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New rearing method and larval diet for the mahogany shoot borer *Hypsipyla grandella* (Lepidoptera: Pyralidae)

*Nancy Barradas-Juanz[1], Francisco Díaz-Fleischer[1], Pablo Montoya[2], Ana Dorantes[1] and Diana Pérez-Staples[1,]**

Abstract

A simplified and improved rearing system was developed for the mahogany shoot borer, *Hypsipyla grandella* (Zeller) (Lepidoptera: Pyralidae) in the laboratory. Improvements were made in neonate rearing, larval containers, adult mating cages and the diet, making the rearing protocols more suitable for eventual large-scale rearing. The larval diet was modified by replacing ingredients with cheaper equivalents that also would be more readily available throughout the year. Cedar seeds in the larval diet were replaced with dried cedar leaves, which are available for longer periods during the year. There were no significant differences in pupal weight, duration of pupal development, fertility (egg hatch) and fecundity (number of eggs oviposited) between organisms reared on the new diet or the original diet. However, larval development took 2 d longer using the new diet. A new method for rearing neonate larvae is proposed, which includes the use of well plates that contain the modified diet. This allowed the rearing of larvae from 1st to 3rd instars without the need for fresh material. Neonate larvae reared on this diet were heavier and survived longer compared to larvae reared on fresh cedar leaves. Jars used for the larval rearing phase were replaced with Petri dishes. Mating and oviposition were accomplished inside the insectaries with air flow directed through the rearing cages. This is the first report of rearing *H. grandella* successfully under artificial conditions for 7 generations. The new rearing protocols will allow the maintenance of larger-sized colonies for further development of bio-rational pest management strategies that could include a sterile insect technique (SIT) component.

Key Words: mahogany (Meliaceae); sterile insect technique; adult rearing; larval rearing; oviposition; artificial diet.

Resumen

Se desarrolló un sistema de cría simplificado y mejorado para reproducir al pyralido barrenador de las meliáceas *Hypsipyla grandella* (Zeller) en laboratorio. Se realizaron modificaciones en la cría de neonatos, en los contenedores de cría larval, a la jaula de apareamiento y a los ingredientes de la dieta, sustituyéndolos por equivalentes de menor precio y mayor disponibilidad. El uso de semilla seca de cedro fue remplazado con hoja seca de cedro que se encuentra disponible más tiempo durante el año. No se encontraron diferencias significativas en cuanto al peso de las pupas, el periodo de desarrollo pupal, ni tampoco en la fertilidad y fecundidad. Sin embargo, el desarrollo larval tomó dos días más en individuos criados con la dieta modificada. El uso de placas de micropozos con la dieta modificada permite el desarrollo de larvas de primer a tercer instar con mayor peso y supervivencia comparadas con larvas criadas con hojas frescas. En la cría de larvas se sustituyó el uso de frascos por cajas Petri. Se logró que los adultos copularan y produjeran huevos fértiles colocando un flujo de aire a través de la jaula. Este es el primer reporte exitoso de cría de una colonia de *H. grandella* en laboratorio durante siete generaciones. Este método de cría facilitará el mantenimiento de colonias para el desarrollo de estrategias de control bioracional como la Técnica del Insecto Estéril.

Palabras Clave: caoba (Meliaceae); técnica del insecto estéril; cría; oviposición; dieta artificial

The mahogany shoot borer, *Hypsipyla grandella* (Zeller) (Lepidoptera: Pyralidae), is one of the most important forestry pests in Latin America and the Caribbean. It mainly damages the new shoots of the red cedar tree (*Cedrela odorata* L.) (Sapindales: Meliaceae). The larvae feed on the meristematic tissue of these shoots, especially on the apical bud, causing bifurcations in the plant and significantly reducing or annulling the commercial value of infested trees (Newton et al. 1993; Taveras et al. 2004). There is currently no effective and recommendable method for managing *H. grandella* populations because the control tactics available often fail to reduce the damage caused by the borer to an economically acceptable level (Newton et al. 1993).

