Biological studies were conducted under controlled conditions of temperature (25 ± 2°C), RH: 60 ± 10%, and photoperiod of 14:10 (L:D) h. Out of 13 diets tested, 2 diets (D9 and D11) were the most suitable. The artificial diets selected had the same composition (green beans, peanuts, sucrose, water, Nipagin, and sorbic acid) except for different antimicrobial agents (D11 has tetracycline, and D9 doesn't). The 68% viability for the egg–adult period of insects reared on these lyophilized artificial diets (LAD) was almost twice as high as the 38% viability obtained with the natural diet. Although adults reared on LAD weighed 17% less than those reared on the natural diet, mean fecundity was higher than on the natural diet (282 eggs/female), reaching 430 eggs/female. The net reproductive rate (Ro) increased over the generations for the diets with lyophilized material and antimicrobial agents. The opposite occurred with the diet of lyophilized material without antimicrobial agents, showing that the insects either adapted or degenerated through generations. Lyophilized diets supported the production of *E. heros* through at least 10 generations, with no degeneration.

Key words: Soybean brown stink bug, lyophilized diet, anticontaminant

Stink bugs are the main group of pests in Brazil, and the Neotropical brown stink bug *Euschistus heros* (F.) is the most abundant species of the pentatomid complex on soybean. The success of *E. heros* is likely due to it feeds on several host plants (Panizzi et al. 2000a; Soria et al. 2011) and its adaptation to warmer and cooler temperatures (Panizzi, 2015). Besides, this insect pest has had additional generations because of no-tillage cultivation systems adopted by growers (Saluso et al. 2011)

Rearing of pentatomids to increase production of egg parasitoids for biological control has become an important matter in Brazil in the past three decades. One of the obstacles for mass-rearing parasitoids of eggs of soybean stink bug has been the lack of a suitable diet for rearing different pentatomid species.

In Brazil, several artificial diets for these insects have been tested. The first diet was developed by Panizzi et al. (2000b), who modified the dry holidic (chemically defined) diet used for rearing *Riptortus clavatus* (Thunberg) (Kamano, 1980) to a dry meridic (partly chemically defined) diet, using components purchased in the Brazilian market. Later, Fortes et al. (2006) compared two types of oil (soybean and sunflower) in the diet used by Panizzi et al. (2000b), and found that for *E. heros*, the diet containing soybean oil gave the best results. Still, the results obtained with artificial diets were poorer than those obtained with the natural diet. More recently, Siqueira (2007) evaluated the dry artificial diet containing soybean oil developed by Fortes et al. (2006), adding lyophilized berries of privet [*Ligustrum lucidum* [W.T. Aiton]) to rear *E. heros*. All diets supported the development of nymphs, although with increased proportions of lyophilized privet berries, the development period from the second to fifth instar was extended and the survival of nymphs decreased. Egg production was also affected in the different treatments.

Our objective was to develop an artificial diet with lyophilized material that could provide the nutritional requirements of *E. heros*, in order to produce insects comparable to those from the field and offspring of quality through out several generations.

Materials and Methods

Laboratory Rearing of *E. heros*

The colony of *E. heros* was established in February 2009 with eggs obtained from the rearing colony of the Insect Biology Laboratory of the Department of Entomology and Acarology of ESALQ, University of São Paulo. The eggs were originally collected in 2008 in a soybean field in Ribeirão Preto, São Paulo State. Every year, new eggs and adults were added to the rearing colony of *E. heros*, obtained from EMBRAPA Soja, Londrina, Paraná State, and from field, respectively. Adult stink bugs were kept in cages (20 × 30 × 40 cm) made of transparent acrylic to prevent food from

drying. On the top of each cage, an opening (30 × 0.5 cm) was also made, where cotton fabric was placed as an oviposition substrate. The cage bottom was made of aluminum. The inside bottom was covered with recycled paper on which was placed the natural diet composed of green beans [*Phaseolus vulgaris* (L.)], peanuts [*Arachis hypogaea* (L.)], and soybean [*Glycine max* (L.) Merrill (Fabales: Fabaceae)] on Petri dishes (9 cm in diameter) or Gerbox covers (11 × 11 cm). The diet was changed every 6–7 d. We used strips of cotton fabric (30 × 25 cm) hung from the top of the cage as an oviposition substrate and shelter. The eggs laid on the fabric were removed daily for use in the maintenance of colonies of *Telenomus podisi* Ashmead and *Trissolcus basalis* Wollaston. To maintain the rearing colony, every 5–6 d the eggs were placed in Petri dishes containing a moistened dental-cotton roll. The nymphs were kept in the Petri dishes until the second instar, when they were removed to plastic trays (25 × 25 × 12 cm) lined on the bottom with recycled paper, containing natural diet. The diet was changed every 6–7 d until the adults emerged. Ten days after the beginning of emergence, adults were transferred to cages as described previously. The rearing room was kept at temperature 25 ± 2°C, RH 60 ± 10%, and photoperiod of 14:10 (L:D) h. We tested 13 diets at different steps of the evaluation, numbered D1–D13 (Table 1), following the sequence in Figure 1.

Comparison of Biological Parameters of *E. heros* on Natural and Artificial Diets

We placed 150 second-instar nymphs of *E. heros* in individual Petri dishes (9 cm in diameter). Nymphs were fed with the natural diet (D1) composed of green beans and peanuts, or the artificial diet (D2) developed by Panizzi et al. (2000b) for rearing *Nezara viridula* (L.) and later modified by the addition of soybean oil, by Fortes et al. (2006) (Table 2). Development and viability of nymphs were compared on the two diets; each insect was considered as one replication. The green bean-peanut diet was replaced every 5–6 d and the artificial diet every 48–72 h, when fungal growth was seen on the diet.

At 24 h of age, adults were weighed and couples were formed from each treatment (the maximum age difference between females and males was 72 h). Each couple was kept in a transparent 350-ml plastic cup (7.5 and 5.5 cm in diameter and 11 cm in height) inverted in a Petri dish 9 cm in diameter, with a tulle strip (2.5 × 10 cm) hung from the inside of the cup as an oviposition substrate. The diet was placed on the plate and a moistened dental-cotton roll was used to supply water by capillary action.

