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Increasing radiation doses in *Anastrepha obliqua* (Diptera: Tephritidae) larvae improve parasitoid mass-rearing attributes

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Abstract

Doses of 40, 80, 120, and 160 Gy were applied to 5-, 6-, 7-, and 8-day-old *Anastrepha obliqua* larvae, which were exposed to the Neotropical-native braconids *Doryctobracon crawfordi* and *Utetes anastrephae* and the Asian braconid *Diachasmimorpha longicaudata*. These tests were performed to know the effect of the increase in host radiation on the emergence of the aforementioned parasitoids and the related consequences of oviposition on the host. The study was based on the fact that higher radiation doses may cause a decrease in the host immune activity. There was a direct relationship between the increase in radiation dose and the parasitoid emergence. Both, the weight and the mortality of the host larvae were not affected by radiation. Although the larval weight of the larvae was lower and the mortality was higher in the younger larvae. Both, the number of scars and immature stages per host puparium originated from the younger larvae were lower than those from older larvae. Only *U. anastrephae* superparasitized more at lower radiation. Superparasitism by *D. longicaudata* was more frequent at 160 Gy. Qualitative measurements of melanin in the larvae parasitized showed that the levels were lower with increasing radiation. As radiation doses increased, the antagonistic response of the *A. obliqua* larva was reduced. Host larvae aged 5- and 6-day-old irradiated at 120–160 Gy significantly improve parasitoid emergence. This evidence is relevant for the mass production of the three tested parasitoid species.

Introduction

Anastrepha obliqua (McQuart) (Diptera: Tephritidae), commonly named 'West Indian fruit fly', is a native Neotropical species that oviposit and develops into a wide range of wild fruits and it is the most significant fruit fly pest of *Mangifera indica* L. ('mango') (Aluja and Birke, 1993; Aluja *et al.*, 2003; Mangan *et al.*, 2011). This pestiferous fruit fly is widespread in Mexico, Central and South America, and the West Indies (Ruiz-Arce *et al.*, 2012; Santos *et al.*, 2020), and it is a serious threat to other mango-producing regions (Jiron, 1996; Montoya *et al.*, 2007; Ruiz-Arce *et al.*, 2012). *A. obliqua* mass rearing has been successfully established at the Moscafrut Biofactory, located in Metapa de Dominguez, Chiapas, Southern Mexico, mainly to implement the sterile insect technique (Rull Gabayet *et al.*, 1996; Orozco-Dávila *et al.*, 2017). The production of millions of *A. obliqua* eggs, larvae, and pupae also represent an opportunity to develop parasitoid mass rearing, to apply an effective biological control of this pestiferous tephritid fruit fly (Ovruski *et al.*, 2000; Montoya *et al.*, 2000*b*; Artiaga-López *et al.*, 2002). *Anastrepha obliqua* is a host that can be used as a natural reservoir for different species of parasitoids, since its larval development occurs in various wild fruits, such as those of the genus *Spondias* (Aluja *et al.*, 1990; Sivinski *et al.*, 2000; Silva *et al.*, 2010). These wild fruits have important physical characteristics for parasitoid oviposition, such as small size, large seed, a narrow and soft pulp layer, and an extremely thin rind (Aluja and Birke, 1993; López *et al.*, 1999; Murillo *et al.*, 2016; Montoya *et al.*, 2017). However, *A. obliqua* has generated physiological defense strategies against parasitoid development that may be the result of a co-evolutionary process (Silva *et al.*, 2002). These authors have reported a wide variety of hemocytes characterized as generators of resistance to parasitoid development inside host larva. Probably, due to this fact, some native and sympatric parasitoid species of *Anastrepha* spp., such as *Doryctobracon crawfordi* (Viereck) and *Opius hirtus* (Viereck), are not viable to develop within *A. obliqua* larvae, although they do so in others *Anastrepha* species (Poncio *et al.*, 2016).

