INFLUENCE OF *ANASTREPHA FRATERCULUS* (DIPTERA: TEPHRITIDAE) LARVAL INSTARS ON THE PRODUCTION OF *DIACHASMIMORPHA LONGICAUDATA* (HYMNEOPTERA: BRACONIDAE) PROGENY AND THEIR SEX RATIO

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ABSTRACT

The aim of this study was to determine the optimal larval age for exposing *Anastrepha fraterculus* (Wiedemann) to *Diachasmimorpha longicaudata* (Ashmead) females to maximize parasitoid female offspring production under laboratory conditions in order to develop an efficient mass-rearing process for this biocontrol agent. Five age ranges of *A. fraterculus* larvae were assessed: 1-3 d-old (first instar), 4-6 d-old (second instar), 7-8 d-old (early third instar), 9-10 d-old (middle third instar), and 11-12 d-old (late third instar). The number of *D. longicaudata* initiating ovipositor probing on a device with third instars of *A. fraterculus* was significantly higher than with others containing younger instars. Although female biased parasitoid offspring was recorded in all treatments using third instars as hosts, significantly more *D. longicaudata* females emerged from *A. fraterculus* pupae originated from middle and late third instars. However, the percentage of unemerged host puparia also increased significantly in both those host instar groups. Superparasitism, recorded as the number of first instar parasitoid head capsules per host, increased significantly as *A. fraterculus* larvae matured from second instars to late third instars. Nevertheless, the maximum average yield of parasitoid progeny was achieved using 9-12 d-old *A. fraterculus* larvae as hosts.

Key Words: fruit flies, parasitoids, sex ratio, superparasitism, biological control, Argentina

RESUMEN

Se determinó la edad óptima de la larva de *Anastrepha fraterculus* (Wiedemann) para ser expuesta a *Diachasmimorpha longicaudata* (Ashmead) con el objetivo de maximizar la producción de descendientes hembras en la cría del parasitoide y así lograr un eficiente proceso de cría masiva del agente. Fueron evaluados 5 rangos de edad de larvas de *A. fraterculus*: 1-3 días (1er estadío), 4-6 días (2do estadío), 7-8 días (3er estadío temprano), 9-10 días (3er estadío medio) y 11-12 días (3er estadío tardío). En las pruebas con el ovipositor se observó un número significativamente mayor de hembras de *D. longicaudata* en la unidad de oviposición con larvas de *A. fraterculus* del 3er estadío que en dispositivos con larvas de estadíos más tempranos. En todos los tratamientos que usaron larvas huéspedes del 3er estadío, la descendencia del parasitoide tuvo predominancia de hembras. Sin embargo, el máximo porcentaje de puparios no eclosionados, también se registró en tratamientos que utilizaron larvas huéspedes del 3er estadío medio y tardío. El superparasitismo, registrado como el número de cápsulas cefálicas de larvas del 1er estadío del parasitoide por pupa del huésped, manifestó un significativo incremento en larvas de *A. fraterculus* del 3er estadío tardío. No obstante, cuando se utilizaron larvas de 9-12 días de edad como huéspedes se alcanzó la máxima producción de progenie del parasitoide.

Translation provided by the authors.

Diachasmimorpha longicaudata (Ashmead) is a solitary, koinobiont larval-prepupal endoparasitoid of several fruit-infesting tephritid flies (Montoya et al. 2000a). It is native to Southeast Asia (Wharton 1989) but has been used in augmentative biological control programs against the Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) in Mexico and Guatemala (Cancino et al. 1995), the Caribbean fruit fly *Anastrepha suspensa* (Loew) in Florida (Sivinski et al. 1996), the Oriental fruit fly *Bactrocera dorsalis* (Hendel) in Hawaii (Purcell 1998), and the Mexican fruit fly *Anastrepha ludens* (Loew) in Mexico (Montoya et al. 2000a). In 1999, *D. longicaudata* was introduced to Argentina via Mexico with the purpose of renewing biological control programs against tephritid pests such as the exotic *C. capitata* and the native South American fruit fly *Anastrepha fraterculus* (Wiedemann) (Ovruski et al. 2003). Although a previous introduction was carried out

