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Colonization and domestication of seven species of native New World hymenopterous larval-prepupal and pupal fruit fly (Diptera: Tephritidae) parasitoids

Martín Aluja[a]*, John Sivinski[b], Sergio Ovruski[c], Larissa Guillén[a], Maurilio López[a], Jorge Cancino[d], Armando Torres-Anaya[a], Guadalupe Gallegos-Chan[a] and Lía Ruíz[d]

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We describe the techniques used to colonize and domesticate seven native New World species of hymenopterous parasitoids that attack flies within the genus *Anastrepha* (Diptera: Tephritidae). All parasitoid species successfully developed on artificially reared Mexican fruit fly, *Anastrepha ludens* (Loew) larvae or pupae. The parasitoid species colonized were the following: *Doryctobracon areolatus* (Szépligeti), *Doryctobracon crawfordi* (Viereck), *Opius hirtus* (Fischer), *Utetes anastrephae* (Viereck) (all Braconidae, Opiinae), *Aganaspis pelleranoi* (Bréthes) and *Odontosema anastrephae* Borgmeier (both Figitidae, Eucoilinae) (all larval-pupal parasitoids), and the pupal parasitoid *Coptera haywardi* (Ogloblin) (Diapriidae, Diapriinae). We provide detailed descriptions of the different rearing techniques used throughout the domestication process to help researchers elsewhere to colonize local parasitoids. We also describe handling procedures such as number of hosts in parasitization units and compare optimal host and female age, differences in parasitism rate, developmental time, life expectancy and variation in sex ratios in each parasitoid species over various generations. In the case of *D. crawfordi* and *C. haywardi* we also provide partial information on mass-rearing techniques such as cage type, parasitization unit, larval irradiation dose and adult handling.

Keywords: hymenoptera; Braconidae; Figitidae; Diapriidae; Tephritidae; *Anastrepha*; biological control; parasitoids; rearing

Introduction

Historically the release of exotic (i.e. non-native) parasitoid species to deal with fruit fly pests has been the norm (Wharton 1989; Aluja 1994; Purcell 1998; Ovruski, Aluja, Sivinski, and Wharton 2000). In comparison, native parasitoids of indigenous pestiferous species have received little attention except for systematic studies and surveys of parasitoids of flies in the economically important genera *Anastrepha* (e.g. Wharton, Gilstrap, Rhode, Fischel, and Hart 1981; Aluja et al. 1990, 2003; Katiyar, Camacho, Geraud, and Matheus 1995; López, Aluja, and Sivinski 1999; Canal and

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Zucchi 2000; Ovruski, Schliserman, and Aluja 2004), *Bactrocera* (e.g. Wharton and Gilstrap 1983) and *Rhagoletis* (e.g. Wharton and Marsh 1978; AliNiazee 1985; Hoffmeister 1990; Gut and Brunner 1994; Feder 1995). The unstated perception has perhaps been that the long-standing co-existence of native parasitoids with flies that have remained pests was evidence that they were unable to exert economically significant levels of control. However, recent interest in the augmentative release of parasitoids (e.g. Sivinski et al. 1996; Purcell 1998; Montoya et al. 2000), with the possibility of strategically increasing the mortality inflicted by native species (Sivinski, Aluja, and López 1997; López et al. 1999; Sivinski, Piñero, and Aluja 2000), has given new impetus to studies of their colonization and mass rearing.

Around 205 species of the Neotropical genus *Anastrepha* have been described to date (Norrbom 2004). In Mexico, 37 species have been reported to date (Hernández-Ortíz and Aluja 1993; Hernández-Ortíz 1998, 2004; Hernández-Ortíz, Manrique-Sade, Delfín-González, and Novelo-Rincón 2002) and the larvae and/or pupae of these species are hosts for a diversity of parasitoids (Aluja et al. 1990, 2003; Hernández-Ortíz et al. 1994; López et al. 1999). Species such as *Diachasmimorpha longicaudata* (Ashmead), *Psyttalia incisi* (Silvestri), *P. concolor* (Szépligeti), *Fopius arisanus* (Sonan), *F. vandenboschi* (Fullaway), *Aceratoneuromyia indica* (Silvestri) and *Pachycrepoideus vindemiae* (Rondani) were introduced into Mexico as biological control agents, beginning in 1954 in an attempt to curb populations of the Mexican fruit fly, *Anastrepha ludens* (Loew) (Jiménez-Jiménez 1961; Wharton 1989; Ovruski et al. 2000). With similar intentions, non-native parasitoids were released in El Salvador, Nicaragua, Costa Rica, Panamá, Colombia, Perú, Brazil and Argentina (Wharton, Gilstrap, Rhode, Fischel, and Hart 1981; Ovruski et al. 2000). However, despite the large numbers of individuals introduced, few parasitoid species have successfully established (Ovruski et al. 2000). The shortcomings of this handful of exotic species has turned attention to the many native parasitoid candidates for augmentative release. Their diversity suggests that suitable species would be available for programs faced with an assortment of pests occurring in a variety of environments (Sivinski et al. 1997; Aluja, López, and Sivinski 1998; Sivinski and Aliya 2003).

To facilitate native parasitoid colonization efforts in other parts of the world, we describe the colonization and domestication of the following seven native *Anastrepha* parasitoids found in Mexico and various other countries in Latin America (in some cases the US) (updates on exact distribution can be found in Ovruski et al. 2000; Ovruski, Wharton, Schliserman, and Aluja 2005): *Doryctobracon areolatus* (Szépligeti), *Doryctobracon crawfordi* (Viereck), *Opius hirtus* (Fischer), *Utetes anastrephae* (Viereck) (all Braconidae, Opiinae), *Aganaspis pelleranoi* (Brèthes) and *Odontosema anastrephae* Borgmeier (both Figitidae, Eucoilinae) (all larval-prepupal parasitoids), and the pupal parasitoid *Coptera haywardi* (Ogloblin) (Diapriidae, Diapriinae). Recent findings on the biology, ecology, and behavior of the latter parasitoid species have been reported by Sivinski (1991), Sivinski et al. (1996, 1997, 2000), Sivinski, Aluja, Holler, and Eitam (1998a), Sivinski, Vulinec, Menezes, and Aluja (1998b), Sivinski, Aluja, and Holler (1999), Sivinski, Vulinec, and Aluja (2001), Aluja et al. (1998), Aluja et al. (2003), López et al. (1999), Guillén, Aluja, Equihua, and Sivinski (2002), Eitam, Holler, Sivinski, and Aluja (2003), Eitam, Sivinski, Holler, and Aluja (2004), Ovruski and Aluja (2002), Ovruski et al. (2004), Ovruski et al. (2005) and Guimarães and Zucchi (2004).

The most common and widely distributed *Anastrepha* native parasitoid species in the Neotropics and subtropics is *D. areolatus* (Ovruski et al. 2000). It is a larval-prepupal braconid parasitoid and broadly distributed from Mexico to Argentina (Wharton and Marsh 1978). When introduced into Florida in 1969, it became one of the most common parasitoids of *A. suspensa* (Loew) (Sivinski et al. 1998a; Eitam et al. 2004). In Mexico, *D. areolatus* and *U. anastrephae* are among the most numerous native species parasitizing larvae of *A. obliqua* (Macquart), a fruit fly that is an economically important pest of mango (*Mangifera indica* L.) and tropical plum (*Spondias purpurea* L.) (Aluja et al. 1996). *Utetes anastrephae* is also a larval-prepupal braconid parasitoid, but in comparison to *D. areolatus*, has the shortest ovipositor of any of the braconids sampled (Sivinski et al. 2001; Sivinski and Aluja 2003). This parasitoid species occurs naturally from Florida to Argentina (Ovruski et al. 2000).

Doryctobracon crawfordi is a larval-prepupal opiine parasitoid commonly associated with *A. ludens* (Plummer and McPhail 1941; López et al. 1999). Reported for the first time by L. de la Barrera (see Herrera 1905), this species apparently prefers more temperate climates (Aluja et al. 1998) and higher altitudes (Sivinski et al. 2000). *Opius hirtus* is another larval-prepupal parasitoid that commonly attacks the relatively rare *A. cordata* Aldrich in *Tabernaemontana alba* Mill. (Apocynaceae) (Hernández-Ortíz et al. 1994). It has also been reported attacking *A. obliqua* in *Tapirira mexicana* Marchand and *Spondias mombin* L (both Anacardiaceae) (Hernández-Ortíz et al. 1994; Sivinski et al. 2000), *A. alveata* Stone in *Ximenia americana* L. (Olacaceae) (López et al. 1999), *Toxotrypana curvicauda* Gerstaecker and *Ceratitis capitata* (Wiedemann) (Wharton 1983). *Aganaspis pelleranoi* and *O. anastrephae* are two figitid larval-prepupal parasitoids that gain access to *A. striata* Schiner and *A. fraterculus* (Wiedemann) in guavas through wounds or holes in the fruit (Ovruski 1994; Sivinski et al. 1997; Ovruski et al. 2004). *A. pelleranoi* is more widely distributed and has a broader host range than *O. anastrephae* (Wharton, Ovruski, and Gilstrap 1998).

One native, pupal endoparasitoid that has potential for fruit fly biological control is *C. haywardi* (Baeza-Larios, Sivinski, Holler, and Aluja 2002a; Guillén et al. 2002). It was originally discovered in Argentina attacking *A. fraterculus* and *A. schultzi* Blanchard pupae (Loiácono 1981). In 1994, *C. haywardi* was found in Veracruz, Mexico, attacking *A. ludens* pupae (López et al. 1999). More recently, this diapriine species was recovered from *A. striata* and *A. serpentina* (Wiedemann) pupae in Venezuela (García and Montilla 2001) and from *A. fraterculus* and *A. sororcula* Zucchi pupae in Brazil (Aguiar-Menezes, Menezes, and Loiácono 2003). Unlike many other pupal parasitoids of Diptera, it has a relatively restricted host range and is known only to parasitize Tephritidae (Sivinski et al. 1998b).