Eggs laid by each female were collected daily, counted, and kept in test tubes of 1.0 × 7.0 cm until the nymphs hatched, to evaluate the viability and duration of the egg period (F_1 generation). The duration of the first instar was determined considering 20 random

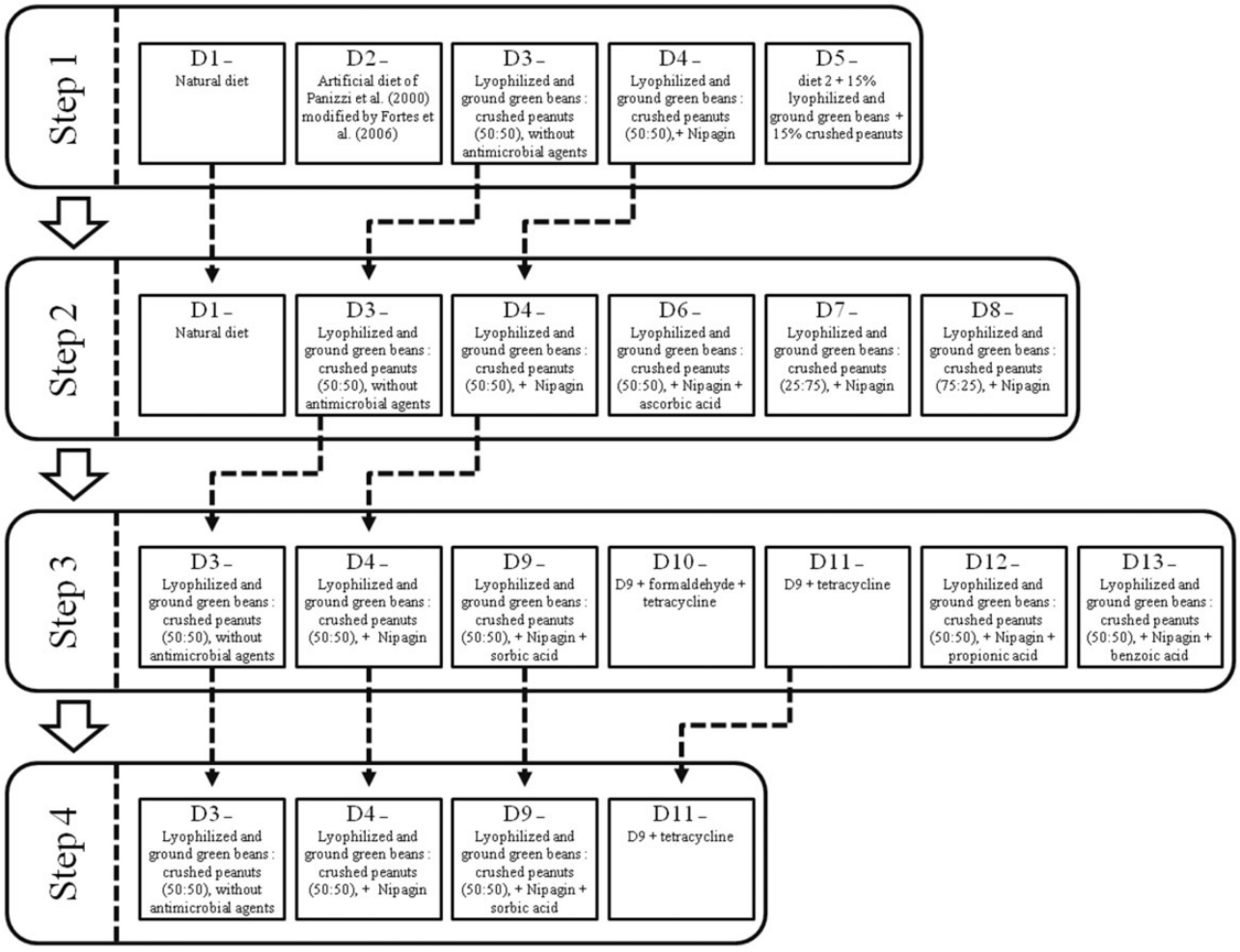

Fig. 1. Flowchart of steps designed to select lyophilized artificial diet for *E. heros* rearing.

Table 1. Diets used in different experiments to develop an adequate diet for *E. heros*

Diets	Composition
D1	Natural diet: green beans and peanuts
D2	Artificial diet of Panizzi *et al*. (2000a,b) modified by Fortes *et al*. (2006)
D3	Lyophilized and ground green beans (35 g), crushed peanuts (35 g), sucrose (5 g), and water (25 ml) without antimicrobial agents
D4	Lyophilized and ground green beans (35 g), crushed peanuts (35 g), sucrose (5 g), and water (25 ml) + Nipagin-8,000 ppm
D5	D2 with added 15% lyophilized and ground green beans and 15% crushed peanuts
D6	Lyophilized and ground green beans (35 g), crushed peanuts (35 g), sucrose (5 g), and water (25 ml) + Nipagin-8,000 ppm + ascorbic acid-800 ppm
D7	Lyophilized and ground green beans (25 g), crushed peanuts (75 g), sucrose (5 g), and water (25 ml) + Nipagin-8,000 ppm
D8	Lyophilized and ground green beans (75 g), crushed peanuts (25 g), sucrose (5 g), and water (25 ml) + Nipagin-8,000 ppm
D9	Lyophilized and ground green beans (35 g), crushed peanuts (35 g), sucrose (5 g), and water (25 ml) + Nipagin-10,000 ppm + sorbic acid-800 ppm
D10	D9 + formaldehyde-3,000 ppm + tetracycline-0.00765 ppm
D11	D9 + tetracycline-0.00765 ppm
D12	Lyophilized and ground green beans (35 g), crushed peanuts (35 g), sucrose (5 g), and water (25 ml) + Nipagin-10,000 ppm + propionic acid-800 ppm
D13	Lyophilized and ground green beans (35 g), crushed peanuts (35 g), sucrose (5 g), and water (25 ml) + Nipagin-10,000 ppm + benzoic acid-2,000 ppm

ovipositions for each treatment, and counting the number of days after the nymphs hatched until they reached the second instar.

Through daily observations, we evaluated the following biological and morphological parameters: development duration and partial viability (per instar) and total egg–adult period, weight of adults at 24 h of age, deformities of legs and wings, sex ratio: ♀/(♀ + ♂), preoviposition and oviposition periods, longevity, number of eggs/female, ovipositions/female, number of eggs/oviposition, and percentage of females that oviposited. The rearing room was kept at a controlled temperature of 25 ± 2°C, RH 60 ± 10% and photoperiod of 14:10 (L:D) h.

Biological data were subjected to analysis of variance and the means were compared using Tukey test at 5% probability, in a completely randomized design. Data and assumptions associated with the model used were analyzed with the program SAS/STAT (2003).