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One control method that has been successfully used for several years against selected pest insects is the sterile insect technique (SIT). The SIT requires the mass-production, sterilization and systematic release of the target insect in adequate over-flooding ratios to reduce the reproduction potential of the natural pest population (Klassen 2005). The objective of the technique is to reduce the birth rate of the target population as opposed to increasing the mortality rate used in other control tactics such as insecticides. The SIT has been used with success against some lepidopteran species, such as the pink bollworm, *Pectinophora gossypiella* Saunders (Lepidoptera: Gelechiidae) in the USA and northern Mexico (Simmons et al. 2007), the false codling moth, *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae) in South Africa (Bloem et al. 2007), the Australian painted apple moth, *Teia anartoides* Walker (Lepidoptera, Lymantriidae) in New Zealand (Suckling et al. 2007) and the codling moth, *Cydia pomonella* L. (Lepidoptera: Tortricidae) in Canada (Bloem & Carpenter 2001). It represents a potential control tactic for the management of *H. grandella*.

For the SIT to be developed and implemented, an uninterrupted supply of physiologically homogenous individuals is required (Hou 1986). Successful laboratory rearing also facilitates the study of their life history traits, behavior, feeding habits as well as the conduct of bioassays to determine susceptibility and resistance to pesticides (Songa et al. 2004; Abbasi et al. 2007), and the development of pheromones (García-Godínez 2002; Pineda-Ríos 2015) and traps. For this reason, an efficient rearing system under controlled conditions is required that includes an artificial diet that permits normal development of the insect (Grijpma 1971; Samaniego & Sterringa 1973; Marti & Carpenter 2008; Chacón et al. 2009).

The main purpose of mass-rearing an insect for use in programs that include a SIT component is the systematic production of high quality insects at an acceptable cost (Hou 1986). Although *H. grandella* has previously been reared under semi-natural conditions including the use of an artificial diet (Vargas et al. 2001; Tavares de Castro et al. 2016), these rearing protocols had severe limitations such as: 1) a larval diet consisting of costly ingredients that are difficult to procure; 2) the use of milled cedar seeds, which are not available year round; 3) the use of large and cumbersome jars for growing the larvae; 4) the necessity of maintaining mating and oviposition cages outdoors, and 5) the use of fresh cedar leaves for neonate larvae. The use of outdoor mating and oviposition cages are particularly limiting if rearing is done in temperate regions (Fasoranti 1985).

The current study proposes solutions for each of these problems. Thus the main objective of this study was to develop a simpler rearing method and a larval diet that will allow large-scale rearing of *H. grandella* under artificial conditions without compromising the quality of the insects produced.

Materials and Methods

BIOLOGICAL MATERIAL

The moths originated from a plantation of red cedar trees, *C. odorata*, which borders the canals of a sugar mill at Mahuixtlán in Coatepec, Veracruz, Mexico (19° 25’ 0.12’’N, −96° 55’ 0.12’’W). Third and older instar larvae were collected from infested branches and tree trunks. Each larva was stored in a plastic bottle (10 × 2.5 cm) with 2 cedar leaves for food, and transported to the INBIOTECA laboratory in Xalapa, Veracruz, Mexico. The larvae were maintained in the laboratory at 24–29 °C, 53–71% RH and a 12:12 h L:D photoperiod (Vargas et al. 2001; Taveras et al. 2004).

The larvae collected from the field were fed on cedar leaves, which had been washed with water and a disinfectant solution (Biopure Internacional, Mexico) until larval development was complete. The pupae were weighed and sexed (Sharma & Singh 1980).

MODIFICATIONS TO THE REARING SYSTEM

The pupae were placed individually into plastic bottles (10 × 2.5 cm) that had moist cotton (3 g) at the bottom. To ensure airflow, a 1.27 cm diam hole was perforated in the bottle lids using a drill (Truper®) and then covered with a piece of mesh cloth of the same size. The bottles with pupae were humidified every 3 d to prevent desiccation.

Once the adults had emerged they were placed in mesh cages (30 × 30 × 30 cm). The door of the cage consisted of a pantyhose with the foot-end cut as an opening. The cages were kept in the laboratory and a direct and constant air current (4.2 m/s in the center) was blown through the cages using a 35 cm fan placed 20 cm from the cage (Fig. 1) (Mo et al. 1998). A Petri dish (15 cm) that contained cotton saturated with potable water was placed in the center of the adult cages. The females oviposited eggs on mesh cloth which had been placed on the walls of the cage. Initially the eggs were white or yellowish but when fertilized they turned red within 24 h.