during the 1960's, the permanent establishment of *D. longicaudata* on *A. fraterculus* was only recently confirmed in northeast (Schliserman et al. 2003) and northwest (Oroño & Ovruski 2007) Argentina. Consequently, the use of *D. longicaudata* in augmentative releases to suppress populations of *A. fraterculus* in citrus-growing areas of northwest Argentina might be successful and ecologically suitable. Knowing which factors influence offspring production is essential to successfully rear *D. longicaudata* for augmentative releases (Montoya et al. 2011). Based on this analysis, a colony of *D. longicaudata* was established on *A. fraterculus* at our laboratory in 2005 (Ovruski et al. 2011). The aim of the present study was to determine the optimal larval age for exposing *A. fraterculus* to *D. longicaudata* females to maximize parasitoid offspring production and thus develop an efficient mass-rearing process for this biocontrol agent.

Materials and Methods

D. longicaudata and *A. fraterculus* colonies were kept at 25 ± 1 ºC; 75 ± 5% RH, under a 12:12 h L:D photoperiod in the laboratory of Biological Control Division at the Planta Piloto de Procesos Microbiológicos Industriales y Biotecnología (PROIMI), located in San Miguel de Tucumán, Argentina. Parasitoids were obtained from a strain reared at the PROIMI laboratory using *A. fraterculus* larvae for over 30 generations (Ovruski et al. 2011). The *A. fraterculus* rearing procedures were carried out as described by Vera et al. (2007).

Five age ranges of *A. fraterculus* larvae were compared to determine the optimal larval age for exposing hosts to *D. longicaudata* females: 1-3 d-old (first instar), 4-6 d-old (second instar), 7-8 d-old (early 3rd-instar), 9-10 d-old (middle 3rd-instar), and 11-12 d-old larvae (late 3rd-instar). *A. fraterculus* instars were determined using the size and shape of the cephalopharyngeal skeleton under a stereomicroscope, as described by Frías et al. (2008). Forty host larvae of each instar group, provided with artificial diet (hydrolyzed brewer's yeast + wheat germ + sugar + agar + water), were exposed to 10 mated *D. longicaudata* females for 1 h inside oviposition devices placed on the floor of cubical Plexiglas cages (20 cm). The oviposition unit was composed of an organdy screen-covered Petri dish (8 cm diameter and 0.8 cm deep). The parasitoid/host ratio was based on data for optimizing *D. longicaudata* mass rearing on *A. ludens* at the Moscafrut Metapa facility in Mexico (Montoya et al. 2000b). Parasitoid females were 6-7 d-old and had no prior oviposition experience. After exposure to the parasitoids, host larvae were transferred onto plastic trays (15 × 11 × 1.5 cm) filled with fresh food. These trays were then placed inside plastic boxes (33 × 23 × 11 cm) containing a 2 cm-vermiculite layer on the bottom as a pupation medium and covered with organdy on the top. The 11-12 d-old larvae were removed from the oviposition device and transferred directly to the plastic boxes described above. All dead larvae were counted and removed. Puparia were sifted from the pupation medium and transferred into plastic cups (8 cm diam × 5 cm deep) containing fresh vermiculite on the bottom. The cups were then tightly covered with a piece of organdy cloth. The number of emerged flies and parasitoids during this period was recorded for a period of 40 d. After this period, sub samples of 10 unemerged puparia were removed and dissected to determine the presence or absence of recognizable immature parasitoid stages (larvae, prepupae, or pupae) or pharate-adult parasitoid cadavers. Instars of *D. longicaudata* were determined using the size and shape of mandibles as described by Ibrahim et al. (1994). The number of first instar parasitoid head capsules per dissected host pupa was also examined. Control tests (no parasitoid exposure) for all larval age ranges were made to determine natural *A. fraterculus* larvae and pupae mortalities, as well as the rate of fly emergence. Treatments and control tests were replicated 12 times. All assays were carried out at the laboratory under the environmental conditions described previously. Parasitoid and fly emergence percentages were based on the total number of recovered pupae in each sample. Parasitoid progeny sex ratio was calculated as the proportion of female offspring. The percentage of larval mortality was calculated as the fraction of dead host larvae from the total number of larvae exposed to parasitoids. The percentage of pupal mortality was estimated as the fraction of non-eclosed puparia from the total number of emerged flies and parasitoids, and unemerged puparia (Montoya et al. 2000b).