To determine the number of eggs per female, two situations were considered, i.e., 100% of females and only females that oviposited, using the first case to graph the cumulative daily oviposition frequency.

The diets were compared by cluster analysis, using the following biological parameters: duration of egg–egg period, egg–adult viability, weight of males and females at 24 h of age, number of eggs per female, percentage of females that oviposited, ovipositions per female, number of eggs per oviposition, longevity of females and males, and deformities of legs and wings.

Fertility life tables were constructed for each step, and the results obtained for insects on the different diets were used to determine the following life tables parameters. Ro is the net reproductive rate defined by

$$R_o = l_x m_x$$

T is the mean interval between generations and can be calculated from

$$T = \frac{\text{Log } \theta \, R_o}{r_m}$$

r_m is the intrinsic growth rate was estimated by solving the equation

$$\sum e^{-r} m^x l_x m_x = 1$$

For theses equations above, l_x is the number of adults still alive at age x and m_x is the age-specific fertility.

Furthermore, it has λ (finite increase ratio) that is determined by

$$\lambda = e^r m$$

DT: the doubling time can be calculated from

$$DT = \frac{\text{Ln}(2)}{r_m}$$

The Jackknife method was used to estimate the parameters (Meyer et al. 1986), and the comparisons were made with the *t* test. Fertility life tables were prepared from the following data: duration and viability of the egg–adult period, preoviposition period, sex ratio, daily number of eggs per female, and daily mortality of males and females.

Table 2. Composition of artificial diet[a] used for *E. heros*

Component	Quantity
Wheat meal	12.5 g
Soy protein	20.0 g
Potato starch	5.0 g
Sucrose	5.0 g
Cellulose	7.5 g
Soy oil	12.5 g
Vitamin solution[b]	5.0 g
Water	30.0 g
Dextrose	5.0 g

[a]Artificial diet from Panizzi *et al*. (2000a,b) and Fortes *et al*. (2006).

[b]Composition of vitamin solution: Niacinamide 1 g, calcium pantothenate 1 g, thiamine 0.25 g, riboflavin 0.5 g, pyridoxine 0.25 g, folic acid 0.25 g, biotin 0.02 ml, vitamin B_{12} 1 g, in 1,000 ml of distilled water.

Supplementing Artificial Diet With Lyophilized Components and Antimicrobial Agents for Rearing *E. heros*

Four steps of evaluation were carried out, using lyophilized material and antimicrobial agents in the artificial diets, and their effects on the biology of *E. heros* (Fig. 1) were evaluated. Lyophilized components of the natural diet were incorporated into the artificial diets. To lyophilize the green beans and peanuts, three samples of 200 g of green beans were divided into pieces of 2.5–3 cm and lyophilized (model Novalyphe-NL150, Savant Instruments, Holbrook, NY). The ideal lyophilization time was determined by weighing the samples every 12 h until constant weight. The lyophilized green beans were then ground. Because lyophilizing removed only a small amount of water (5.9%), peanuts were added to the diet without lyophilizing, but after they were crushed in a blender.

To prepare the diet, the lyophilized and ground green beans, crushed peanuts, and sucrose were mixed in a plastic container and the antimicrobial agent diluted in water was added. The mixture was macerated until a homogeneous paste was obtained. The paste was placed in a mold (Gerbox seed-box cover) of $12 \times 12 \times 0.5$ cm lined with aluminum foil, cut into squares of about 5–6 mm, and wrapped with the same foil. Afterward, the diet was oven-dried at 60°C for 4 h and the dry diet was stored in a freezer at −34°C. The pH and protein were analyzed in the samples of lyophilized diet without antimicrobial agents, and in the individual components (lyophilized and ground green beans and crushed peanuts).

The optimum ratio of lyophilized and ground green beans: crushed peanuts (50:50) in the artificial diet was determined from observation of the ratio that led to a reduction in the duration of the second instar-adult period and greater viability for the period, compared with the individual components of the diet. Thus, the ratios of the diet components: lyophilized and ground green beans (35%) plus crushed peanuts (35%), sucrose (5%), and water (25%) were evaluated for the first time in this step. The lyophilized green beans plus crushed peanuts comprised 70% of the diet, in equal proportions (50:50). The Nipagin (methyl parahydroxybenzoate, Mingtai, Bah-Der City, Taoyuan Hsien, TW) concentration (8,000 ppm) was also determined in preliminary tests, by observing that 16,000 ppm caused 100% mortality of the stink bugs, while 4,000 ppm had no effect on fungal growth.

In step 1, we compared the natural diet with artificial diets containing lyophilized material and antimicrobial agents, this experiment consisted of 5 treatments and 100 replications. In step 2, we observed the effect of the proportion of lyophilized and ground green beans: crushed peanuts on the artificial diet, with 6 treatments and 150 replications. In step 3, we investigated the effects of different doses of antimicrobial agents added to the lyophilized artificial diet on the biology of *E. heros* and the development of pathogens. This part of the experiment consisted of 7 treatments and 100 replications. After selecting the best diets in step 3, we conducted step 4, to analyze the biological parameters of *E. heros* in the fourth generation (F_4) in the treatments that allowed the insects to reach this generation (Fig. 1). This last step consisted of 4 treatments and 150 replications. In all experiments, each nymph corresponded to one replication.

In all steps, the couples were added as described in the item 'comparison of biological parameters on natural and artificial diet'. Rearing conditions, biological parameters evaluated, experimental design, statistical analysis, group analysis, and life table were the same as in the item 'comparison of biological parameters on natural and artificial diet'. In each generation, more than 40 emerged individuals were considered an adequate number to maintain the populations. In each generation, we assessed the sex ratio, weight of adults (males and females), and deformities of legs and wings.

Results

Comparison of Biological Parameters of *E. heros* on Natural and Artificial Diets

The dry artificial diet developed by Panizzi et al. (2000b) and supplemented with soybean oil by Fortes et al. (2006) (D2) significantly affected ($F = 24.20$; $P < 0.0001$) the development of the egg–adult period of *E. heros*, which was 39.5 d, longer than the 32.2 d on the natural diet (D1). The effect occurred in the last instars (fourth and fifth), because from the egg phase to the third instar there were no differences among the treatments.

The viability of the second instar-adult period on the natural diet was 82%. The viability for the same period on the artificial diet in this study was 41%. On the natural diet, males and females were heavier and showed a lower percentage of deformities of legs and wings, and the females lived longer; the sex ratio was not affected.