For egg collection, adults were removed from the cage through the access door. The entire cage was submerged in a white plastic bucket with water and left to soak for 15 min. Removal of the eggs from the walls of the cage was facilitated using a piece of mesh cloth (10 × 10 cm). The eggs were collected using a syringe without the needle and placed in Petri dishes (15 cm). Neonate larvae up to third instar were reared on tender cedar leaves following the methods described by Vargas et al. (2001).

Third instar laboratory-reared larvae were placed in either: 1) glass jars (65 mm in diam × 90 mm in depth) that contained approximately 10 g of modified diet (n = 27) or, 2) small plastic petri dishes (60 × 15 mm) with approximately 4 g of modified diet (n = 26). To avoid the emergence and spread of bacterial or fungal infections the diet in the containers was replaced weekly until completion of larval development. The pupae were weighed and sexed and then transferred to plastic bottles with moist cotton. Emerged adults were transferred to meshed cages (30 × 30 × 30 cm) at a 1:1 male:female ratio for mating.

MODIFICATIONS TO THE LARVAL DIET

The artificial diet for rearing *H. grandella* as described by Vargas et al. (2001) (original diet) was modified (modified diet) as presented in Table 1. The diet along with all the equipment used was autoclaved for 20 min at 121 °C and 1,200 kPa of pressure. Third instar larvae were placed in both diets (see below) and the experiment was replicated 3 times using a total of 171 larvae with the original diet and 150 with the modified diet.

Each third instar larva was placed in a sterile Petri dish (60 × 15 mm) with 4 g of either the original or modified diet, sufficient for feeding the larva for 1 week. To avoid infections the diet was replaced with fresh food when it showed signs of decomposition. The larvae were fed until pupation. The pupae were weighed with an analytical balance (A&D, model HR-200). The emerged adults were separated according to treatment and placed in the oviposition cages. The cages were checked every third d and when fertile eggs occurred, they were collected as described above.

REARING OF NEONATE LARVAE

A first experiment was carried out in Sep–Nov 2013 followed by a second in Mar–Apr 2014. Newly hatched laboratory-reared larvae less than 24 h old were used in these experiments.

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Fig. 1. Oviposition cage. The fan provides airflow through the cage.

First, the weight of the neonate larvae and their survival to third instar was compared between the traditional method using fresh leaves in Petri dishes (15 cm) for rearing neonate larvae (Vargas et al. 2001) (31 larvae per Petri dish, *n* = 312) and the method proposed by Griffith & Smith (1977) using well plates (1 larvae per well, 24 per well plate, *n* = 193) but using the same modified larval diet described above. In each well plate, a drinking straw was used to produce 2 g of modified diet pellets (Mo et al. 1998). After placing the larvae in the wells with a brush, each well was covered with an autoclaved marble to prevent escape of the larvae.

Secondly, the weight of neonate larvae and their survival to third instar was compared between the traditional fresh leaves method (24 larvae per Petri dish, *n* = 48), the well plate method described above (1 larvae per well, 24 per well plate, *n* = 48), and a third method where the same amount of modified diet as used in the well plate was arranged in a circle along the borders of a Petri dish (15 cm) (24 larvae per Petri dish, *n* = 48).

Table 1. Composition of the original and modified artificial diets for the rearing of larvae of *Hypsipyla grandella*.

Ingredients	Original Diet (g)	Ingredients	Modified Diet (g)
Wheat germ	30.00	Wheat germ	30.00
Sugar	7.50	Sugar	7.50
Casein	5.00	Powdered milk	15.0
Agar	5.00	Agar	5.00
Brewer´s yeast	7.50	Brewer´s yeast	7.50
P-hydroxyl benzoate	0.25	Benzoate	0.25
Cholesterol	0.05	Cholesterol	—
Wesson salt mixture	2.50	Regular veterinary salt	2.50
Sorbic acid	0.50	Nipagin	0.50
Milled cedar seeds	0.50	Milled cedar leaves	0.50
Distilled water	212.50 mL	Distilled water	215.50 mL
Chlortetracycline	0.07	Tetracycline	0.07
Mixed vitamins	3.75	Mixed vitamins	3.75

STATISTICAL ANALYSIS

A Generalized Linear Model with Poisson distribution and Log as linking function was used for the analysis of larval and pupal development data. Pupal weight was analyzed using a two-way ANOVA, using diet and replicate as factors. A Generalized Linear Model with Binomial distribution and Log as linking function was used for the analysis of fertility and fecundity data. The statistical packages JMP version 8.0 and R version 3.0.2 were used.