The number of female ovipositor probes in the oviposition device was recorded when the parasitoids were released into the cages. Behavioral observations were made every 15 min and each observation lasted 20 s. An ovipositor probe was confirmed each time a female parasitoid inserted its ovipositor through the organdy screen of the oviposition device (Duan & Messing 2000).

Data on parasitoid emergence, sexual ratio of parasitoid offspring, number of first instar parasitoid head capsules per host, and number of female ovipositor probes were compared statistically using one-way analyses of variance (ANOVA) ($P < 0.05$). Fly emergence and larval and pupal mortalities recorded from the experimental treatments and controls were compared by a two-way ANOVA ($P < 0.05$). Data on the stages of parasitoid cadavers recorded from dissected unemerged host puparia were subjected to

multivariate analyses of variance (MANOVA) ($P < 0.05$). Fisher LSD tests ($P = 0.05$) were used to determine significant differences between means from both the ANOVA and MANOVA. Proportional data were transformed to arcsine square root before analyzed. Only untransformed means (±SE) are presented in the text. A Pearson correlation test ($P < 0.05$) was used to determine the degree of association between the sexual ratio of parasitoid offspring and the number of first instar parasitoid head capsules per host pupa.

Results

The age of host larvae affected parasitoid emergence ($F_{(4, 55)} = 69.7, P < 0.0001$) and offspring sexual proportion ($F_{(4, 55)} = 82.6, P < 0.0001$). Older *A. fraterculus* larvae yielded a higher proportion of adult parasitoids than younger host larvae (Fig. 1). Also, parasitoid offspring sex ratio increased significantly with older *A. fraterculus* larvae. Exposure to middle and late *A. fraterculus* third instars produced the highest proportion of female offspring, whereas exposure to second instar larvae produced a higher proportion of male offspring (Fig. 1). The proportions of dead larvae and unemerged puparia differed between the ages of host larvae exposed to parasitoids ($F_{(4, 110)} = 27.918, P < 0.0001$ for larval mortality, and $F_{(4, 110)} = 22.137, P < 0.0001$ for pupal mortality). The percentage of dead host larvae decreased significantly from younger larvae to older larvae, while the proportions of unemerged puparia increased (Fig. 2). The mortality of unexposed host larvae (control) was significantly lower compared to the larvae exposed to parasitoids ($F_{(4, 110)} = 21.205, P < 0.0001$) (Fig. 2). The percentages of unemerged puparia recorded in the parasitoid treatments were also significantly higher than in the controls ($F_{(4, 110)} = 20.275, P < 0.0001$) (Fig. 2). The high quality of the host larvae used in the study was confirmed by the emergence of adult flies recorded in the controls, which was significantly higher (> 92%) than for the treatments (from 3 to 53%) ($F_{(4, 110)} = 49.364, P < 0.0001$).

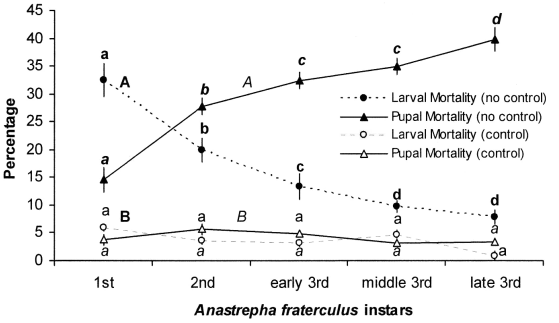
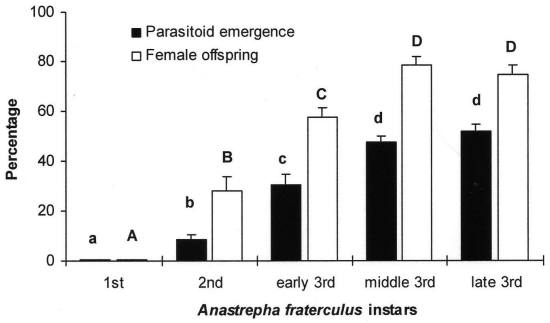