For the other biological parameters studied, the preoviposition period, oviposition per female, number of eggs/oviposition, and number of eggs/female, the natural diet overcame the artificial diet, including the percentage of females that oviposited (96.7% on the natural diet and 50% on the artificial diet), besides the high percentage of deformed eggs (personal observation) obtained on the artificial diet. In general, the few nymphs obtained in F_1 with the artificial diet did not reach the fourth instar.

The oviposition rhythm differed between the insects reared on the two diets. On the artificial diet, as longevity was lower, 100% of the eggs were laid before 90 d, whereas on the natural diet, 100% of the eggs were laid before 150 d.

Supplementing the Artificial Diet With Lyophilized Components and Antimicrobial Agents for Rearing *E. heros*

Step 1: Comparison of the Effects of Natural and Artificial Diets Containing Lyophilized Material and Antimicrobial Agents on the Biology of E. heros.. The LAD proved to be more advantageous (greater phagostimulant effect) for *E. heros* than the artificial diet (D2), because on D2, many insects died without feeding, while on LAD, the insects continued to feed for a longer time, showing gregarious behavior similar to that observed on the natural diet (D1). The total protein content of LAD was 25.8%, although the protein levels of the individual components ranged from 20.8% for crushed peanuts to 33.8% for lyophilized green beans.

As one of the problems of the artificial diet is contamination by fungi and/or bacteria, the use of antimicrobial agents was necessary. However, it is crucial to determine the appropriate dose, because preliminary tests showed that at the concentration of 16,000 ppm, Nipagin caused 100% mortality, and while the concentration of 4,000 ppm did not affect the insects, it was not effective in controlling fungi. Therefore, the choice of the appropriate dose of antimicrobial agent was essential, as there is a fine line between pathogen control and impaired insect rearing (Alverson and Cohen, 2002).

The diets affected the duration of the egg–adult period ($F = 3.93$; $P = 0.0022$). There was, as expected, an increase in this period on diets D2 (artificial) and D4 (LAD with antimicrobial agent), and the differences were more significant after the third instar ($F = 2.74$; $P = 0.0212$). The egg stage was also affected, and increased when the insects were reared on the artificial diet (D2).

Viability was higher on the diets containing lyophilized green beans and crushed peanuts, while the artificial diet D2 had the lowest viability, only 17%. The viability of eggs (30%) was also affected on D2, showing the nutritional inadequacy of the diet. Numerically, the lyophilized diets D3 and D4 resulted in higher viability, 63.8 and 63.5%, respectively.

The weight of adults was also affected by the artificial diets (female = 85.6 and male = 76.4 mg) ($F = 2.46$; $P = 0.0360$). Again, D2 was less adequate, although diet D5 was comparable to D2 (female = 86.4 and male = 75.4 mg). Similarly, insects reared on diet D2 showed a higher percentage of deformed wings and legs. The sex ratio and preoviposition period did not differ among the diets. The longevity of males and females, percentage of females that oviposited, and number of eggs/oviposition were not affected by the diets; however, oviposition/female and the total number of eggs/female were higher with diets D1, D3, and D4, with 28.7, 13.7, and 16.4 ovipositions/female, and 300, 174, and 200 eggs/female, respectively.

Table 3. Duration and viability of instars and egg–adult period of *E. heros* reared on artificial diet containing LAD[a] and antimicrobial agents in different doses

Treatments	Duration (days)						Egg–adult
	Egg phase	First instar	Second instar	Third instar	Fourth instar	Fifth instar	
D3	7.5 ± 0.10[b] a[c]	3.0 ± 0.00 a	4.9 ± 0.09 b	6.3 ± 0.10 c	6.3 ± 0.07 b	9.2 ± 0.06 ab	37.3 ± 0.14 b
D4	7.8 ± 0.05 a	3.0 ± 0.00 a	4.9 ± 0.11 b	6.6 ± 0.14 bc	6.6 ± 0.09 ab	9.1 ± 0.06 b	38.0 ± 0.14 bc
D9	7.7 ± 0.06 a	3.0 ± 0.00 a	5.3 ± 0.17 ab	6.8 ± 0.12 bc	6.8 ± 0.10 a	9.3 ± 0.06 ab	38.8 ± 0.15 bc
D10	7.6 ± 0.12 a	3.0 ± 0.00 a	5.1 ± 0.08 ab	7.2 ± 0.10 a	6.8 ± 0.09 a	9.4 ± 0.08 a	39.1 ± 0.20 a
D11	7.5 ± 0.09 a	3.0 ± 0.00 a	5.1 ± 0.14 ab	6.5 ± 0.14 bc	6.5 ± 0.08 ab	9.3 ± 0.07 ab	37.9 ± 0.17 bc
D12	7.7 ± 0.07 a	3.0 ± 0.00 a	5.4 ± 0.15 ab	6.6 ± 0.11bc	6.5 ± 0.09 ab	9.0 ± 0.07 c	38.3 ± 0.15 bc
D13	7.8 ± 0.05 a	3.0 ± 0.00 a	5.7 ± 0.13 a	6.8 ± 0.11 ab	6.6 ± 0.10 ab	9.2 ± 0.09 ab	39.1 ± 0.18 a
	Viability (%)						
D3	58.8 ± 4.97 a	91.7 ± 2.40 a	97.0 ± 1.91 ab	99.0 ± 1.09 ab	100.0 ± 0.00 a	99.0 ± 1.00 a	51.2 ± 6.53 a
D4	58.2 ± 3.65 a	91.7 ± 2.40 a	96.0 ± 1.63 ab	100.0 ± 0.00 a	100.0 ± 0.00 a	94.8 ± 2.63 a	48.6 ± 6.51 ab
D9	51.8 ± 5.59 a	91.7 ± 2.40 a	97.0 ± 1.91 ab	100.0 ± 0.00 a	99.0 ± 1.10 a	92.7 ± 3.12 a	42.3 ± 7.50 ab
D10	25.1 ± 5.65 b	91.7 ± 2.40 a	98.0 ± 2.00 a	98.0 ± 1.20 ab	100.0 ± 0.00 a	91.7 ± 1.62 a	20.3 ± 11.88 b
D11	62.8 ± 5.45 a	91.7 ± 2.40 a	98.0 ± 1.15 a	95.9 ± 1.63 ab	97.9 ± 1.23 a	97.8 ± 1.23 a	51.8 ± 5.66 a
D12	47.6 ± 4.49 a	91.7 ± 2.40 a	97.0 ± 1.00 ab	92.8 ± 3.55 b	100.0 ± 0.00 a	96.7 ± 2.00 a	38.0 ± 8.10 ab
D13	54.2 ± 5.98 a	91.7 ± 2.40 a	90.0 ± 2.00 b	94.4 ± 1.06 ab	100.0 ± 0.00 a	95.3 ± 3.37 a	40.3 ± 6.83 ab

Temp., 25 ± 2°C; RH: 60 ± 10%; photoperiod of 14:10 (L:D) h.