Results

REARING METHOD

In the experiment comparing the rearing of larvae in jars and Petri dishes, no significant differences were found in terms of pupal weight (F = 0.529; df = 1,24; P = 0.473), nor in the number of d necessary for larval (GLM; χ^2 = 0.037; df = 1,24; P = 0.847) or pupal development (GLM; χ^2 = 0.154; df = 1,21; P = 0.695). The same sex ratio (1M:1F) was obtained in both treatments. However, larval mortality was higher in the jars (74%) than in the Petri dishes (23%).

MODIFICATIONS TO THE LARVAL DIET

The average weight of pupae was 0.132 ± 0.002 g when larvae were reared on the modified diet and 0.134 ± 0.002 g with the original diet (Fig. 2). Significant differences were found among replicates (F =

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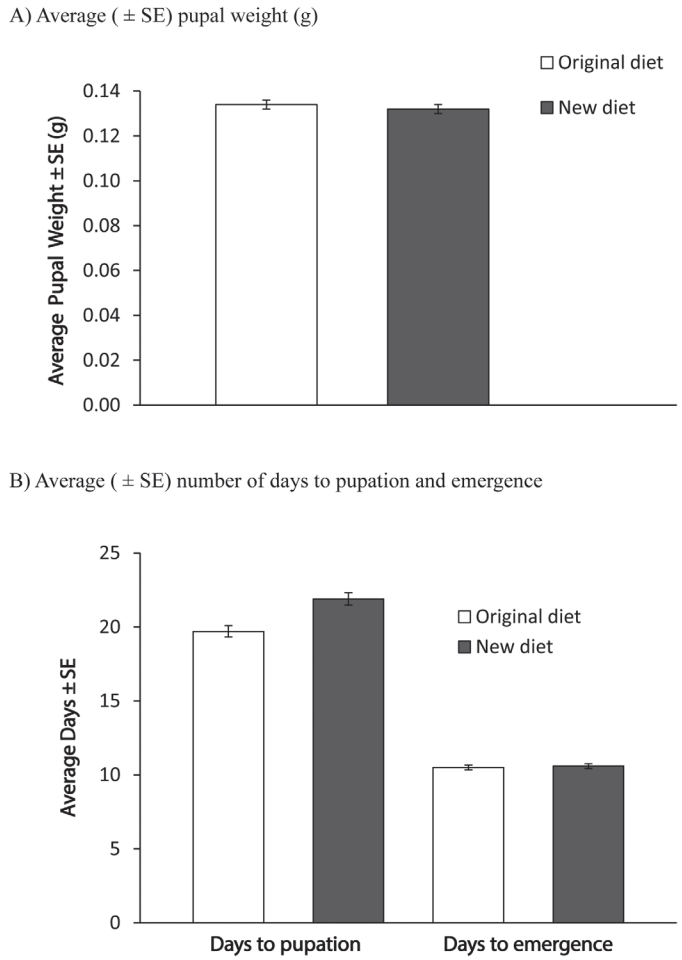


Fig. 2. A) Average (± SE) pupal weight (g), and B) average (± SE) number of d to pupation and emergence of insects reared on original or modified diets.

67.673; df = 3,314; $P < 0.001$) but not between treatments ($F = 3.652$; df = 3,314; $P = 0.057$). The period from egg to third instar larva lasted 14–15 d (4 d as an egg and 10 d as a larva up to third instar). The average period of larval development after the third instar was 21.9 ± 0.4 d for larvae reared on the modified diet and 19.7 ± 0.4 d for larvae reared on the original diet (Fig. 2), with significant differences found between treatments (GLM; $\chi^2 = 16.831$; df = 3,314; $P < 0.001$) and among replicates (GLM; $\chi^2 = 19.229$; df = 3,314; $P < 0.001$).

The period of pupal development was on average 10.6 ± 0.2 d using the modified diet and 10.5 ± 0.2 d using the original diet (Fig. 2), with significant differences found among replicates (GLM; $\chi^2 = 14.083$; df = 3,287; $P < 0.001$) but not between treatments (GLM; $\chi^2 = 0.120$; df = 3,287; $P = 0.729$). An equal sex ratio was obtained for both treatments. A total of 1,604 eggs were collected, of which 215 were infertile and 1,389 were fertile; from these a total of 628 larvae emerged. There were no significant differences in fertility or fecundity of females that were reared as larvae on either diet (GLM; z = 1.353; df = 1,12; $P = 0.176$ and GLM; z = −0.115; df = 1,12; $P = 0.909$, respectively) (Fig. 3).