Materials and methods

Source of insects

In every case with the exception of *C. haywardi*, we obtained parasitoids by harvesting mature fruit from the tree or retrieving fallen fruit from the ground and transporting it to our laboratories in Xalapa, Veracruz, where they were processed following the methods described in Aluja et al. (1998), López et al. (1999) and Sivinski et al. (2000). In the case of *C. haywardi*, specimens stemmed from pupae that

Table 1. Location and host plant from which the individuals stemmed that were used to establish the first successful colonies.

Locality	Host plant	Fruit fly host	Parasitoid species
Llano Grande[1] and Tejería[2], Municipality of Teocelo, State of Veracruz, Mexico	*Spondias mombin* L. (Anacardiaceae)	*Anastrepha obliqua*	*Doryctobracon areolatus* *Utetes anastrephae*
		A. obliqua pupae	*Coptera haywardi*
	Psidium guajaba L. (Myrtaceae)	*A. fraterculus* and/or *A. striata*	*D. crawfordi, Aganaspis pelleranoi, Odontosema anastrephae*
	Citrus sinensis L. (Rutaceae)	*A. ludens*	*D. crawfordi*
La Mancha[3], Santiago Tuxtla[4] and San Andrés Tuxtla[5], State of Veracruz, Mexico	*P. guajaba* L. (Myrtaceae)	*A. fraterculus* and/or *A. striata*	*O. anastrephae*
Vicinity of Tapachula[6], State of Chiapas, Mexico	*Ximenia americana* L. (Olacaceae)	*A. alveata*	*O. anastrephae*
Playa Escondida[7] and Sontecomapan[8], Los Tuxtlas, State of Veracruz, Mexico	*Tabernaemantana alba* Mill. (Apocynaceae)	*A. cordata* larvae	*Opius hirtus*

[1](19°22'08'' N, 96°51'57'' W), [2](19°22'07'' N, 96°54'59'' W), [3](19°35'23'' N, 96°22'49'' W), [4](18°28'31'' N, 95°18'40'' W), [5](18°26'42'' N, 95°11'53'' W), [6](14°54'21'' N, 92°15'33'' W), [7](18°36'47'' N, 95°03'45'' W), [8](18°25'07'' N, 95°12'48'' W).

were collected underneath fruit naturally infested in the field or from lab reared pupae artificially exposed to parasitization in the field (details in López et al. 1999). Details on fruit fly (fruit) and parasitoid host (fruit fly larvae) species and the geographical location where the specimens for founding the colonies were collected are provided in Table 1.

Laboratory conditions

During the initial phases of the colonization and domestication processes, we maintained parasitoid colonies at the Fruit Fly and Parasitoid Laboratory of the Instituto de Ecología, A.C., Xalapa, México, at $25 \pm 1^{\circ}$C, $70 \pm 5\%$ RH, and a photoperiod of 12:12 h. Over time (i.e. several years of observations), much insight into the particular idiosyncrasies of each species was gained, and as a result, we moved established colonies of *D. crawfordi* and *C. haywardi* into a laboratory maintained at a lower temperature ($23 \pm 2^{\circ}$C). As previously noted, both species are common in areas above 800 m, with lower year-round temperatures. All the other species, typically found in warmer climates, were maintained in laboratories at $25 \pm 1^{\circ}$C. A separate laboratory, kept at $27 \pm 1^{\circ}$C, $70 \pm 5\%$ RH, 12:12 h photoperiod) was used to rear *A. ludens* adults, while larvae and pupae were kept at $30 \pm 1^{\circ}$C, $75 \pm 5\%$ RH in an additional room without light (i.e., full darkness). This species was used as a host for all the parasitoid species. Yet another laboratory was used to mass-rear *D. crawfordi* in Metapa de Domínguez, Chiapas ($24 \pm 2^{\circ}$C, $70 \pm 10\%$ RH, 12:12 h photoperiod).

Rearing of A. ludens *larvae as parasitoid hosts*

Our *A. ludens* strain was originally provided by the Comité Estatal de Sanidad Vegetal (DGSV-SAGARPA) in Xalapa, Veracruz, where it had been kept for over 200 generations. We placed 200 mL of *A. ludens* pupae in $30 \times 30 \times 60$-cm Plexiglas cages. Between 2,500 and 3,000 adults emerged 1–2 days later and were fed *ad libitum* with a mixture of hydrolyzed protein (Greif Bros. Corporation, Delaware, OH) and locally available refined sugar (no particular brand). Water was provided *ad libitum* by using 300-mL plastic bottles with a cotton wick. After 8 days, flies were provided with an artificial oviposition medium placed inside the cage, which originally consisted of a 10-cm dome-like, hollow, dark green hemisphere made of green cheesecloth (dyed with commercial fabric dye (Mariposa®, Colorantes Importados, S.A. de C.V., México D.F., Mexico) and paraffin (McPhail and Guiza 1956). This oviposition device was later replaced by a 12-cm diameter Petri-type plastic dish covered with green linen cloth and filled with transparent silicon or 'fuseleron' (Devcon®, Junta Flex, ITW Poly Mex SA de CV, Mexico). The plastic dish was placed upside down on top of the fly-holding cage so that females could insert their aculeus through the cloth and lay eggs into the 'fuseleron'. Once flies reached 8 days of age, eggs were collected daily over an 8-day period and washed in a solution of 2 g of sodium benzoate (Baker, J.T. Baker S.A. de C.V., Xalostoc, Edo. de México) dissolved in 1 L of purified water. After washing, eggs were placed on pieces of filter paper (Whatman No. 1, Whatman Int., Ltd., Maidstone, England) in Petri dishes, incubated for 4 days and then placed (2 mL per unit) in a $11 \times 26 \times 32$-cm plastic washbowl containing an artificial diet (ingredients in Appendix 1). Once the desired larval stage was reached (2nd and 3rd stage depending on parasitoid species), exposure to parasitoids was carried out according to the technique used for each particular species (details follow).

In the particular cases of the *D. crawfordi* and *C. haywardi* strains sent from Xalapa, Veracruz to the Laboratorios de Desarrollo de Métodos, Campaña Nacional Contra Moscas de la Fruta in Metapa de Domínguez, Chiapas, Mexico for mass-rearing purposes, parasitoids were exposed to irradiated *A. ludens* larvae (pupae in case of *C. haywardi*) produced locally (Domínguez, Hernández, and Castellanos 2002). For *D. crawfordi* we used larvae irradiated at 40 Gy and in the case of *C. haywardi*, irradiation dose for pupae was 30 Gy (Cancino, Ruiz, Sivinski, Gálvez, and Aluja 2008). Since irradiated larvae support parasitoid development but do not mature into fertile flies, removal of unattacked hosts from the colony is greatly simplified (Sivinski and Smittle 1990). Larvae (32,000) and pupae (25,000) were placed in 1-L containers and irradiated, in an atmosphere containing oxygen, using a Gammacell irradiator with a cobalt-60 source (Cancino et al. in press) located at the Medfly mass rearing facility in Metapa de Domínguez, Chiapas.

Cages for holding parasitoids

Various sizes of Plexiglas cages, covered with fiberglass and aluminum screen, were used to house parasitoids. Screen mesh size and cage size depended on the size of the parasitoid species kept inside (details in Table 2). In the case of Plexiglas cages, one side of each cage was covered with plastic wrap (Kleen Pack®; Kimberly Clark de México S.A. de C.V.) held in place by three strips of masking tape (Shurtape®,

Table 2. Summary of rearing procedures and handling conditions used during the domestication and colonization of seven native *Anastrepha* parasitoid species (all parasitoid colonies were maintained at 25 ± 1°C, 70 ± 5% RH, 12:12 h photoperiod) (see Figures 1–4 for further details on rearing cages and parasitization devices such as FF, SD and M-PD).

Species[1]	Rearing Plexiglas cage size	No. of parasitoids per rearing cage: Female	No. of parasitoids per rearing cage: Male	Host stage attacked	Host age (days)	Type of parasitization devices (and No. hosts per unit)	Host exposure periods (h)	No. of exposed hosts per parasitoid female and per hour
Doryctobracon crawfordi	30 × 30 × 30[1,2]	30	15	Larva	8	Fruit filled with guava – FF (50) Sandwich-type oviposition	36	0.05 larvae
						device one – SD1 (250) Sandwich type oviposition	36	0.23 larvae
						device two – SD2 (250)	7	19 larvae
D. areolatus	25 × 25 × 25[1]	30	15	Larva	8	FF (50)	36	0.05 larvae
						SD1 (250)	36	0.23 larvae
Utetes anastrephae	25 × 25 × 25[1]	40	20	Larva	7 – 8	FF (50)	48	0.03 lavae
						Modified Petri dish – M-PD (250)	24	0.26 larvae
						SD2 (250)	7	0.91 larvae
Opius hirtus	30 × 30 × 60[1]	40	20	Larva	8	FF (50)	36	0.04 larvae
						SD1 (250)	24	0.26 larvae
						SD2 (250)	7	0.91 larvae
Aganaspis pelleranoi	30 × 30 × 30[1]	30	15	Larva	9	Uncovered Petri dish – UP (250)	24	0.35 larvae
						UP (250)	7	1.19 larvae
Odontosema anastrephae	30 × 30 × 30[1]	30	15	Larva	9	UP (250)	24	0.35 larvae

Table 2 (*Continued*)

Species[1]	Rearing Plexiglas cage size	No. of parasitoids per rearing cage: Female	Male	Host stage attacked	Host age (days)	Type of parasitization devices (and No. hosts per unit)	Host exposure periods (h)	No. of exposed hosts per parasitoid female and per hour
Coptera haywardi	$30 \times 30 \times 30$[1]	30	15	Pupa	1 – 2	Covered pupae – CP (500)	168	0.10 pupae
		125	125	Pupa	2	Naked pupae – NP (800)	72	0.09 pupae

[1]The fiberglass screen that covered the cage frame had a 0.3-mm mesh size. [2]When *D. crawfordi* was mass-reared (details in text) we used an aluminum cage frame covered with a metallic screen (1-mm mesh size).