[a]LAD diet composition: lyophilized and ground green beans: crushed peanuts (70%), sucrose (5%), and water (25%). [b] ± SE (standard error).

[c]Means followed by the same letter in a column do not differ by Tukey test at 5% probability.

The frequency of oviposition on artificial diets was less satisfactory, because more than 90% of ovipositions occurred between 40 and 60 d, whereas on D1 and D4, most occurred between 75–90 d, because females lived longer when they were reared on these diets.

Artificial diets with lyophilized green beans (LAD) were the best: they were superior to D2 in all parameters and comparable to D1. Diet D4, with Nipagin-8,000 ppm, generated offspring until the third generation (F3), and was selected for the subsequent step of testing.

Step 2: Effect of Ratio of Lyophilized and Ground Green Beans: Crushed Peanuts Added to Artificial Diet on the Biology of E. heros. The duration of the egg–adult period for insects on the natural diet (D1) was equal to that of insects reared on LAD diets with different ratios of lyophilized and ground green beans: crushed peanuts. No differences were observed for viability, which was low for all diets, ranging from 7.5 to 18.2% for D8 and D1, respectively.

The treatments had no effect on the weight of females. However, the weight of males was lower than that of females in all treatments (from 76.8 to 89.6 mg for males and 97.9 to 84.8 mg for females), whereas on D7, males emerged with lower weight (76.8 mg). No significant differences were observed for deformities of legs and wings, sex ratio, longevity, preoviposition period, percentage of females that oviposited, oviposition/female, and number of eggs per female.

The frequency of oviposition and cumulative oviposition showed a decrease in laying rate after 60 d on D6 and D8. On other diets (D3, D4, and D7), the survival of females (up to 90 d) was comparable to that on the natural diet, where the interval between ovipositions was longer (2–3 d); while on LAD diets, the frequency was every 1 or 2 d. Thus, females reared and maintained on diets with lyophilized components had a shorter longevity than those on the natural diet, without affecting their fertility. Males had greater longevity than females.

The best ratio of lyophilized and ground green beans: ground peanuts was obtained within the range of 25–50:75–50, because offspring were obtained until the third generation, with the proportion of 25:75 (D7). However, this ratio was disregarded, due to the oily appearance of the diet. There were no differences in the biological parameters evaluated. Treatments D3 and D4 were selected for the next step, because Ro was similar to that of insects on the natural diet (D1) (Table 5).

Step 3: Effects of Different Doses of Antimicrobial Agents Added to Lyophilized Artificial Diet on Biology of E. heros *and Pathogen Development*. The main contaminants in the artificial diets were the fungi *Penicillium* spp. and *Aspergillus* spp., which were identified in the Laboratory of Pathology and Microbial Control of Insects, ESALQ-USP.

In this experiment, the number of 'substitutions' of diets for the second-instar adult period ranged from 0 to 1 on artificial diets with LAD and antimicrobial agents, to 8 'substitutions' in LAD (D3) without antimicrobial agents.

The antimicrobial agents affected the development of *E. heros* ($F = 12.76$; $P < 0.0001$), extending its development, mainly in the treatments with formaldehyde (D10) and benzoic acid (D13) (Table 3).

Egg–adult viability (Table 3) for LAD diets, in this experiment, exceeded the levels obtained in previous tests, including the natural diet. However, it varied among the treatments, with the highest viability occurring in diets D3 and D11. The egg phase was the most critical for all treatments, and the diet with formaldehyde (D10) gave the poorest results.

The weight of adults at 24 h of age was also significantly affected by the antimicrobial agents, and formaldehyde (D10) was the least suitable antimicrobial agent in the diet. Adults reared on LAD without an antimicrobial agent (D3) weighed the most, followed by D12, D4, and D11. In general, the weight of females exceeded that of males, and the longevity of males exceeded that of females in all experiments (Table 4).

LAD diets with antimicrobial agents had no effect on the deformation of legs or on the longevity of females and males. There was effect of the diet on wing deformations ($F = 9.25$; $P < 0.0001$); insects from D3 (control) showed the lowest incidence of deformation, and the highest incidence of deformation was observed in the D10 and D9 diets with 34 and 25%, respectively. The sex ratio was, on average, 0.5 for all treatments, and the preoviposition period ranged from 9 to 11 d (Table 4).

Table 4. Adult weight at 24 h of age, deformations of legs and wings, longevity, sex ratio, preoviposition period, females that oviposited, number of ovipositions per female, number of eggs per oviposition, and number of eggs per female of *E. heros* reared on artificial diet containing LAD[a] and antimicrobial agents in different doses