Fig. 2. Influence of *A. fraterculus* instars on the percentage (mean ± SE) of larval and pupal mortality recorded in experimental treatments (hosts exposed to *D. longicaudata* females) and controls (unexposed hosts). Plot markers followed by the same small letter indicate no significant differences (Fisher LSD test, P = 0.05). Different capital letters (either bold or italic) indicate significant differences between treatments and controls (ANOVA, $P = 0.05$).

Significantly more *D. longicaudata* females ($F_{(4, 55)} = 172.76, P < 0.0001$) were observed probing the oviposition unit containing third instars of *A. fraterculus* than devices containing first or second host instars (Fig. 3). Significant differences (Wilks' $\lambda = 0.0339, F_{(32, 178.61)} = 8.384, P < 0.0001$) were recorded among the five ages of *A. fraterculus* larvae regarding the stages of parasitoid cadavers found after the unemerged host puparia had been dissected (Table 1).

First instar head capsules of *D. longicaudata* were mainly found in parasitized unemerged pupae. The number of first instar parasitoid head capsules per host increased significantly ($F_{(4, 55)} = 32.823, P < 0.0001$) as the *A. fraterculus* larvae



Fig, 1. Effect of the age of *A. fraterculus* larvae on the proportion of emerged *D. longicaudata* adults (mean ± SE) and on progeny sex ratio (% emerged females of the total parasitoid offspring). Bars with the same letter indicate no significant differences (Fisher LSD test, $P = 0.05$).

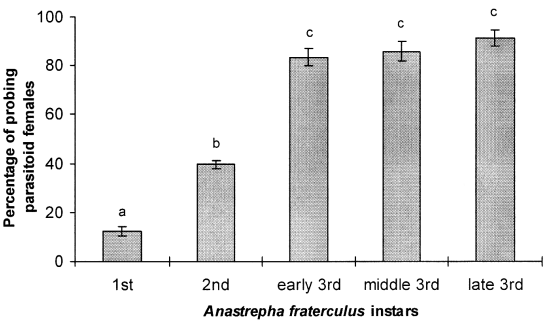


Fig. 3. Probing responses of *D. longicaudata* females (mean ± SE) on artificial oviposition devices containing different instars of *A. fraterculus* (mean ± SE). Bars with the same letter indicate no significant differences (Fisher LSD test, $P = 0.05$).

TABLE 1. COMPOSITION OF THE STAGES OF PARASITOID CADAVERS IN THE DISSECTED UNECLOSED PUPARIA OF *ANASTREPHA FRATERCULUS* PARASITIZED BY *DIACHASMIMORPHA LONGICAUDATA*.

Host instar[a]	Percentage (mean ± SE) of immature parasitoid cadavers[b]						Percentage (mean ± SE) of pharate adult cadavers[b]
	Egg	1st instar	2nd instar	3rd instar	prepupa	pupa	
L_1	0 a	0 a	0 a	0 a	0 a	0 a	0 a
L_2	45.9 ± 10.7 b	40.8 ± 4.9 b	2.1 ± 2.1 a	1.4 ± 1.5 a	1.4 ± 1.4 a	0 a	0 a
L_{3E}	2.9 ± 2.9 ac	37.7 ± 4.4 b	8.7 ± 3.8 b	6.0 ± 3.4 b	18.6 ± 3.1 b	19.3 ± 4.4 b	6.9 ± 3.2 b
L_{3M}	8.7 ± 4.2 c	49.8 ± 4.5 c	2.1 ± 1.4 ab	0.9 ± 0.9 a	22.1 ± 2.5 c	12.9 ± 4.3 b	3.5 ± 1.9 ab
L_{3L}	1.3 ± 1.3 ac	72.2 ± 3.3 d	0 a	1.1 ± 1.1 a	20.2 ± 2.7 bc	3.1 ± 2.1 a	2.2 ± 1.5 ab