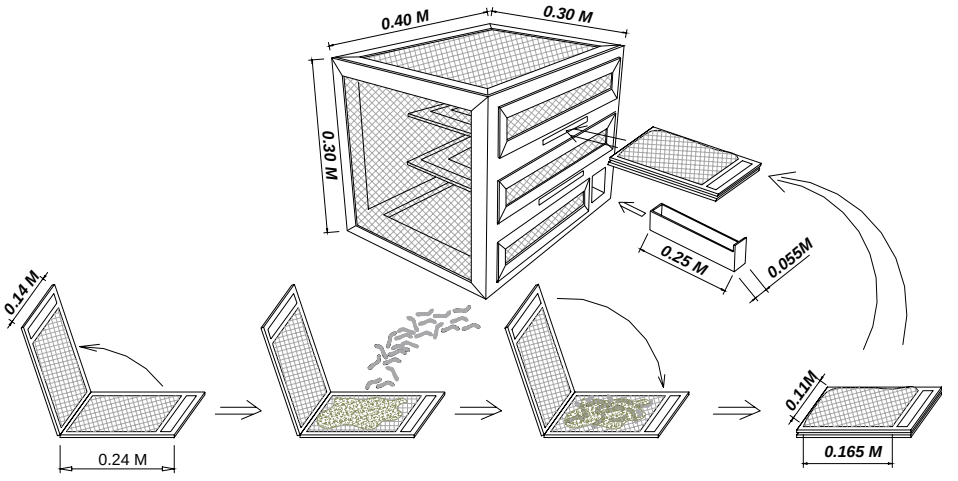

Figure 1. 'Metapa' cage used in initial *D. crawfordi* mass-rearing efforts. 'Cassette-type' oviposition units (compact disk cases) filled with larvae (2000 third instar larvae mixed with a small amount of rearing diet) were slid into cage openings in walls. Each cage contained 1,500 parasitoids.

Shurtape Technologies, Inc., Hickory, NC). A 150-mL container holding one or two orange, mango or guava (depending on availability) branches with five to eight leaves each, was placed in every cage to provide resting sites and adequate conditions for mating activities. In the case of *C. haywardi*, 10×10-cm pieces of black paper were used to form small (5×8 cm) resting shelters that were placed on cage floors (1–2 per cage). In each clean, sealed cage, we placed a predetermined number of newly emerged males and females from a given parasitoid species (details in Table 2).

For mass-rearing purposes (case of *D. crawfordi*), we used a $40 \times 30 \times 30$-cm cage with an aluminum frame, covered with a metallic mesh (1 mm) known as the 'Metapa' cage (Figure 1). In the cage front, there are two 15×1.5-cm openings that project inside of the cage by means of two 17×11.5-cm hollow aluminum squares (width of 2 cm) covered with the same 1-mm metallic mesh used to cover all cage walls. Inside the hollow squares, we slid the oviposition units, which consisted of empty compact disk cases ($10 \times 5 \times 1$ cm, length $\times$ width $\times$ depth) in which the top had been replaced by organdy cloth held tightly to the frame. Between the disk case bottom and the cloth cover, we placed 2,000 third instar *A. ludens* larvae mixed with some of the diet the larvae had been reared in (Figure 1). Each cage contained 1,500 parasitoids (sex ratio close to 1:1) that were allowed to parasitize larvae over a period of 4 h daily over 10 days. After this, they were replaced with a new cohort.

Feeding, and handling of adults

Adults were fed with diluted honey (70% honey, 30% water) (Miel Carlota®; Herdez S.A. de C.V., Cuernavaca, Morelos, Mexico). Pieces of cotton (Zuum®; Universal Productora S.A. de C.V., México D.F.) saturated with this liquid diet were placed in Petri dishes (10 cm in diameter) and offered to the parasitoids *ad libitum* (see Bautista, Harris, and Vargas 2001). Food was changed on a weekly basis. Water was

also administered on a piece of cotton and was changed two times per week. At the same time that food and water were changed, dead parasitoids were removed from the cages to avoid problems with fungi, bacteria, mites, and other insect pathogens. To keep parasitoids from escaping the cages while maneuvering objects within them, we temporarily shut the lights in the laboratory and used a 22-W lamp to attract the parasitoids towards the light.

Diagnostic features for quick recognition of the sexes

To facilitate quick recognition of the sexes, the following diagnostic features were used. In the case of braconid species, differences among the sexes were obvious because the female, besides being larger than the male, has an exerted ovipositor that is clearly visible (Sivinski et al. 2001; Sivinski and Aluja 2003). In the case of figitids, the most obvious character for identifying the sexes is the size and shape of the antenna, since the ovipositor is not apparent in females. Male antennae are filiform and 1.6–1.8 times longer than female antennae which are moniliform (Ovruski and Aluja 2002). In the case of *C. haywardi*, sex can also be distinguished by clearly different antennal lengths. Female and male antennae measure, respectively (mean $\pm$ SE), 1.7 ± 0.1 mm ($N = 20$) and 3.0 ± 0.2 mm ($N = 20$).

General conditions for the reproduction, management and care of parasitoids

Once field-collected larvae had pupated and adult parasitoids emerged, the domestication phase ensued. It initially consisted of adapting adults of each species to the artificial housing and rearing conditions associated with the laboratory. The first step was to identify and manipulate environmental conditions, such as temperature, required by each species. In addition, preliminary observations of mating and oviposition behaviors were conducted to determine which species parasitized larvae and which attacked pupae and what circumstances enhanced mating. To confirm that *C. haywardi* exclusively parasitized pupae (and not late third larval instars), females were offered two guavas containing 50, third instar *A. ludens* larvae. These fruit were removed before the larvae had pupated. At the same time, parasitoids were exposed to pupae (0–2 days old) for 7 days (168 h).

Description of oviposition units utilized to colonize each species of parasitoid

We tried to fabricate the cheapest and most natural oviposition devices to entice females to accept the artificial laboratory conditions (details in Figures 2–4). In what follows we describe the oviposition devices that worked best for us after several failed attempts.

Oviposition substrates for larval-prepupal parasitoids

Fruit filled with larvae (FF). Our objective was to simulate a naturally infested fruit that would be attractive to wild parasitoids, particularly in the initial stages of the domestication process. Commercial guava (*Psidium guajava*) was chosen as the preferred parasitization unit because: (a) almost all species of larval-prepupal parasitoids described in this work were found parasitizing fruit fly larvae in guavas in

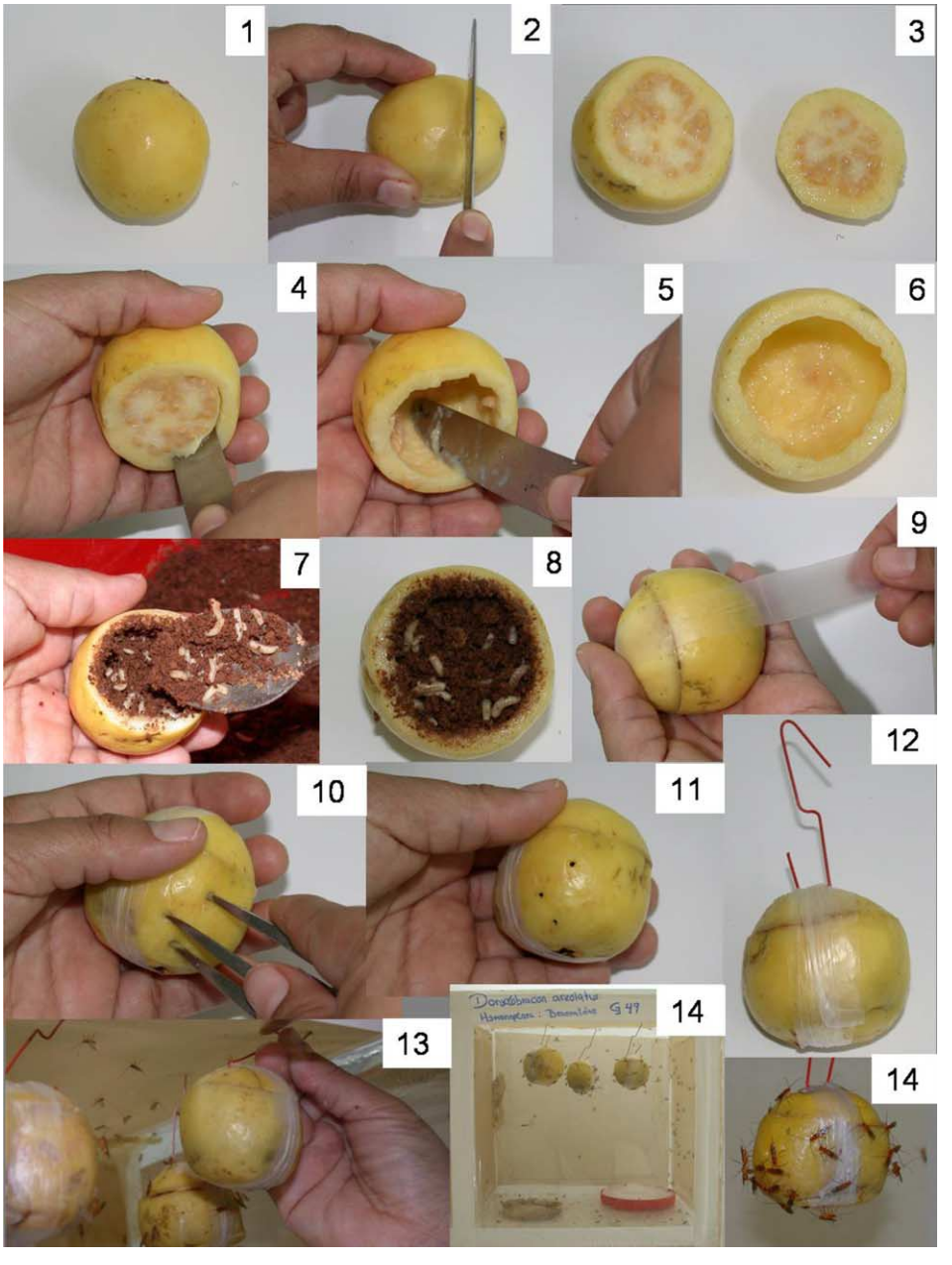

Figure 2. Description of the 'fruit filled with larvae (FF)' oviposition substrate used during the initial colonization stages of larval-prepupal parasitoids. (1–3) Cutting of fruit, with proximal quarter functioning as 'lid' and rest as 'base'. (4–6) Removal of pulp to create cavity (hollow 'base'). (7–8) Filling of hollowed 'base' with larvae mixed with diet. (9) Joining of 'base' and 'lid' with aid of 1.5 × 10-cm parafilm strip ('belt'). (10) Pricking of holes into of fruit. (11–12) Paper clip inserted into parafilm 'belt' to hang fruit from cage roof. (13) Fruit hanging from cage roof. (14) Parasitoids ovipositing in FF unit.