Treatments	Weight (mg) at 24 h of age		Deformations (%)		Longevity (days)		Sex ratio
	Female	Male	Legs	Wings	Female	Male	
D3	94.5 ± 1.72[b] a[c]	80.0 ± 1.88 a	14.7 ± 3.05 a	5.3 ± 01 c	44.3 ± 4.4 a	77.8 ± 7.11 a	0.5 a
D4	83.8 ± 1.79bc	76.6 ± 1.18 a	26.4 ± 7.64 a	11.0 ± 4.13 bc	51.8 ± 5.34 a	75.4 ± 7.68 a	0.6 a
D9	79.4 ± 1.86 c	67.0 ± 1.17 bc	15.7 ± 4.42 a	24.7 ± 4.23 ab	37.4 ± 3.99 a	92.9 ± 10.57 a	0.5 a
D10	66.1 ± 1.33 d	61.4 ± 1.35 c	20.5 ± 4.65 a	34.1 ± 5.01 a	45.9 ± 7.96 a	58.4 ± 11.27 a	0.5 a
D11	83.4 ± 1.76 bc	76.6 ± 1.53 a	14.4 ± 3.73 a	8.9 ± 1.74 c	39.3 ± 6.48 a	98.1 ± 9.62 a	0.6 a
D12	87.3 ± 1.66 ab	78.1 ± 1.85 a	13.8 ± 1.97 a	8.1 ± 1.21 c	56.7 ± 6.16 a	82.6 ± 10.25 a	0.5 a
D13	78.9 ± 2.19 c	68.3 ± 1.67 bc	18.5 ± 3.68 a	16.1 ± 3.90 bc	48.8 ± 6.51 a	70.5 ± 9.45 a	0.5 a
	Preoviposition period (days)		Females that oviposited (%)	Oviposition/ female	Number of eggs/oviposition	Number of eggs/female	
						All females	Only females that oviposited
D3	9.4 ± 0.41 c		100.0 ± 0.00 a	20.4 ± 2.56 a	12.8 ± 0.64 a	260.1 ± 34 18 a	260.1 ± 34.18 a
D4	9.7 ± 0.33 bc		100.0 ± 0.00 a	24.2 ± 2.77 a	12.3 ± 0.48 a	286.0 ± 28 53 a	286.0 ± 28.53 a
D9	10.6 ± 0.39 ab		92.0 ± 7.14 a	16.5 ± 2.19 a	11.8 ± 0.58 a	171.5 ± 22.72 a	186.4 ± 20.90 a
D10	10.9 ± 0.38 a		100.0 ± 0.00 a	16.5 ± 4.06 a	11.6 ± 0.76 a	206.9 ± 53 51 a	206.9 ± 53.51 a
D11	9.6 ± 0.24 bc		100.0 ± 0.00 a	16.0 ± 2.79 a	13.4 ± 0.49 a	211.6 ± 37.08 a	211.6 ± 37.08 a
D12	9.9 ± 0.21 abc		90.0 ± 6.84 a	26.2 ± 3.27 a	12.0 ± 0.43 a	285.2 ± 38.55 a	316.9 ± 35.85 a
D13	9.9 ± 0.21 abc		86.4 ± 4.80 a	22.7 ± 3.30 a	12.1 ± 0.44 a	232.9 ± 43 81 a	269.6 ± 41.10 a

[a]Composition of LAD diet: lyophilized and ground green beans: crushed peanuts (70%), sucrose (5%) and water (25%).
[b] ± SE (standard error).
[c]Means followed by the same letter in a column do not differ by Tukey test at 5% probability.
Temp., 25 ± 2°C; RH: 60 ± 10%; photoperriod of 14:10 (L:D) h.

LAD diets resulted in greater fecundity, with no effect of the antimicrobial agents on the percentage of females that oviposited (86–100%), oviposition/female (16–26), number of eggs/oviposition (12–13), and number of eggs/female (171–286), based on 100% of females. These results show the good nutritional quality of the diet containing lyophilized material (Table 4).

All diets extended the oviposition period, concentrating 90% of oviposition within 54 d in D3 (control) and 90 d in D10 (the worst treatment) containing formaldehyde, without; however, affecting fecundity.

In the laboratory, 10 generations (F_{10}) were obtained on diets D9 and D11, which maintained the desired characteristics, such as sex ratio around 0.5, lower percentage of deformations of legs and wings, and weight of adults close to 70 mg. D3 (control without antimicrobial agents) and D4 were eliminated in the sixth generation (F_6), because the number of offspring was insufficient to continue observations and therefore involved a high probability of inbreeding, and also because of the high incidence of fungi on diet D3. D10 and D13 were eliminated before F_1, because formaldehyde is being removed from the market and has carcinogenic properties, in addition to its negative effects on *E. heros*. The treatment with propionic acid (D12) was eliminated after generation F_3 as it produced males with low weight, which would have affected mating, causing high rates of infertility and producing fewer offspring (personal observation).

Step 4: Evaluation of Diets (LAD) Adequate for Rearing *E. heros*. LAD diets (D3, D4, D9, and D11) produced more than 40 individuals until the third generation (F_3), and were therefore compared in the fourth generation (F_4).

The egg–adult period on LAD diets in this step (33–34 d) was similar to that obtained on D1 of the first experiment 'comparative biology' (32 d), showing that the insects eventually adapted to each diet. In contrast, the insects that fed on the natural diet (D1) and LAD without antimicrobial agents, degenerated over several generations, and it became necessary to introduce wild-caught insects to reinvigorate the population.

The diets did not affect the duration of the egg phase, which was 7 d for all treatments; or the duration of the first and second instars, which lasted 3 and 5.7–5.9 d, respectively. There was no difference in the viability of the treatments; the egg phase was critical, as it showed < 90% viability.

Weight, longevity of males, and sex ratio were unaffected by the antimicrobial agents. Longevity showed the same trend observed in step 3, that is, males lived longer than females, and females in turn had higher survival on LAD diets with antimicrobial agents than on LAD without antimicrobial agents (D3). D3 without antimicrobial agents generated heavier females, with a lower percentage of deformations of legs and wings.

The biological parameters of preoviposition period, percentage of females that oviposited, number of eggs/oviposition, and number of eggs/female were unaffected by the antimicrobial agents. Oviposition rate per female was similar in all treatments. On the poorest-performing diet (D4), 90% of the eggs were laid within 60 d, and the rest between 75 and 100 d.

Fecundity on the natural diet (D1) as well as on the LAD diet without antimicrobial agents (D3) decreased over time, whereas on the LAD diets with antimicrobial agents (D9 and D11), the mean fecundity increased significantly compared with the natural diet (282 eggs/female), reaching 430 eggs/female. On LAD diets, *E. heros* adapted to the diet through the generations, showing no signs of degeneration as on the natural diet.

Table 5. ertility life table of *E. heros*, reared on natural diet, artificial diet of Panizzi *et al.* (2000) as modified by Fortes *et al.* (2006), and artificial diets containing lyophilized green beans (LAD)[a] and different doses of antimicrobial agents (Jackknife Method)