[a]L_1, 1st instar; L_2 2nd instar; L_{3E}, early 3rd-instar; L_{3M}, middle 3rd-instar; L_{3L}, late 3rd-instar.
[b]Values in the same column with the same letter are not significantly different (Fisher LSD test, $P = 0.05$).

matured from second instars to late third instars (Fig. 4). This increase was significantly associated with an increase in parasitoid female offspring ($r = 0.7776$, $N = 60$, $P = 0.0002$).

DISCUSSION

The biased offspring sex ratio towards females of *D. longicaudata* as *A. fraterculus* larvae aged could be influenced by an interaction between following biological factors: 1) suitable host age and high quality of host larvae that might stimulate parasitoids to lay more female eggs; 2) high level of superparasitism; 3) differential survival of each sex during parasitoid post-embryonic development in superparasitized hosts; 4) higher number of fertilized eggs laid in the presence of conspecific females foraging on the same artificial oviposition device.

Regarding the first statement, the results of this study clearly demonstrated that all the instars of *A. fraterculus* were not equally suitable for *D. longicaudata*, and that parasitoids develop successfully in older instars. As suggested by Montoya et al. (2011), *D. longicaudata* females could be able to regulate their offspring sex ratio in response to host conditions. Chemical cues emanated from host larvae can be exploited in the process of host acceptance by *D. longicaudata* (Wong & Ramadan 1992). Chemosensory cells located at the tip of the ovipositor might allow *D. longicaudata* females to detect changes in pH and hormone levels in the host larva haemolymph (Greany et al. 1977). Thus, the internal physiological conditions of maturer instars would thus elicit egg fertilization in *D. longicaudata* females (Wong & Ramadan 1992). The behavioral data of the present study showed that a very high level of ovipositor-probing responses (>85%) only occurred in devices containing third instar larvae of *A. fraterculus*. It seems that distinct physical and/or chemical cues are be involved in the host acceptance of *D. longicaudata* females. Arthur (1981) pointed out that host size, shape, movement and surface texture, the presence of kairomones associated with host larval frass, body fat, and labial and mandibular glands, as well as haemolymph, may elicit host acceptance behaviors by female parasitoids. It has been demonstrated that the cues of third instar larvae of *A. suspensa* and *C. capitata* that elicit ovipositor-probing behavior in gravid *D. longicaudata* are primarily vibration and/or sound produced by larvae feeding or crawling inside the substrate (Lawrence 1981; Duan and Messing 2000).

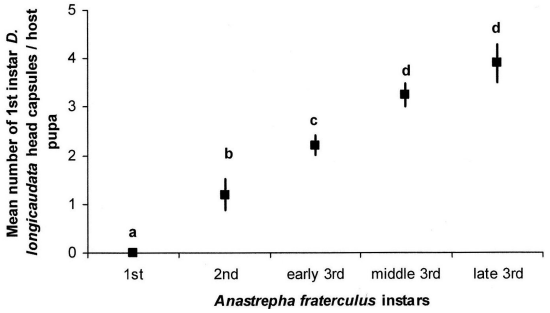


Fig. 4. Mean (± SE) number of first instar *D. longicaudata* head capsules per host pupa found in the five assayed instars of *A. fraterculus*. Plot markers followed by the same letter indicate no significant differences (Fisher LSD test, $P = 0.05$).