Figure 3. Modified Petri dish (M-PD) oviposition unit used to rear *U. anastrephae*, the parasitioid species with the shortest ovipositor (left). For comparative purposes (i.e. distinguish differences in thickness of oviposition unit), a 'sandwich-type oviposition device' is also shown (right).

the field (López et al. 1999) and (b) because guava can be obtained year round in local markets and supermarkets at a reasonable price. Guavas were cut open transversally along the peduncle, about one-quarter down the length of the fruit as measured from the proximal end (Figure 2). The proximal quarter sections functioned as 'lids' for the filled fruits and the remainder of the fruit served as 'bases' for filling. Mesocarp and endocarp (pulp) were extracted in the bases to

Figure 4. Preparation of the 'sandwich-type oviposition devices (SD)'. (A) Exposure of naked larvae without fruit skin. (B) One-mm (thickness) guava epicarp (skin) pieces placed on top of chiffon cloth covering larvae placed to entice female parasitoids to land on oviposition unit and parasitize larvae.

create cavities that could be filled with larvae and diet. Guavas had to be mature (yellow and soft, but not watery) and emit the characteristic odor associated with this fruit (i.e. not sealed with wax). However, if wax residues were encountered, they were removed by gently washing the fruit with diluted soap. The optimal size for guavas was 45–55 g and 4–5 cm in diameter. Larger fruit typically yielded smaller

numbers of parasitoids because females were unable to reach larvae feeding deep within the fruit (Sivinski 1991). The short ovipositor of *U. anastrephae* (Sivinski et al. 2001) restricts females to parasitizing larvae in small fruit such as *Spondias mombin* (López et al. 1999). As a consequence, we were forced to use small (25–30 g and 3–4 cm in diameter) larvae-filled guavas to colonize this species. We filled each fruit with ca. 50, laboratory-reared, second or third instar *A. ludens* larvae, and hung three or four guavas per rearing cage (Figure 2). Larval stage was associated to parasitoid species as described in Table 2. Once guava 'bases' were filled with larvae, they were covered with their corresponding 'lids' and the different parts tightly joined with 1.5×10-cm strips of parafilm (Parafilm 'belts') (Parafilm® Laboratory Film, American National Can Tm, Chicago, IL). Four to five holes were pricked into the fruit with a 1-mm metal needle to allow for aeration. Plastic paper clips were inserted into the Parafilm 'belts' to hang fruit from the cage ceilings where parasitoid density was usually highest. A variant of this technique was used in the case of *O. anastrephae* and *A. pelleranoi*, whose females prefer to enter into fruit interiors to search for fruit fly larvae (Ovruski 1994; Sivinski et al. 1997). For these species a 2-mm orifice was left in the upper portion of each guava (between the 'lid' and the 'base') to serve as an entrance for female parasitoids. Because adults of these two figitid species prefer to forage on the ground (Ovruski et al. 2004), fruit were not hung, but rather placed on cage floors.

Modified Petri dish (M-PD). This technique was only used in the case of *U. anastrephae*, which as noted before, has the shortest ovipositor of the species we were attempting to colonize. The oviposition unit consisted of 10-cm diameter Petri dishes, which we made shallower by scraping down ca. 50% of the walls (height was lowered from 0.9 to 0.4 cm) (Figure 3). We placed *A. ludens* larvae mixed with the diet on which they had been reared on the lowered Petri dish 'bottom plate' and tightly covered it with a stretched-out piece of Parafilm (original size was 5×5 cm). We chose to use Parafilm, because we had observed that the organdy cloth, which worked well in the case of other species, apparently did not provide the necessary mechanical aculeus stimulation that *U. anastrephae* females needed before parasitizing larvae.

Sandwich-type oviposition devices (SD). Once the parasitoids had reproduced for several generations using the 'fruit filled with larvae' technique (FF), the next step in the colonization process was to develop an artificial oviposition substrate for parasitoid females that was inexpensive and easy to handle. For this reason, we began to adapt adult parasitoids to 'sandwich-type devices' (SD) which were similar to the Petri dish methodology employed for mass rearing exotic opine parasitoids such as *D. longicaudata* and *D. tryoni* (Cameron) in Hawaii (Wong and Ramadan 1992). We used two kinds of SD devices (Figure 4).

Sandwich-type oviposition device one (SD1). This parasitization unit was suitable during the initial rearing stages of *D. crawfordi*, *D. areolatus*, and *O. hirtus* (Figure 2). It consisted of a 11.5×1.6-cm (diameter $\times$ height) plastic 'dish' with a bottom made of a 15×15-cm piece of chiffon cloth. On the cloth surface we placed ca. 250 *A. ludens* larvae mixed with the diet on which they had been reared. The age of the larvae depended on the species of parasitoid being reared (details in Table 2). The dish containing larvae and diet was covered with another 15×15-cm piece of chiffon cloth that was tied to the base by a 11.7×0.8-cm (diameter $\times$ height) plastic 'ring'

put in place by pushing against the base (i.e. pressure exerted with index fingers). After the 'sandwich' was built, we completely covered the chiffon cloth top with a layer of guava epicarp (skin) ca. 1 mm in thickness. The thin skin pieces were obtained by finely slicing the guava epicarp with a razorblade or sharp knife. The ultimate goal was to entice females to oviposit by mechanical and olfactory stimulation with the fragrant guava epicarp.

Sandwich-type oviposition device two (SD2). The parasitization unit was the same as described under SD1, but in this case larvae were exposed in naked form (i.e. not mixed with diet). Furthermore, we did not place a layer of guava epicarp but instead soaked the chiffon cloth with liquid guava pulp. This method turned out suitable to entice wild *D. crawfordi*, *D. areolatus*, *O. hirtus*, and *U. anastrephae* females to oviposit.

Uncovered Petri dish (UP). We discovered that the females of the figitids *A. pelleranoi* and *O. anastrephae* were suffering severe ovipositor damage while attempting to parasitize larvae in the oviposition units covered with chiffon cloth. Furthermore, because females of these species like to enter fruit in search of the larvae feeding inside, we used an uncovered unit. We used the bottom part of a Petri dish half filled with diet mixed with larvae. At the same time, half a guava was added to the artificial diet with larvae. The fruit, including seeds, was macerated into pieces and thoroughly mixed with the diet. In general, endocarp and mesocarp were utilized because the fruit's fragrance appeared to attract females and stimulate oviposition behavior.

Oviposition substrate for pupal parasitoid

Initial exposure of *A. ludens* pupae to *C. haywardi* was done in 500-mL plastic containers containing a ca. 10-cm layer of moistened soil (50–70% water content) and some leaf litter. Soil was brought from the original collection locality of Tejería, Veracruz (López et al. 1999), and was predominantly clay (Guillén et al. 2002). Approximately 500 recently formed pupae (1–2 days from pupation) were placed in the plastic container and mixed with the soil (referred to as CP method, i.e. covered pupae, in the text). Then, a mature guava placed on a galvanized wire screen was inserted into the container to lure parasitoids to the pupae underneath. The wire screen measured 10×10 cm with 1×1-cm mesh openings. Pupae were exposed to parasitism over a period of 7 days (Table 2). The guava was only inserted into the oviposition unit during the first three generations, after which time the parasitoids seemed to respond well to *A. ludens* pupae alone. After the 21st generation, soil and leaf litter were eliminated and only 'naked' pupae (referred to as NP-method, i.e. naked pupae, in the text) were exposed during 3 days on a 11.5×1.6-cm (diameter $\times$ height) plastic dish (Table 2).

Maintenance of parasitized larvae and pupae

In the case of M-PD (modified Petri dish), SD1 (sandwich-type oviposition device one (larvae mixed with diet)), and UP (uncovered Petri dish) exposures, larvae were cleaned of diet and guava residues by placing them in a fine mesh plastic colander and rinsing them under running tap water. Once clean, larvae were placed in 500-mL plastic containers with 2.5 cm^3 of moistened vermiculite where they formed puparia.

All containers were labeled, protected with a top made of chiffon cloth, and maintained under laboratory conditions ($25 \pm 1^{\circ}C$, $70 \pm 5\%$ RH) until fly or parasitoid adults emerged. In the case of FF (fruit filled with larvae) exposures, fruit was placed in 200-mL plastic vials, which in turn were placed inside 500-mL plastic containers with 2.5 cm^3 of moistened vermiculite. This was done to allow larvae to exit the fruit, a process that many times caused the fruit to disintegrate, spilling larvae and diet onto the floor of the 200-mL vial. On day 4, any diet or fruit residues were rinsed from pupae and larvae as described above and transferred to a 500-mL plastic container with moistened vermiculite, where they remained until adult emergence. The double container technique allowed us to avoid fungal and bacterial contamination that usually ensues if the vermiculite is mixed with fruit and diet residues.