NEONATE LARVAL REARING

Neonate larvae reared on the modified diet in the well plates gained more weight than neonate larvae reared on the traditional fresh red cedar leaves (average 3rd instar weight (well plates) 0.063 ± 0.003 g and (fresh leaves) 0.051 ± 0.004 g) (GLM; $\chi^2 = 5.062$; df = 2,183; $P = 0.024$) (Fig 4a). The percentage survival of neonate larvae to the third instar was 76.6% for the well plate method versus 50% for the fresh leaf method (Fig. 4b).

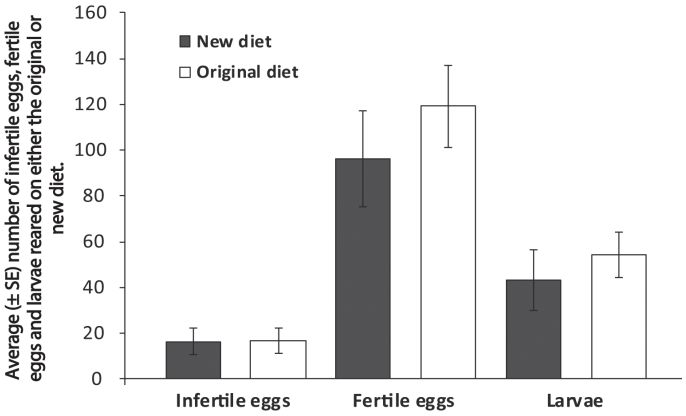


Fig. 3. Average (± SE) numbers of fertile eggs, infertile eggs and hatched larvae of insects reared either on the original diet or on the new modified diet.

For the second experiment with neonate larvae the average 3rd instar weight was 0.074 ± 0.006 g for the well plate method with the modified diet, 0.053 ± 0.004 g for the traditional fresh leaf method, and finally 0.058 ± 0.005 g for the Petri dishes with the modified diet (GLM; $\chi^2 = 6.374$; df = 2,112; $P = 0.041$) (Fig. 5a). The percentage survival of neonate larvae to the third instar was 88% for the well plates, 82 % for the fresh leaves and 60% for the Petri dish method (Fig. 5b).

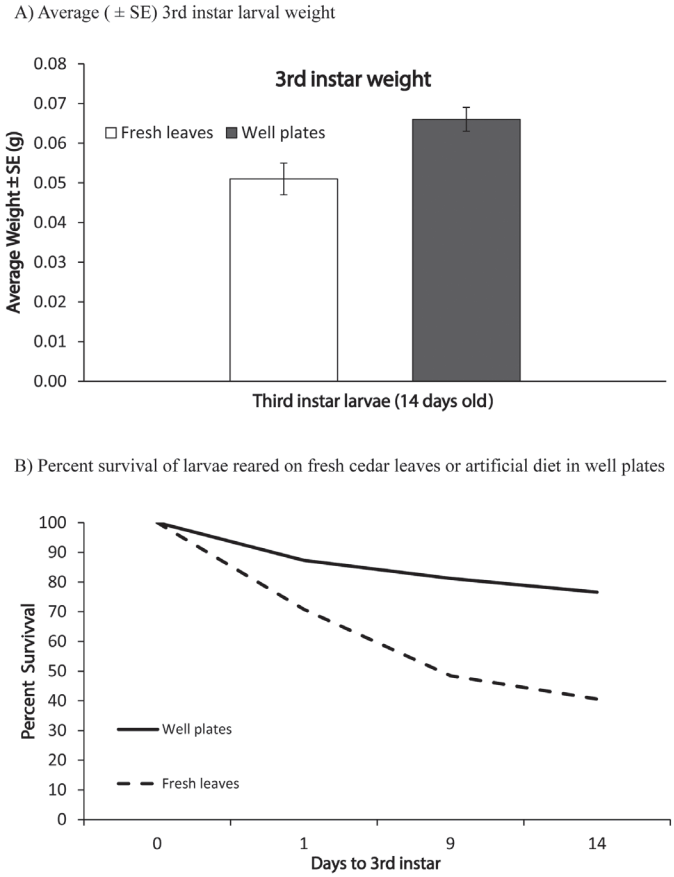


Fig. 4. A) Average (± SE) 3rd instar larval weight, and B) percent survival of larvae reared on fresh cedar leaves or on artificial diet in well plates.