The second aforementioned assumption is supported by the close association found between parasitoid female offspring production and the number of first instar parasitoid head capsules per host. Middle and late *A. fraterculus* instars yielded significantly more *D. longicaudata* female offspring that younger instars, but they also recorded the highest rates of superparasitization. This data is in agreement with the observations of Wong et al. (1990) on *Diachasmimorpha tryoni* (Cameron), another fruit fly larval endoparasitoid. These authors found that both progeny female-biased sex ratio and superparasitism increased when late third-instars of *C. capitata* were used as hosts in the *D. tryoni* mass-rear-

ing process. Similarly, Wang & Messing (2003) also recorded more *D. tryoni* females emerged from superparasitized host puparia. Also, González et al. (2007) demonstrated that under mass-rearing conditions, the *D. longicaudata* offspring sex ratio always favoured females when moderate to high levels of superparasitism were recorded in third-instars (8-9-d old) of *A. ludens*. As suggested by Montoya et al (2000b), superparasitism in *D. longicaudata* might be an adaptative behavior that could increase the chance of parasitoid female offspring survival inside host larvae.

According to the third factor, differential sex mortality during parasitoid larval development has been mentioned as a probable cause influencing offspring sex ratios in solitary parasitoid species (Heimpel & Lundgren 2000). Montoya et al. (2011) previously proposed this hypothesis to explain parasitoid female biased sex ratios recorded in *A. ludens* pupae superparasitized by *D. longicaudata*. Ovipositing *D. longicaudata* females would be expected to lay eggs of the better sex competitor (probably parasitoid female) under conditions of superparasitism (Montoya et al. 2011). Supernumerary *D. longicaudata* larvae might be eliminated through physiological suppression mechanisms (Montoya et al. 2000b), although physical competition could also cause the death of *D. longicaudata* first instar larvae (Lawrence 1988).

Regarding the fourth assumption, a female-biased sex allocation pattern has been frequently recorded in *D. longicaudata* under crowded mass rearing situations on *B. dorsalis* (Wong & Ramadan 1992; Vargas et al. 2002) and *A. ludens* (Montoya et al. 2000b, 2011; González et al. 2007), as well as under experimental laboratory rearing conditions on *Bactrocera papayae* Drew & Hancock (Petcharat & Petcharat 1997) and *C. capitata* (Ovruski et al. 2003; Paranhos et al. 2008).

Another issue to consider is that the difference in host mortality between experimental and control treatments could be due to parasitism. Although no parasitoids were recorded from the treatment including 1-3 d-old host larvae (L1), the high larval mortality detected in *A. fraterculus* first instars might be caused when ovipositing parasitoid females insert their ovipositor in unsuitable larvae. Previous assays with first instar *A. fraterculus* parasitized individually by *D. longicaudata* showed a 90% mortality among these larvae (S.O., unpublished data). Dissections of puparia from second instar hosts exposed to parasitoids revealed that about 86% of the unemerged parasitized host pupae only contained cadavers of eggs and first instar parasitoids. As suggested previously by Lawrence et al. (1978), hormonal interference may influence the survival rate of immature stages of *D. longicaudata*. It has been demonstrated that first instar *D. longicaudata* need a suitable hormonal environment inside the host to advance to the second instar stage, which occurs when the host has pupated (Lawrence 1982, 1986). The high percentage of puparia that did not yield adult flies observed in the treatment using third instar larvae could be interpreted as a negative effect of superparasitism. This is in agreement with studies by Montoya et al. (2000b) on *D. longicaudata* parasitizing third instars of *A. ludens*, who suggested that high host mortality could be due to the effect of superparasitism.

As a conclusion, the results of the present study demonstrate that exposure to 9-12-d-old *A. fraterculus* larvae significantly increases *D. longicaudata* female emergence to a maximum of 80% under laboratory conditions. Moreover, these results also reveal that although the intensity of superparasitism was higher using middle and late-third instars of *A. fraterculus* as hosts, the maximum average yield of parasitoids (52.1%) was also achieved with these host instars. This information is important for optimizing female production in the mass rearing process of *D. longicaudata* on *A. fraterculus*. As pointed out by Montoya et al. (2011) regarding fruit fly biological control programs using *D. longicaudata*, an increased production of parasitoid females implies the mass release of more females, with a subsequent increase in the cost-effective relation of a biocontrol program.

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