Handling of emerged parasitoids and flies

Once parasitoids and flies had emerged, they were transferred to a clean, empty Plexiglas cage and provided with food and water. The size of the cage and the number of males and females per cage depended on the species (see Table 2). Daily inspection of containers with pupae was critical to make sure that emerging adults did not escape or suffer stress because of lack of food and water. Length of pupal period and associated timing of parasitoid emergence was species-specific and may occur before, after, or in synchrony with host emergence. In the case of parasitoids that emerge before their host (i.e. *U. anastrephae*), there was no need to separate adult parasitoids from adult flies since unemerged *A. ludens* pupae were simply removed and discarded once the adult parasitoids had emerged. In the case of parasitoid species whose emergence is more synchronous with host emergence (i.e. *O. hirtus*, *D. crawfordi*, and *D. areolatus*), we were forced to separate adult parasitoids from adult flies. This was done utilizing a standard aspirator. Adult parasitoids that were very sensitive to 'rough' handling (i.e. aspirator) like *D. areolatus*, or that were destined for behavioral studies, were separated using 10-mL glass vials into which insects walked. When parasitoids had a more prolonged pupation interval than their hosts (i.e. *A. pelleranoi*, *O. anastrephae*, and *C. haywardi*), the emerged adult flies and empty puparia were separated to leave only parasitized pupae. Separation of flies and empty puparia was critical to avoid fungal growth and to significantly lower the risk of contamination by mites.

Determination of percent parasitism, sex ratio, and pupal viability per generation

To measure percent parasitism, two 10-mL samples of parasitized *A. ludens* larvae (approximately 220 larvae) were processed per generation. The first sample was taken when parasitoid females reached, 4 and the second one when they reached 10 days of age. In the case of *C. haywardi*, instead of larvae, two random samples of 100 pupae were processed. The handling procedure for these larvae and pupae was the same as that described earlier. Once parasitoid adults had emerged, number and sex were recorded. Relative percent parasitism was estimated by dividing the total number of parasitoids that emerged by the total number of larvae exposed in the parasitization unit as we were not interested in an exact determination of the 'killing power' of each parasitoid species at this juncture (i.e. a certain proportion of larvae/pupae were parasitized and killed and therefore ended up not yielding an adult

parasitoid). Pupal viability was determined as the total number of pupae that yielded flies and parasitoids divided by the total number of unemerged and emerged pupae.

Demographic studies. Doryctobracon areolatus, D. crawfordi, *and* O. hirtus

Adults used in these tests stemmed from colonies that were 14 generations old. *Utetes anastrephae*, *A. pelleranoi* and *O. anastrephae* had been reared over nine generations, and *C. haywardi* over 24 generations. For these studies, braconid larval-prepupal species were only reared using *A. ludens* larvae in the FF (fruit filled with larvae) method, while figitid larval-pupal species were reared using *A. ludens* in the UP (uncovered Petri dish) method (Table 2). In all cases, 30 host larvae were exposed daily to 15 parasitoid pairs (i.e. 15 females and 15 males totaling 30 individuals per cage) for 24 h during their entire adult lifespan in Plexiglas rearing cages containing water and honey (details on size in Table 2). After exposure to parasitoid attack, host larvae were placed in plastic trays (500 mL) and provided with fresh larval diet. Three days after, formed pupae were separated from diet and transferred to other 500-mL trays with 150 mL of moistened vermiculite. All trays were taken into a room at $25 \pm 1^{\circ}$C, $70 \pm 5\%$ RH, and full darkness, where they remained until fly and parasitoid adults emerged. After all died (no food or water was provided), they were counted and sexed. In the case of the pupal parasitoid *C. haywardi*, 30 pairs (30 females and 30 males totaling 60 individuals per cage) were exposed daily to 20 two-day-old *A. ludens* pupae in 5×1.5 (diameter $\times$ height) plastic Petri dishes covered with 1 cm of vermiculite. Cages in this case, were $10 \times 10 \times 10$ cm in size, with glass walls and aluminum frame. Each study (i.e. one per species) was replicated five times.

Life table parameters (l_x, fraction of the original cohort surviving to age x; p_x, period survival; q_x, period mortality; d_x, fraction of the original cohort dying at age x; e_x, expectation of life; M_x, average number of male and female offspring produced by female at age x; m_x, female offspring per female at age x; Carey 1993, 1995) were calculated from daily mortality records and offspring data for cohorts of all larval-prepupal and pupal parasitoids. These values were used to determine reproductive parameters such as gross fecundity rate (GFR in text), net fecundity rate (NFR in text), cohort lifespans, and offspring sex ratios (as female proportions) and population parameters such as R_o (net reproductive rate), r (intrinsic rate of increase), λ (finite rate of increase), and T (mean generation time) (Carey 1993; Vargas et al. 2002). These demographic parameters helped us to estimate the relative population growth vigor of the first colonized cohorts.

Experiments to determine optimal pupal age to rear C. haywardi

We conducted two types of experiments with mated, 7-day-old females: no choice and choice tests. In each case we tested six treatments, each corresponding to an age class of the host (*A. ludens* pupae). Pupal age classes (days) tested were: 0–2, 3–5, 6–8, 9–11, 12–14, and 15–17. We used 30 pupae per age class, 10 per age included in every age class (i.e. in age class 0–2, there were 10 pupae each of ages 0 ($<$24 h or prepupa), 1 and 2 days). In the no choice experiment, we released 15 *C. haywardi* females together with 30 pupae of a determined age class in a 500-mL plastic container that was halfway filled with sterilized clayey soil (pupae were superficially buried). Exposure period was 24 h and the experiment was replicated five times for every age

Table 3. Parasitization rates (mean percent parasitism), proportion of emerged females, and pupal viability in all seven native *Anastrepha* fruit fly parasitoids as the domestication and colonization process proceeded.

Parasitoid species	Rearing method	% Parasitism[e] (Mean ± SEM)	% Emerged females (Mean ± SEM)	% Pupal viability[e] (Mean ± SEM)
D. crawfordi[a]	FF	38.7 ± 2.9	54.5 ± 2.8	55.9 ± 2.9
	SD1	20.8 ± 1.4	47.9 ± 2.5	58.8 ± 3.3
	SD2	37.9 ± 2.1	44.7 ± 2.6	43.6 ± 2.3
U. anastrephae[b]	FF	26.1 ± 4.3	58.7 ± 2.9	72.8 ± 2.6
	M-PD	20.4 ± 4.1	45.4 ± 3.3	50.4 ± 4.9
	SD2	25.2 ± 3.9	50.6 ± 3.7	39.7 ± 4.4
O. hirtus[a]	FF	24.7 ± 2.1	55.6 ± 2.7	61.0 ± 3.6
	SD1	16.5 ± 1.0	45.4 ± 2.2	55.5 ± 1.4
	SD2	13.7 ± 1.3	56.8 ± 3.9	64.7 ± 2.2
D. areolatus[a]	FF	24.3 ± 1.6	60.1 ± 1.9	56.1 ± 2.8
	SD1	11.1 ± 1.2	58.5 ± 2.6	54.4 ± 1.6
A. pelleranoi[a]	UP-24h	26.4 ± 1.8	58.3 ± 2.7	50.0 ± 2.4
	UP-7h	35.6 ± 2.9	46.8 ± 2.9	65.6 ± 4.3
O. anastrephae[c]	UP-24h (bisexual)	30.5 ± 2.6	61.1 ± 3.2	52.9 ± 4.1
	UP-24h (unisexual)	24.4 ± 1.2	100.0	56.2 ± 1.8
C. haywardi[d]	CP	3.8 ± 0.3	57.1 ± 1.3	8.4 ± 0.3
	NP	4.5 ± 0.3	48.2 ± 2.8	8.3 ± 0.4

[a]Data from first 14 generations; [b]data from first nine generations; [c]data from first 20 generations, [d]data from first 12 generations. FF (fruit filled with larvae), M-PD (modified Petri dish), SD1 (sandwich-type oviposition device one [larvae mixed with diet]), (sandwich-type oviposition device two [naked larvae]), UP (uncovered Petri dish filled with larvae mixed with diet and fruit pulp; 7 and 24 h refer to exposure period), CP (pupae covered with soil), NP (pupae exposed naked [without soil cover]).

class. In all cases (each replicate) we used a new cohort of females (i.e. no repeated measures on same cohort). In the multiple choice experiment, we released 90 females together with 180 pupae encompassing all age classes (30 pupae per age class) in a 15 × 10-cm (diameter × height) plastic container that was also half-filled with sterilized clayey soil. To distinguish pupae of every age class, they were individually marked with a dot of acrylic paint (six colors used) (Colores Acrílicos Indelebles Politec, Distribuidora Rodin, Mexico). Exposure period in this case was 36 h and we replicated each experiment five times. The pupae were handled as already described before until all parasitoids emerged and were counted.

Statistical analyses

Owing to the fact that colonization efforts where not simultaneous and that we typically only had access to a small number of individuals of any given species at any particular time, we could not run any formal statistical analyses comparing the performance of the various rearing methods. Nevertheless, overall trends can be ascertained by visually comparing data summarized in Tables 3 and 5. In the case of the experiment to determine optimal *A. ludens* pupal age to rear *C. haywardi*, we ran a one-way ANOVA comparing percent parasitism, sex ratio and proportion of

unemerged puparia (sometimes the host is killed due to single or multiple parasitoid stings). *Post-hoc* mean comparisons were done by means of a Tukey honest significant difference test (HSD) at an a of 0.05. Proportions were arcsine square root transformed prior to analysis, but untransformed means are presented in the text.

Results

Colonization and adult handling conditions

A summary of parasitoid rearing and handling procedures is provided in Table 2. In what follows, we report the most relevant results of the colonization efforts on a per species basis to facilitate domestication and colonization efforts in other parts of the world. We place emphasis on sex ratios, percent parasitism and mean proportion of pupae yielding a parasitoid given that these parameters greatly influence the success rate of the domestication/colonization process early on.