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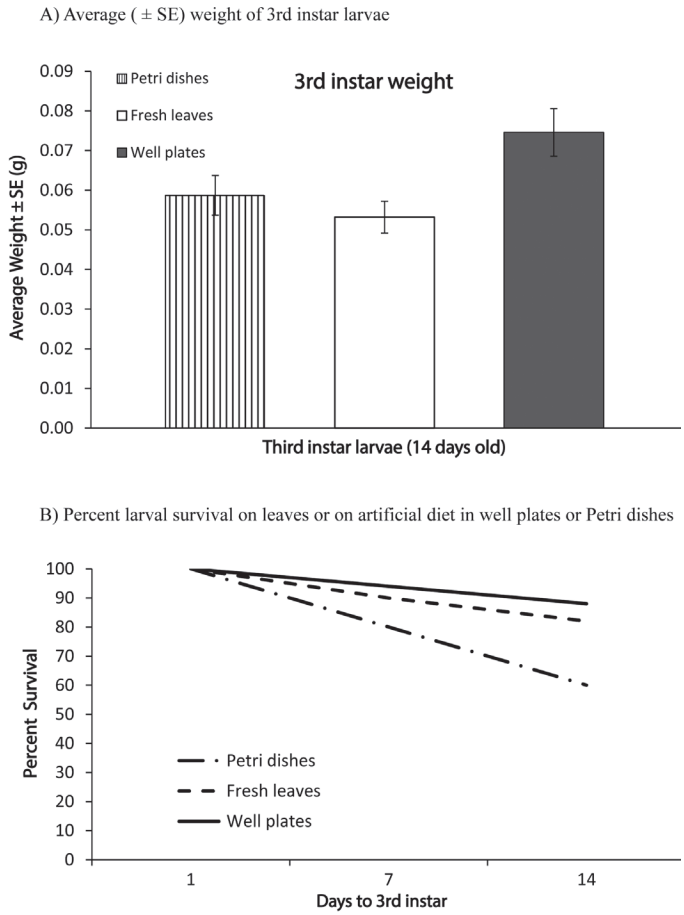


Fig. 5. A) Average (± SE) weight of 3rd instar larvae, and B) percent survival of larvae reared on fresh cedar leaves or on artificial diet either in well plates or in Petri dishes.

Discussion

Using the new modified diet, we eliminated the use of costly ingredients that are difficult to procure, and avoided the use of milled cedar seeds, which are not available year round. Furthermore, with this modified diet and modifications made to the rearing system *H. grandella* was able to complete its cycle and to produce fertile descendants entirely under laboratory conditions for 7 generations. To our knowledge, this had not been previously reported for this species.

Replacing jars with Petri dishes as well as reducing the size of the oviposition cages were critical to make the rearing methods more efficient. Both modifications decreased the amount of space required, which enabled the production of more individuals in a smaller space. This is an important cost-reduction factor when establishing a mass-rearing program.

Hypsipyla grandella completed its development cycle from egg to adult in 46–47.5 d at a temperature of 24–29 °C. These development periods were much longer than the life cycles of 30 and 36 d (at 30 and 25 °C, respectively) previously reported for this species (Taveras et al. 2004). The development period, however, was similar to the cycle of 35–45 d (24–26 °C) reported for *H. robusta* (Mo et al. 1998). The life cycle in natural populations of *H. grandella* can vary from 30 to 60 d (Griffiths 2001; Newton et al. 1998). It is also possible that this species enters diapause and extends the development period to 104 d (Howard & Mérida 2004).

Larval development after the third instar lasted 21.9 and 19.6 d using the modified and original diets, respectively. These results are similar to the 17–19 d (30 and 25 °C respectively) reported by Taveras et al. (2004). The longer development period of the larvae using the modified diet is however compensated by the lower cost and logistical benefits by replacing the expensive components that are not available all year round.