Taking into account the aforementioned evidence of high physiological resistance in *A. obliqua*, the expectations of its use as a host in parasitoid rearing would face a serious problem. The low level of parasitoid emerged from *A. obliqua* puparia under laboratory conditions is an indicator that weakens the objective of proposing it as a host for parasitoid mass rearing (Eben *et al.*, 2000; Cancino *et al.*, 2009). However, the use of nuclear techniques, such as radiation, in tephritid fruit fly hosts can be effective to reduce host immune resistance and analyze the viability for parasitoid mass rearing (Cancino *et al.*, 2012). It has been proved in both pestiferous fruit flies, *Ceratitis capitata* (Wied.) and *Anastrepha ludens* (Loew), that the application of radiation doses favors parasitoid development, and it can even be considered a direct relationship with the increase in radiation dose (Cancino *et al.*, 2020; Suárez *et al.*, 2020). Radiation dose between 20 and −30 Gy applied to *A. obliqua* larvae used as hosts for *Diachasmimorpha longicaudata* (Ashmead), a larval-pupal southeast Asian-native parasitoid, successfully allowed parasitoid development, and also prevented host emergence from non-parasitized larvae (Cancino *et al.*, 2009). However, the effect of higher radiation doses is unknown. In view of the above facts, it is undeniable that host radiation for parasitoid mass rearing is relevant and its use is intensifying. Therefore, assays were carried out to determine the best radiation dose (>20–30 Gy) to expose irradiated *A. obliqua* larvae of the most suitable age to *D. crawfordi*, *Utetes anastrephae* (Viereck), and *D. longicaudata* females to achieve maximum levels of parasitoid mass production with good quality individuals. So, mass production quality parameters such as larval host weight, host mortality, parasitoid emergence, and parasitoid offspring sex ratio were estimated. In addition, the number of scars in host puparia, the superparasitism, and the presence of melanization in parasitoid larvae were also assessed. The Neotropical *D. crawfordi* is not able to develop successfully in *A. obliqua* larvae in nature (Poncio *et al.*, 2016), while the other native parasitoid *U. anastrephae* is closely associated with *A. obliqua* larvae in wild environments (Sivinski *et al.*, 1997). On the contrary, the exotic *D. longicaudata*, which is a generalist parasitoid, successfully attacks larvae of different *Anastrepha* species (Montoya *et al.*, 2017). For these reasons, it was hypothesized that increasing radiation dose under rearing conditions will reduce the *A. obliqua* larvae immune response, which will increase the parasitoid yield. The results are of great interest to use *A. obliqua* larvae for parasitoid mass production and its subsequent use in parasitoid mass releases under open-field conditions.

Materials and methods

Insect source

Samples of *D. crawfordi* (<350 generations), *U. anastrephae* (<300 generations), and *D. longicaudata* (<550 generations) were taken from parasitoid colonies kept under mass-rearing conditions at the Biological Control Department of the Moscafrut Plant. Adults parasitoids were provided with honey and water *ad libitum* and were kept at 24 ± 1°C; 65 ± 5% RH; and 12:12 h L:D. Ages from 5- to 8-day old *A. obliqua* larvae were used as parasitoid hosts. The age of the larva in days was determined according to the initial egg date sown in the diet. The host larvae were mass-reared on a corn-based diet containing corncob fractions (15%), torula yeast (5.83%), cornflour (8%), sugar (8.33%), guar gum (0.10%), sodium benzoate (0.23%), methyl *p*-hydroxybenzoate (0.11%), citric acid (0.63%), and water (61.77%) and kept under mass-production standard procedure at the Moscafrut Plant (Artiaga-López *et al.*, 2002). Batches of host larvae with fly emergence percentages <90 were discarded and not used in the tests.