Doryctobracon crawfordi. The domestication process of this species was initiated in October 1994, using the FF (fruit filled with larvae) method over 10 generations. Then gradually, between the 10th and 15th generations, we exposed the parasitoids to the SD1 (sandwich-type oviposition device using larvae mixed with diet) method. The length of the larval exposure period was the same in both cases (Table 2). The sex ratio for both FF and SD1 parasitoids varied throughout the colonization process. For example, for FF parasitoids, the smallest proportion of females occurred in the first four generations (0.4–0.9:1). From generation 5 to 42 and with only one exception (generation six, 0.7:1), the sex ratio consistently favored females (1.1–7.0:1). Similarly, the lowest proportion of SD1 females was observed in the first eight generations (0.3–0.9:1), whereas the highest appeared after generation 9 (1.1–2.6:1; generations 9–14). Starting with generation 14, the SD1 technique was replaced by method SD2 (sandwich-type oviposition device using naked larvae). The sex ratio in the SD2 strain varied sharply from generation to generation over the 44 generations recorded (most likely due to variations in host quality). The lowest proportions of SD2 females were 0.2:1, whereas the highest proportions were 6:1 (mean values in Table 3). Percent parasitism levels during the first 14 generations using the three rearing methods varied between 15.5–62.3% (FF), 9.1–41.8% (SD1) and 20.0–56.8% (SD2) (mean values in Table 3).

Doryctobracon areolatus. This parasitoid species presented various challenges during the early stages of the domestication/colonization process. Among the most difficult ones to overcome was a propensity to enter what appeared to be a reproductive diapause from late November until almost March (coldest time of the year), despite the fact that we controlled temperature and lighting conditions inside the laboratory. As a result, from 1993 to 1997 we were only able to keep temporary colonies (all eventually died out) by using plums (*Spondias purpurea* and *S. mombin*) and mangos (*Mangifera indica*) naturally infested by *A. obliqua* (collected in the field) and parasitized by *D. areolatus.* Later, in July of 1997, we were able to successfully establish two *D. areolatus* colonies using artificially reared *A. ludens* larvae as a host, taking advantage of an unusually high parasitism rate in *A. obliqua* developing in the above mentioned fruit species. One of the colonies was maintained employing the SD1 (sandwich-type oviposition device using larvae mixed with diet) method while the

other colony was maintained using the FF (fruit filled with larvae) technique (see Table 2 for details). As was the case with *D. crawfordi*, sex ratios in both successfully colonized strains tended to be initially male-skewed. However, in subsequent generations the proportion of males and females was gradually equalized or favored females. The lowest proportion of FF females was observed in first and second generations (0.7–0.8:1) and thereafter (up to generation 69) it reached a maximum of 3.5:1. Overall, sex ratios of SD1 parasitoids were female skewed but intergenerational variation was greater than that observed in FF parasitoids (Table 3). Parasitism rates during generations 1–69 (68 in the case of the SD1 method) using the FF method varied from 8.2 to 36.4% between first and 69th generation, whereas employing the SD1 technique varied from 1.4 to 25.9% between first and 68th generation (Table 3).

Opius hirtus. Domestication of the first strain of this species was initiated in October 1994, through FF (fruit filled with larvae) exposures. However, the colony was lost in generation 6 (March 1995). We believe that failure hinged principally on the fact that females were probably not mating because of saturation of the environment with sexual pheromones (a very strong fruit-like bouquet was perceived near the cage). We therefore doubled cage size and introduced citrus branches with ample foliage as resting sites (tips of branches were inserted into 60-mL glass vials covered with cotton to prevent the parasitoids from drowning). After the original failure, a new colonization attempt was initiated in January 1996 with a few (<20) parasitoids obtained from a rare *Anastrepha* species (*A. cordata*) collected in the few remaining patches of tropical evergreen rainforest in Southern Veracruz, Mexico. Due to the difficulties involved in finding parasitoids in nature and considering our initial failure, we maintained three strains along the domestication/colonization process. Initially, we used the FF technique and then (generation six), started a new line using the SD1 (sandwich-type oviposition device using larvae mixed with diet) method (Table 2). Three generations later, we started a third line, by switching to the SD2 (sandwich-type oviposition device using naked larvae) method. In the latter case, we reduced the exposure period 5-fold with respect to the other rearing methods. Because in nature *O. hirtus* females are faced with very low host densities, we wanted to reduce the risk of larvae being marked with a marking pheromone that would have caused females to quickly leave the 'resource patch'. Sex ratios in the FF strain tended to be initially (generations 1–7) male-skewed (0.4–0.9:1), but in subsequent generations (8–37), favored females (1.3–5.6:1). In general, sex ratios of SD1 parasitoids were more male-skewed than FF parasitoids. In the case of the SD2, sex ratios were highly variable over time (0.2–5.3:1 over 115 generations). Mean parasitism was highest under the FF rearing method (Figure 2). Parasitization rates varied between 9.5–59.1, 7.2–26.4, and 6.8–31.4% in the FF, SD1, and SD2 lines, respectively. Pupal viability was highest in the FF and SD2 lines (Table 3).

Utetes anastrephae. This parasitoid presented a particularly difficult challenge because of its extremely short ovipositor and the fact that it is usually reared from only very small fruit in nature (e.g. *S. mombin*, *Tapirira mexicana*; López et al. 1999; Sivinski et al. 2000, but see Eitam et al. 2004 for exceptions to the rule). The first unsuccessful colonization attempt was made in October 1996 using the FF rearing method (after fourth generation no adults emerged). In September 1999, another attempt was made using ca. 800 female parasitoids collected from *A. obliqua* larvae infesting *S. mombin*. The original colony was divided into FF (fruit filled with larvae) and M-PD (modified Petri dish) strains. After four generations, we initiated a third strain (SD2

(sandwich-type oviposition device using naked larvae)) with M-PD material. Exposure periods in the M-PD and SD2 strains were reduced 2–7-fold with respect to the FF strain to avoid superparasitism caused by easier access to larvae (Table 2). Sex ratios in the FF strain were slightly male-skewed in the first two generations (0.8–0.9:1), but then remained relatively stable over the next 11 generations, with a consistent tendency for more females to emerge than males (1.1–6.5:1). In contrast, sex ratios in the M-PD and SD2 strains were highly variable between generations. The lowest proportion of females fluctuated between 0.3 and 0.9:1 in both M-PD and SD2 strains, while the greatest proportions fluctuated between 1.1–2.3:1 and 1.0–7.0:1 in the M-PD (first 11 generations) and SD2 (first nine generations) (mean $\pm$ SE values in Table 3). Parasitization rates varied between 6.8–51.8, 1.8–56.4, and 3.6–60% in the FF, M-PD, and SD2 rearing methods, respectively (mean $\pm$ SE values in Table 3). Finally, we found that pupal viability in insects stemming from FF lines was higher than those stemming from M-PD and SD2 lines (Table 3).

Aganaspis pelleranoi. A colony of this figitid parasitoid was initiated in September of 1994, using adults obtained from field-infested *P. guajava*. At first, parasitoids were reared with the variant of the FF (fruit filled with larvae) technique described in Section 2, but few individuals were obtained per generation. Therefore, beginning with the fifth generation, this technique was replaced by the UP (uncovered Petri dish) rearing method, allowing us to reduce exposure periods 3-fold (Table 2). In general, and with few exceptions (e.g. generation one), sex ratio in UP-24h (24 h refers to the exposure period in hours) parasitoids favored females over the first 14 generations. In the case of the UP-7h strain, sex ratios were highly variable, ranging between 0.2 and 8:1 (78 generations considered). Parasitization rates varied between 20.0–68.2 and 11.8–43.6% in the UP-7h and UP-24 h lines, respectively. Also, pupal viability in UP-7h lines was higher than in UP-24h lines (Table 3).

Odontosema anastrephae. The first unsuccessful attempt at colonization was started in November of 1995. For the first two generations, we employed the variant FF (fruit filled with larvae) method, but extremely low yields forced us to switch to the UP (uncovered Petri dish) technique using 36-h exposure periods. However, extremely low oviposition activity by females and an extremely male-biased sex-ratio (as low as 0.2:1), lead to the demise of the colony after eight generations. After a 3-year search for sufficient wild material, we were finally able to start a new colony between September and November 1998, using the UP rearing technique. A second *O. anastrephae* colony was started in February 2000, with wild material stemming from guavas. Interestingly, starting with generation 11 (December 2000) essentially only females emerged (such a pattern has remained steady over more than 75 generations). On occasion, one or two males emerged (sex ratio of 1: 0.008), but when such was the case, we immediately removed them given our interest in maintaining a theliotokous line. In both lines, exposure period was gradually reduced to 6 h (details in Table 2). In the case of the bisexual *O. anastrephae* colony, sex ratios varied greatly between generations, fluctuating between 0.1:1 (first four generations) and 1.1–8.0:1 in the remaining generations (29 generations considered). Parasitism rates varied between 7.7–79.5 and 12.0–37.8%, in the unisexual and bisexual colonies, respectively. Pupal viability in the bisexual and unisexual *O. anastrephae* colonies was similar (Table 3).

Coptera haywardi. This endoparasitic pupal parasitoid was first colonized in November of 1994 by means of the CP-method (covered pupae (artificially buried in soil)). Starting with the 21st generation, we replaced this rearing technique with the NP-method (naked pupae (soil removed)), which is still currently used because of its practicality. Sex ratio in CP-line favored females in all generations (1.1–2: 1), except one (generation 15, 0.7:1; data stem from generations 4 to 20). In the case of the NP-line, sex ratios varied more, fluctuating between 0.5 and 2.5:1 (34 generations considered). Parasitism rates varied initially between 2.2–6.0 and 2.8–7.4% in the CP and NP lines (first 12 generations obtained using each rearing method). Currently (generations 35–42 in NP method), parasitism rates have reached $21.4 \pm 1.1\%$ (range 11.3–27.1%, $n = 16$), and sex ratios fluctuate between 0.4 and 2.5:1. Data on mean parasitism rates, mean proportion of emerged females and pupal viability for the first 12 generations are shown in Table 3.