Treatments	Ro[b]	T	DT	rm	λ
Natural diet versus artificial diet					
D1	86.31 a[c]	58.22 a	09.04 a	0.076 a	1.0795 a
D2	5.48 b	71.52 b	27.58 b	0.024 b	1.0245 b
STEP 1: Comparison of natural diet and artificial diet containing lyophilized components and antimicrobial agents					
D1	57.17 a	62.79 b	10.73 d	0.064 a	1.0667 a
D2	03.27 d	65.11 b	35.61 a	0.019 d	1.0187 d
D3	29.52 b	63.89 b	12.99 c	0.053 b	1.0546 b
D4	37.27 b	74.06 a	14.13 bc	0.049 b	1.0500 b
D5	9.33 c	60.42 b	18.36 ab	0.037 c	1.0380 c
STEP 2: Effect of the ratio of lyophilized and ground green beans: crushed peanuts added to artificial diet on the biology of *E. heros*					
D1	15.76 a	69.28 bc	16.97 ab	0.040 a	1.0411 a
D3	3.93 b	78.54 ab	30.49 ab	0.019 b	1.0190 b
D4	3.20 b	63.17 c	33.42 a	0.019 b	1.0191 b
D6	2.63 b	78.82 a	20.14 ab	0.014 bc	1.0138 bc
D7	1.96 bc	84.79 a	−103.01 b	0.009 bc	1.0092 bc
D8	0.37 c	71.83 abc	−42.98 b	−0.010 c	0.9903 c
STEP 3: Effect of different doses of antimicrobial agents on the biology of *E. heros* and the development of pathogens in the diet					
D3	66.04 ab	59.94 bc	9.89 c	0.070 a	1.0726 a
D4	81.49 a	65.77 a	10.34 c	0.067 a	1.0688 ab
D9	38.37 c	58.97 c	11.17 b	0.062 b	1.0639 c
D10	21.92 d	67.29 a	14.87 a	0.046 d	1.0475 d
D11	65.01 ab	60.75 abc	10.04 c	0.069 a	1.0714 ab
D12	57.33 b	62.94 ab	10.74 bc	0.064 ab	1.0665 bc
D13	44.86 bc	64.96 a	11.77 b	0.059 bc	1.0605 c
STEP 4: Evaluation of diets (LAD) for rearing *E. heros*					
D3	136.11 a	57.82 b	8.14 b	0.085 a	1.089 a
D4	81.32 b	55.26 b	8.66 ab	0.080 ab	1.083 ab
D9	132.21 ab	65.63 a	9.30 a	0.074 b	1.077 b
D11	135.26 ab	63.55 a	8.95 a	0.077 b	1.080 b

[a]LAD diet composition: lyophilized and ground green beans: crushed peanuts (70%), sucrose (5%), and water (25%);
[b]Net reproductive rate (R_o), mean interval between generations (T), doubling time (DT), intrinsic rate of increase (r_m), finite rate of increase (λ);
[c]Means followed by the same letter in a column do not differ by bilateral Student *t* test;

The Cluster Analysis

In step 1, based on the cluster analysis of the four developmental stages fed on the artificial diet with lyophilized green beans (LAD) (Fig. 2), both D3 and D4 were chosen as controls for the subsequent steps. Both diets contained lyophilized green beans; D3 contained antimicrobial agents and D4 also contained Nipagin.

In the second step, we selected the natural diet D1 (control), which gave the best results, LAD D4 and D3 with a 50:50 ratio (lyophilized and ground green beans: crushed peanuts).

In step 3, the natural diet (D1) was considered as the mean of the values of the first three experiments. Diets D9, D11, D3, and D4 were retained through the generations, because all of them allowed *E. heros* to reach the third generation (F_3), and as the insects achieved the fourth generation, they were compared with evaluate their biological performance.

In step 4, which corresponds to the fourth generation (F_4) of insects obtained in step 3, the analysis resulted in selection of D9 and D11, which supported reproduction of *E. heros* in the laboratory until the 10th generation of adults, and diets D3 and D4 were discarded at the 6th generation in the laboratory.

Fertility Life Table of *E. heros* Reared on Different Artificial Diets With Lyophilized Green Beans (LAD) and Antimicrobial Agents

In the first experiment, Ro on the natural diet (D1) was 86, i.e., the population of *E. heros* increased 86 times in each generation, versus 5.5 times for insects reared on the artificial diet. The other life-table parameters, rm (intrinsic growth rate) and finite increase ratio (λ), which represent the number of female individuals added to the population by each female, were higher on the natural diet (D1), showing that the natural diet was adequate, with a decrease in the mean interval between generations (T) and the doubling time (DT) (Table 5).

In step 1, when different ratios of lyophilized green beans and antimicrobial agents were compared, D1 was also superior to other D1 diets for parameters Ro, rm and λ, followed by diets D3 and D4 (Table 5).

In step 2, D1, although at a higher ratio, showed the best nutritional adequacy for *E. heros* (Table 5), followed by diets D3 and D4. The highest values of λ, rm and Ro confirmed this higher nutritional quality, with lower values of T and DT.

In step 3, LAD diets at the 50:50 ratio were the most complete, and D4 was the best, followed by D3 (LAD diet without antimicrobial agents) and D11 (LAD diet containing Nipagin, sorbic acid and tetracycline). The results for Ro, rm and λ, DT and T support this assertion, according to the Jackknife analysis (Table 5).

Although the insects reared on diet D1 were degenerating over the generations, those reared on LAD diets were able to adapt to them. The fertility life-table value showed that D3 (without antimicrobial agents), D9 and D11 were the best and were all superior to D4 (with Nipagin-8,000 ppm) (Table 5).

Based on biological data in the fertility life table and the cluster analysis carried out in step 4, the D3 and D4 diets were discarded