The average pupal weight obtained with the modified diet was greater than that reported for pupae of *H. grandella* reared with the original diet (Vargas et al. 2001), i.e., 0.124 and 0.081 g when maintained at 25 and 30 °C, respectively (Taveras et al. 2004). Our pupae were, however, lighter (0.148 g) than the average weights (0.164 g) reported for *H. grandella* (Sterringa 1973) reared on the modified diet of Hidalgo-Salvatierra (1971), and the average weight (0.150 ± 0.026 g) reported for *H. robusta* using the Couilloud & Guiol (1980) diet (Mo et al. 1998). These differences are most likely related to the different ingredients used to prepare these diets, the rearing methods which were slightly different, as well as different genetic backgrounds of the various populations and/or different environmental factors prevailing in the area where the populations were originally collected. The modified diet fulfilled the objective of successfully rearing larvae within the standard weight range (Cohen 2004).

The pupal period lasted 11 d when larvae were fed on both diets, which is similar to the period of 10.7 d reported for *H. grandella* by Sterringa (1973) and is within the period of 10 to 13 d (at 30 and 25 °C, respectively) reported by Taveras et al. (2004). It is also within the range of 8–10 d reported as pupal durations for wild populations of *H. grandella* (Newton et al. 1993; Briceño 1997; Griffiths 2001; Vargas et al. 2001; Howard & Mérida 2004). Likewise, the pupal period in this study is similar to the 11.5 d obtained for *Leucania separata* Walker (Lepidoptera, Noctuidae) reared on an artificial diet at 25 °C (Hirai 1976).

Deterioration of the diet in a mass-rearing factory can be an important cause of reduced fecundity and fertility of the reared insects (Jiménez-Pérez & Wang 2001). However here, no differences were found in these 2 parameters between the 2 diets. The new diet therefore fulfilled its role without producing adverse effects on the reproductive capacity of the insect. Both diets produced males and females in equal numbers, which agrees with the data reported by Mo et al. (1998) for laboratory-reared *H. robusta* and also with the data reported for wild populations (Newton et al. 1993; Hilje & Cornelius 2001). This is consistent with the sex ratio reported for other lepidopterans reared on an artificial diet such as the beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera, Noctuidae) (Elvira et al. 2010), the diamondback moth, *Plutella xylostella* L. (Lepidoptera, Plutellidae) (Hou 1986) and *L. separata* (Hirai 1976).

The use of well-plates allowed the successful rearing of neonate larvae that fed on the modified diet until the third instar, and it produced heavier larvae that survived better than with the traditional method of using fresh cedar leaves or using the neonate larval diet of Griffith & Smith (1977). Likewise, the well-plate method allowed the development of neonate larva to the fourth instar for the European cabbage butterfly, *Pieris rapae* (L) (Lepidoptera: Pieridae), the common armyworm, *Pseudaletia convecta* (Walker) (Lepidoptera: Noctuidae), the Indian mealmoth, *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae), the codling moth, *C. pomonella*, and the native budworm (*Helicoverpa punctigera* (Wallengren) (Lepidoptera: Noctuidae) (Griffith & Smith 1977). The use of the diet placed in well-plates, as reported here, eliminated the use of fresh leaves during the entire rearing process.

Rearing insects in isolation usually involves the transfer of neonates to escape-proof containers. The use of a large number of containers is inconvenient and expensive (Griffith & Smith 1977). A few trials to

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increase the rearing larval density were carried out, however none of the experiments were successful (data not presented), mainly due to cannibalism (NBJ, personal observation). Therefore, we suggest that larvae of *H. grandella* be reared at a density of 1 larva per container. Despite this limitation, we encourage further investigations to attempt an increase in larval rearing density, which would be more cost effective for mass-rearing conditions.

The ingestion of balanced nutrients plays a very important role in the growth and reproductive cycle of insects and allows continued rearing over several generations (Elvira et al. 2010). Before a diet can be considered appropriate for mass-rearing, it must be evaluated over several generations to determine whether the biological parameters vital for the survival, reproduction and normal behavior of the insect are maintained (Cohen 2001; Martínez-Martínez 2004). Further experiments should evaluate if laboratory insects have similar behaviors compared to their wild counterparts, including chemical signals, biotic interactions and responses to abiotic factors that are not present in laboratory conditions.

Through the following modifications: 1) a smaller rearing cage, 2) a modified larval diet, and 3) a modified neonate rearing method, we achieved uninterrupted rearing of *H. grandella* for 7 generations under controlled laboratory conditions. This rearing method represents an important step towards colony establishment, which will allow the detailed study of this pest and development of biorational control methods such as the SIT.

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