Results of the experiments to determine optimal pupal age are summarized in Table 4. Under choice conditions, parasitism in pupal age classes 0–2, 3–5, and 6–8 was significantly higher than in age classes 9–11, 12–14, and 15–17 (one-way ANOVA, $F_{5,24} = 78.43$, $P < 0.0001$). Similar results were obtained in the no-choice experiment (one-way ANOVA, $F_{5,24} = 31.46$, $P < 0.0001$). Mean parasitism in the optimal pupal age class varied between 60 and 70% in the no-choice experiment and between 36 and 55% in the choice one (further details in Table 4). With respect to sex ratios, in both choice and no-choice experiments, mean proportion of females was similar in parasitoids emerging from pupae within the first 5 age classes (i.e. 0–2, 3–5, 6–8, 9–11, 12–14) but different when compared to the sixth age class (15–17 days) (one-way ANOVA, $F_{5,24} = 3.50$, $P = 0.0161$ and $F_{5,24} = 6.26$, $P = 0.0008$ for the choice and no-choice conditions, respectively) (Table 4). There were no statistically significant differences among age classes with respect to the proportion of unviable (i.e. unemerged) pupae in the choice experiment ($F_{5,24} = 0.99$, $P = 0.4425$). The situation

Table 4. Percent parasitism, sex ratio (proportion of females) and proportion of uneclosed pupae in the experiments designed to determine the optimal host age (*Anastrepha ludens* pupae) for *Coptera haywardi*. Experiments conducted under choice (pupae of varying ages offered simultaneously to ovipositing females) and no-choice conditions (females offered pupae of only one age class).

	Choice experiment (mean ± SEM)			No-choice experiment (mean ± SEM)		
Age class of *A. ludens* pupae	% Parasitism	% Females	% Unemerged pupae	% Parasitism	% Females	% Unemerged pupae
0–2	55.3 ± 2.3a	52.7 ± 4.1a	37.3 ± 5.8a	60.7 ± 5.0a	45.1 ± 7.0a	30.7 ± 5.9ab
3–5	46.0 ± 3.9ab	52.2 ± 7.2a	44.7 ± 5.9a	68.0 ± 5.1a	55.2 ± 12.8a	17.3 ± 4.6a
6–8	36.7 ± 5.5b	32.3 ± 5.9a	57.3 ± 6.4a	60.9 ± 8.0a	55.7 ± 6.2a	28.7 ± 8.1ab
9–11	20.0 ± 2.9c	42.2 ± 17.4a	58.0 ± 9.6a	43.3 ± 10.7a	76.1 ± 2.2a	41.3 ± 5.5ab
12–14	3.3 ± 1.1d	20.0 ± 20.1a	66.7 ± 8.8a	12.7 ± 2.7b	48.3 ± 20.5a	52.0 ± 5.7b
15–17	0.0 ± 0.0d	0.0 ± 0.0b	52.7 ± 16.1a	0.0 ± 0.0b	0.0 ± 0.0b	51.7 ± 2.2b

Means within a column followed by the same letter are not significantly different (Tukey HSD test, $P = 0.05$).

changed in the case of the no-choice experiment, since significant differences were detected ($F_{5,24} = 5.91$, $P = 0.0011$) (Table 4).

Demographic parameters

Reproductive and population parameters for larval-prepupal and pupal parasitoid species are summarized in Table 5. Highest GFR, NFR, R_o, r, and λ were recorded in the diaprid *C. haywardi* and in the braconid *D. crawfordi*. Mean generation time (T) was longest in the case of *C. haywardi* and *A. pelleranoi*, while it was short and similar in *D. areolatus*, *D. crawfordi*, and *O. hirtus*. Mean life spans in all larval-prepupal parasitoid species were quite short (<15 days). In contrast, in the pupal parasitoid *C. haywardi* lifespan was almost twice as long (Table 5). Survivorship curves for all species are shown in Figure 5.

Discussion

Our multiyear effort aimed at domesticating and colonizing various native fruit fly parasitoids resulted in many practical lessons that will hopefully facilitate similar efforts elsewhere in the world. Clearly, there were a number of major hurdles to overcome before successful establishment of stable colonies was achieved: (1) availability of large enough numbers of wild parasitoids to start a colony in the cases of rare species like *O. hirtus*. (2) Availability of a stable supply of high quality larval or pupal hosts. (3) Finding a fruit species that is available year round and that emits volatiles attractive to as many parasitoid species as possible and that can therefore be used to entice females to lay eggs under highly artificial laboratory conditions (e.g. guava in our case). (4) Building oviposition units that expose sufficient larvae to the attack of females with varying ovipositor sizes. (5) Overcoming the initially highly male-biased sex ratio, presumably due to lack of mating that in many cases led to the demise of the incipient colony. (6) Overcoming apparent pheromone saturation in the small rearing cages that can lead females to not mate or do so reluctantly. (7) Finding ideal environmental conditions to suit the idiosyncrasies of each species. (8) Cost considerations as the domestication and colonization processes are labor and material intensive and therefore end up being expensive.

As noted by Vargas et al. (2002), knowledge on parasitoid demographic parameters is critical when trying to select candidate species for fruit fly biological control. Our data here, added to the wealth of knowledge already accumulated on the basic biology and ecology of native *Anastrepha* parasitoids (e.g. Sivinski et al. 1997, 2000; Aluja et al. 1998, 2003; Eitam et al. 2003, 2004) highlights the potential that species such as *D. crawfordi*, *O. hirtus* and *C. haywardi* have for augmentative release programs in regions with variable climatic and host density conditions. Furthermore, in many Latin American countries, in addition to dealing with pestiferous *Anastrepha* species, the presence of *C. capitata* is often the main concern for growers. Two of the species colonized here (i.e. *A. pelleranoi* and *C. haywardi*) are the only native parasitoids shown so far to be able to attack this important agricultural pest (Ovruski et al. 2004, 2005).

Being able to choose among many parasitoid species opens up the possibility to release the one best adapted to the particular climatic and ecological conditions of a

Table 5. Basic demographic parameters for seven native *Anastrepha* larval-prepupal and pupal parasitoids successfully colonized.

Demographic Parameter (Mean ± SEM)	Parasitoid species						
	D. areolatus[1]	*D. crawfordi*[1]	*U. anastrephae*[2]	*O. hirtus*[1]	*A. pelleranoi*[2]	*O. anastrephae*[2]	*C. haywardi*[3]
Offspring sex ratio (female proportion)	58.58 ± 6.45	50.43 ± 2.98	49.41 ± 2.58	60.25 ± 3.84	55.02 ± 3.79	30.90 ± 4.97	56.41 ± 2.47
Cohort lifespan (days)	9.82 ± 0.41	11.09 ± 0.11	10.50 ± 1.37	11.23 ± 3.02	7.94 ± 1.11	5.34 ± 0.35	28.04 ± 1.87
GFR (gross fecundity rate) (offspring/female)	6.61 ± 1.75	29.15 ± 8.30	2.87 ± 0.40	6.27 ± 2.04	13.57 ± 1.84	13.26 ± 2.20	85.13 ± 6.63
NFR (net fecundity rate) (offspring/female)	2.19 ± 0.41	10.68 ± 1.40	2.64 ± 0.34	2.14 ± 0.37	5.17 ± 0.74	5.54 ± 0.81	63.70 ± 0.76
R_o (net reproductive rate) (female offspring/generation)	1.39 ± 0.16	5.36 ± 0.66	1.34 ± 0.20	1.27 ± 0.13	2.84 ± 0.53	1.44 ± 0.21	35.24 ± 0.78
r (intrinsic rate of increase) (per female per day)	0.03 ± 0.01	0.24 ± 0.04	0.07 ± 0.04	0.03 ± 0.01	0.13 ± 0.03	0.09 ± 0.03	0.25 ± 0.01
λ (finite rate of increase) (per day)	1.04 ± 0.01	1.27 ± 0.05	1.08 ± 0.04	1.03 ± 0.01	1.15 ± 0.03	1.09 ± 0.03	1.28 ± 0.01
T (mean generation time) (days)	8.65 ± 0.87	7.69 ± 1.44	3.08 ± 0.39	8.46 ± 0.68	7.49 ± 0.45	4.07 ± 0.73	14.37 ± 0.39

[1]Individuals stemmed from colonies that were 14 ([1]), 9 ([2]) and 24 ([3]) generations old, respectively.