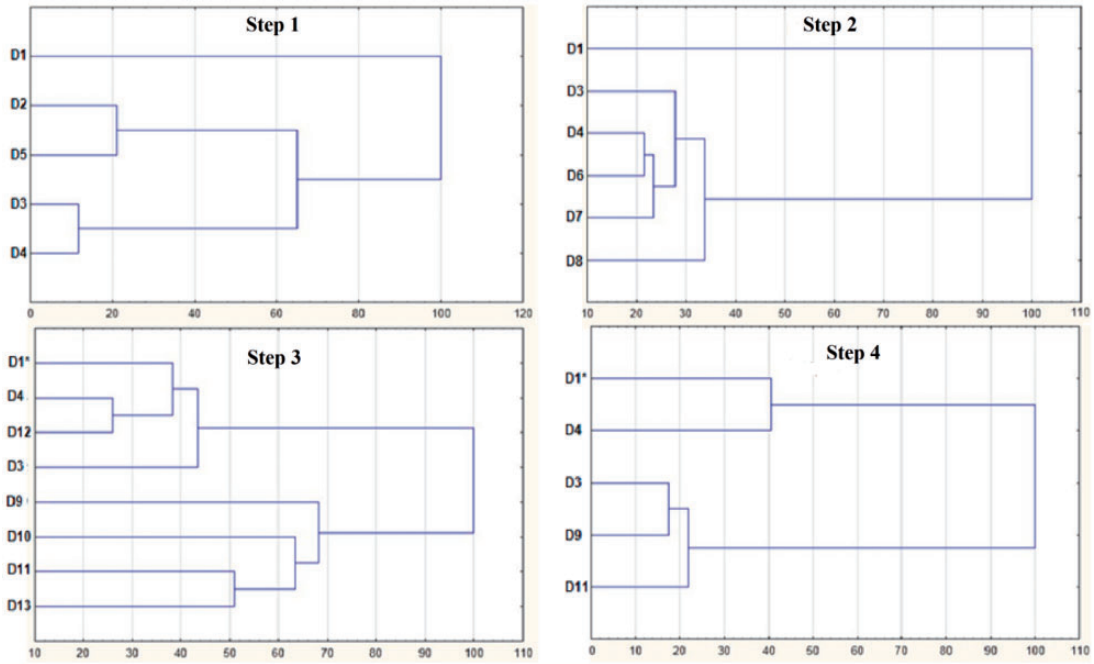

Fig. 2. UPGMA Phenogram for mean Euclidean distance of four steps in the improvement of the artificial diet containing lyophilized green beans (LAD) and antimicrobial agents for *E. heros*. Temp.: 25 ± 2°C; RH: 60 ± 10%; photoperiod of 14:10 (L:D) h. D1* in steps 3 and 4, the mean values determined in experiment 1 and steps 1 and 2 were used.

after the sixth generation (F_6), because the constant presence of fungi on the diet (without antimicrobial agents) hampered its management, and D4 showed lower fecundity than D9 (LAD 50:50 + Nipagin-10,000 ppm + sorbic acid-800 ppm) and D11 (D9 + tetracycline-0.00765 ppm), which supported the production of a large number of offspring until the 10th generation (F_{10}).

Discussion

Comparison of biological parameters showed that the results were similar to those found by Fortes et al. (2006) and Siqueira (2007). The viability on artificial diets was lower, especially for the egg phase and fourth and fifth instars, while the duration and initial viability of *E. heros* (first to third instars) were not affected. This probably occurred because these early instars use nutritional resources accumulated in the eggs, which apparently were sufficient to support the early development of the stink bug. On the artificial diets, males lived longer, possibly because they did not expend energy, since with the premature death of females they had no opportunity to copulate.

The weight of insects on the natural diet was higher than the results found by Fortes et al. (2006) and Siqueira (2007) for this same diet, and similar to those on the artificial diet. The artificial diet affected the weight of the insects, since, in general, according to Slansky (1982), hemipteran females are heavier than males; and in this study, the weights of males and females were similar.

The extension of the development period of nymphs, the reduction of viability from the fourth to fifth instars, together with the lower weight and longevity of adults, increased the proportion of deformations of legs and wings, and decreased fertility in relation to the natural diet, indicating that the artificial diet was inadequate for *E. heros*. This inadequacy may be associated with a deficiency or imbalance of nutrients or to a lack of phagostimulant in the diet, as many of the insects did not feed (Parra, 2010).

The bioassay performed in step 1 showed that, apparently, the lyophilized diet is a phagostimulant and contains nutrients similar to those of natural foods. The lyophilized material retains the integrity of the diet components, and the high protein content may explain the success of the artificial diets with this lyophilized material (LAD).

For the diet with lyophilized components without antimicrobial agents, the low longevity could be related to fungal contamination, which hinders the rearing of various species of insects, as reported by Singh and Bucher (1971), Thompson and Sikorowski (1978), Sikorowski et al. (1980), Sikorowski and Goodwin (1985), Douglas and Sikorowski (2009a,b).

In step 2, the 25:75 ratio of lyophilized and ground green beans: crushed peanuts extended the egg–adult period from the fifth instar on, which may have occurred because high amounts of peanuts affected the development of the nymphs, as the surface of the diet appeared oily, hindering the nymphs from moving around. The diet with 75:25, with a high amount of lyophilized green-bean pulp, had a very dry consistency, which may also have affected the feeding of nymphs. In this step, males showed greater longevity than females. Apparently, this behavior was related to the frequency of copulation, which did not occur due to the early death of females.

In step 3, the antimicrobial agents, mainly formaldehyde, proved inappropriate for the diet of *E. heros*. Negative effects of high doses of antimicrobial agents on insects have been reported, and Alverson and Cohen (2002) found that Nipagin affected the survival of nymphs, dry weight of adults, egg production, development period, and time of initiation of oviposition of *Lygus hesperus* (Knight, 1917) (Hemiptera: Miridae) at concentrations of 1,000, 1,500, and 2,000 ppm. However, although antimicrobial agents have negative effects, studies showed that for the development of nymphs, adding these agents to the diet allows it to be replaced at longer time intervals, or replacement may even be unnecessary, which could lower production costs of stink bugs in Applied Biological Control programs.

Furthermore, all diets tested in step 3 extended the oviposition period, but did not affect fecundity. This finding suggests that the antimicrobial agents increased the longevity of females, and therefore the number of ovipositions and eggs per female, probably related to the effect of antimicrobial agents on symbionts that are occasionally present (Siqueira 2007; Douglas and Sikorowski 2009a).

In step 4, we selected four diets (D3, D9, D11, and D4) based on the evaluation of biological parameters (for each step), and the possibility of obtaining more than 40 individuals in the third generation (F_3) on these diets. From there, the cluster analysis and the fertility life table indicated that diets D9 and D11 were the most suitable. Although adults on the diets of lyophilized material weighed 17% less than those reared on the natural diet, fecundity was higher than the level obtained with the natural diet.

Therefore, LAD diets (D9 and D11) containing 25.8% protein, slightly acidic pH (6.2) and that supported the rearing of *E. heros* through the generations with high Ro (132 and 135 for diets D9 and D11, respectively) can be recommended for mass rearing of this stink bug. Rearing of *E. heros* on diet D11 continued and reached the 22nd generation in the laboratory (J.R.P. Parra,; personal information). Therefore, diets D9, composed of green beans, peanuts, sucrose, water, Nipagin, and sorbic acid, and D11, composed of green beans, peanuts, sucrose, water, Nipagin, sorbic acid, and tetracycline, could be used for production of natural enemies without being limited by degeneration through the generations (Corrêa-Ferreira, 1993)

Diets containing lyophilized material proved to be suitable. The lyophilized diets D9 and D11 allowed the production of *E. heros* through at least 10 generations, with no degeneration and with the potential for production of the egg parasitoids *T. podisi* and *T. basalis*, for Applied Biological Control programs to control the stink bug in soybean crops.

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