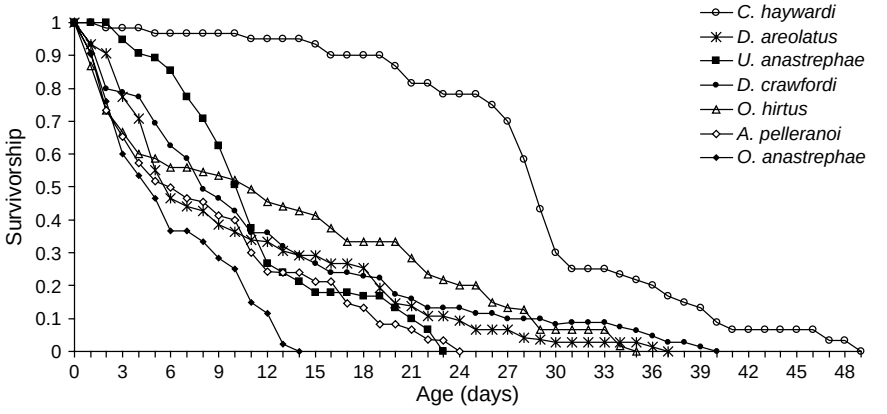

Figure 5. Survivorship (lx) curves for *Doryctobracon areolatus*, *D. crawfordi*, *Opius hirtus*, *Utetes anastrephae*, *Aganaspis pelleranoi*, *Odontosema anastrephae* and *Coptera haywardi*.

fruit growing region (e.g. temperature, rainfall, host density, larval host) which can greatly influence the efficacy of the control agent released or strategy implemented (Ovruski et al. 2000; Sivinski et al. 2000). In Mexico, a good example of the latter is represented by the native *D. crawfordi* and the recently introduced (1954–1955, quoted in Jiménez-Jiménez 1956) exotic species *D. longicaudata* which, given the short time of their interaction (<50 years), have not been able to partition the niche in which they forage in nature (Miranda 2002). Both have long ovipositors (Sivinski et al. 2001) and thus are able to attack third instar *A. ludens* larvae in large fruit such as *Citrus sinensis*, *C. paradisi* and *M. indica* in perturbed environments (López et al. 1999) where they exhibit similar distributions in tree canopies (Sivinski et al. 1997). Of the two species, *D. longicaudata* has already been successfully released augmentatively to reduce populations of *A. ludens* and *A. obliqua* in mango plantations in warm, lowland areas of the Soconusco region in Chiapas, Mexico (Montoya et al. 2000). Interestingly, here we found that *D. crawfordi* was not only the species exhibiting the highest *r* values of all larval-prepupal parasitoids studied (Table 5), but its intrinsic rate of population increase was twice as high as the one reported for *D. longicaudata* reared on *Bactrocera dorsalis* (Hendel) under laboratory conditions (Vargas et al. 2002). As documented by Sivinski et al. (2000), *D. crawfordi*, in contrast to *D. longicaudata*, prefers more humid, temperate environments and does not enter diapause (which is the case with *D. longicaudata*; Aluja et al. 1998). According to Miranda (2002), each species should be released singly in different environments owing to the fact that they compete for the same resource. A particularly interesting potential release site for *D. crawfordi* is in areas where the native *A. ludens* host (*Casimiroa greggii* [S. Watts]) is abundant (e.g. canyons and mountain slopes in Tamaulipas and Nuevo León, Mexico), allowing fly populations to increase and cause damage to commercial citrus groves planted nearby. *D. crawfordi* is indigenous to those areas (González-Hernández and Tejada 1979), rendering augmentative releases of this native species instead of the exotic *D. longicaudata*, more environmentally friendly (Simberloff and Stiling 1996).

Despite the fact that *D. areolatus* was one of the native species with one of the lowest *r* values, it nevertheless exhibits certain ecological advantages over *D.*

crawfordi. For example, it is the most widely distributed native fruit fly parasitoid in the Neotropics (i.e. Florida to Argentina) and exhibits a close association with *A. obliqua* in native fruit species within the Anacardiaceae (Ovruski et al. 2000). As is the case with the exotic *D. longicaudata*, *D. areolatus* also prefers warm and drier environments at lower altitudes (Sivinski et al. 2000; but see below). Based on the fact that Vargas et al. (2002) reported a 4-fold higher intrinsic rate of increase in *D. longicaudata* when compared to what we found here for *D. areolatus*, the logical inference would be that the exotic species is a better candidate for augmentative releases. But recent evidence gathered in Florida where both species coexist (Eitam et al. 2004), indicates that at least in that part of the world, the distribution of *D. longicaudata* was negatively related to variance in monthly temperatures (it was most abundant in southern Florida along the Atlantic and Gulf coasts). These authors also reported that *D. longicaudata* may depend on a constant supply of hosts. In contrast, *D. areolatus*, a species that is able to diapause over extended periods (11 months; *D. longicaudata* did so only over a 7-month period) (Aluja et al. 1998), was the dominant species in most interior locations (Eitam et al. 2004). Based on the findings of Eitam et al. (2004), in Florida *D. areolatus* is apparently a superior searcher, while *D. longicaudata* a superior intrinsic competitor. So, as was the case with the previous example (*D. crawfordi*/*D. longicaudata*), augmentative releases of *D. longicaudata* need to be tailored to local conditions and are not warranted in every location.

Opius hirtus exhibited similar *r* and fecundity values as *D. areolatus*, but together with *D. crawfordi*, was one of the larval-prepupal species that lived longest. Of all the braconid species that we successfully colonized, it is the least common and most specialized parasitoid (Sivinski et al. 2000; Aluja et al. 2003). Recently, García-Medel, Sivinski, Díaz-Fleischer, Ramírez-Romero, and Aluja (2008) showed that it is very effective at parasitizing hosts at very low densities and that it is able to coexist with other species such as *D. longicaudata*. As indicated by LaSalle (1993), many times rare parasitoid species exert a significant regulatory effect on pests. All the above renders *O. hirtus* an interesting candidate for more wide scale tests.

The fourth species of native braconid parasitoid that we were able to colonize was *U. anastrephae*. In nature, this species is specialized at attacking *A. obliqua* and *A. fraterculus* in small fruit within the Anacardiaceae (e.g. *Spondias* spp.) and Myrtaceae (e.g. *Psidium* spp., *Eugenia* spp., *Myrcianthes* spp.), respectively (Sivinski et al. 1997; López et al. 1999; Ovruski et al. 2004). The detailed studies by Sivinski et al. (1997) discovered an apparent partitioning of the niche in *S. mombin* trees, with *U. anastrephae* being most abundant in interior parts of the canopy preferentially infesting smaller fruit, while *D. areolatus* was most abundant in exterior parts of the canopy and infested larger fruit. Program managers would have to ascertain if any of these characteristics are of interest when deciding about new potential candidates for augmentative releases.

Of the two figitid species we were able to successfully colonize, *A. pelleranoi* offers various interesting attributes. On the one hand, and in contrast to the braconid species, it preferentially forages on the ground where it attacks larvae in fallen fruit (Sivinski et al. 1997; Ovruski et al. 2004). It does so in a wide range of hosts that varies greatly with respect to physical and chemical characteristics (Wharton et al. 1998; Ovruski et al. 2000). On the other hand, it is one of the few native parasitoid species in the New World that is able to attack *C. capitata* (Baeza-Larios et al. 2002a; Ovruski et al. 2004, 2005).

Coptera haywardi (the only pupal parasitoid the colonization of which we describe here), was the species exhibiting the longest survival and highest fecundity, and exhibited *r* values similar to those found in *D. crawfordi*. We also show here that it can attack pupae of highly contrasting age classes (i.e. 0–2 to 9–11 days of age). Furthermore, *C. haywardi* produced high rates of pupal mortality (85–92%). Similar observations were previously reported by Sivinski et al. (1998b) with *A. suspensa* (Loew) and Guillén et al. (2002) with *A. ludens* pupae. Considering all the above, and the fact that *C. haywardi* is an endoparasitoid that only attacks tephritid flies (Sivinski et al. 1998b), among them *C. capitata* and several species within *Anastrepha*, it represents an ideal candidate to substitute generalist, cosmopolitan species such as *P. vindemiae*, *Spalangia endius* Walter and *S. cameroni* Perkins, which are known primarily as parasitoids of synantropic flies (e.g. in poultry sheds) (Morgan 1986).

We conclude that, given the relatively fast adaptation of these organisms to laboratory conditions, it is feasible to mass rear most of them. As a matter of fact, in the case of *D. crawfordi*, *A. pelleranoi*, and *C. haywardi*, successful attempts at mass-rearing have already taken place in the fruit fly and parasitoid mass-rearing facilities of the Medfly Program in Metapa de Domínguez, Chiapas, Mexico and, in the case of *C. haywardi*, the La Aurora rearing facility in Guatemala City, Guatemala (see Baeza-Larios, Sivinski, Holler, and Aluja 2002b). Furthermore, as reported by Cancino et al. (2008), with the exception of *A. pelleranoi* and *O. anastrephae*, native parasitoids can be successfully reared using irradiated larvae or pupae. As discussed above, demographic parameters from well-established colonies such as ours might guide mass-rearing and control programs. They indicate, all other things being equal, which parasitoids might increase at the greater rate and thus are cheaper to produce. Furthermore, the effectiveness of the parasitoid species successfully colonized here should not be limited to Mexico, but rather they should be amenable to introduction, augmentation and conservation in many other tropical areas (e.g. Costa Rica, Colombia, Venezuela, Brazil, Bolivia, Argentina) where fruit flies such as *A. fraterculus*, *A. obliqua*, *A. ludens*, *A. serpentina*, *A. striata*, and *A. sororcula* are important pests. Gates et al. (2002) have highlighted three important benefits of the use of native parasitoids in biological control: (1) avoidance of costly and prolonged trips abroad in search of candidate species, (2) avoidance of cumbersome importation and quarantine protocols, and (3) avoidance of potential non-target effects on local fauna (also see Simberloff and Stiling 1996). Importantly, and given the massive rate of deforestation prevalent in Latin America, on top of searching for native species as potential fruit fly biological agents, we also need to foster the conservation of natural habitats, to enhance local parasitoid reservoirs and prevent the local extinction of rare species such as *O. hirtus* (Aluja 1996, 1999; Aluja et al. 2003).

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Appendix1. Composition of the artificial diet used to rear *A. ludens* larvae under laboratory conditions.

Amount	*Ingredients*
100 g	Dried Yeast (Type B-Torula), Lake States, Rhinelander, WI, USA
100 g	Natural wheat germ, Nutrisa SA de CV, México DF
100 g	Refined sugar
150 g	Sugar cane bagass (from local sugar refinery) OR Corn cob fractions, Mt. Pulaski, Products, Inc.
8 g	Sodium benzoate, Baker (J.T. Baker SA de CV, Xalostoc, Edo. de México)
2 mL	Hydrochloric acid, Baker (J.T. Baker SA de CV, Xalostoc, Edo. de México)
2 u	Viterra Plus capsules, Pfizer (Pfizer SA de CV, Toluca, Edo. de México)
750 mL	Distilled water

These amounts are recommended for seeding of 2 mL of eggs and the production of 2,500 to 3,000 larvae.