Parasitoid emergence and parasitoid offspring sex ratio

Tests were accomplished to achieve high parasitoid emergences with a female-biased sex ratio under an optimal radiation dose and a suitable host larval age for parasitoid mass rearing using *A. obliqua* as the host. Samples of 5-, 6-, 7, and 8-day-old *A. obliqua* larvae were taken from the mass-rearing larval diet. The diet was removed from the host larvae by washing them with fresh water. Five samples of 100 larvae for each age were individually placed in $7.5 \times 4\ \text{cm}^2$ (diameter by height) cylindrical plastic containers. Each sample of the larval host age was respectively exposed to gamma radiation at 40, 80, 120, and 160 Gy in a 127 Gamma Beam panoramic Irradiator (Nordion®, Ottawa, ON, Canada) with a cobalt 60 dry storing source at a rate of $4.60\ \text{Gy min}^{-1}$ and 22°C. The irradiated larvae were placed again into containers with a rearing diet. Non-irradiated larvae were also included in the study as a control test (0 Gy). For each treatment, equivalent to a certain irradiation dose and larval host age, 100 *A. obliqua* larvae mixed with a rearing diet were individually exposed to 30 mated, 5–10-day-old *D. crawfordi*, *U. anastrephae*, and *D. longicaudata* females, all with previous oviposition experience. The larvae were exposed to parasitoids inside a $10 \times 1.5\ \text{cm}^2$ (diameter by height), the base of Petri dish which was covered with organdy screen, fastened with an elastic band. This was introduced into a cubical wooden structure cage ($27 \times 27 \times 27\ \text{cm}^3$) covered with a plastic screen. A ratio of 3.3:1, 100 hosts/30 parasitoid females ratio was used. The host exposure time was 60 min; after exposure, the host larvae were kept on the rearing diet according to their corresponding treatment in a $7.5 \times 4\ \text{cm}^2$ (diameter by height) cylindrical plastic container with a lid. When host larvae were aged 9-day-old, they were washed with fresh water only to eliminate the rearing diet. Subsequently, the parasitized larvae were returned to the container with vermiculite at the bottom ready for pupation. The treatments continued in the containers at 26°C and 60–80% RH for 15 days until *U. anastrephae* and *D. longicaudata* adult emergence, and for 20 days until *D. crawfordi* emergence. Each treatment was replicated ten times. For data assessment, the parasitoid emergences and the parasitoid offspring sex ratio were estimated. Parasitoid emergence was calculated as the number of emerged adults divided by the total number of offered pupae × 100, while offspring sex ratio was calculated as the fraction of daughter over son parasitoid offspring.

Effect of radiation doses on A. obliqua larvae

Larval host weight and host mortality were evaluated to know the effects of higher radiation doses on these two quality parameters. The larval host weight was determined from a 100-larva sample by treatment on a semi-analytical scale (PIONEER PA512C, OHAUS® Ohaus Corporation, Parsippany, NJ, USA). The host mortality was recorded 72 h after *A. obliqua* larvae exposed to parasitoids were placed inside pupation medium. Dead host larvae were counted and removed from each container. Each treatment was replicated ten times.

Table 1. Summary of two-way generalized linear models on the effect of radiation dose (RD) and larval host age (LHA), and their interactions on quality control parameters in *D. crawfordi*, *U. anastrephae*, and *D. longicaudata* reared on *A. obliqua* larvae

		D. crawfordi			*U. anastrephae*			*D. longicaudata*		
Parameters	Fixed factors	*df*	χ^2	*P*	*df*	χ^2	*P*	*df*	χ^2	*P*
Parasitoid emergence[a]	RD	4	151.59	<0.0001*	4	224.81	<0.0001*	4	144.42	<0.0001*
	LHA	3	90.77	<0.0001*	3	55.49	<0.0001*	3	67.32	<0.0001*
	RD × LHA	12	36.34	0.0003*	12	68.63	<0.0001*	12	23.33	0.0251*
Parasitoid offspring sex ratio[a]	RD	4	43.37	<0.0001*	4	3.31	0.505	4	9.10	0.06
	LHA	3	12.71	0.005*	3	8.28	0.040*	3	9.97	0.018*
	RD × LHA	12	12.22	0.421	12	10.85	0.541	12	15.55	0.21
Larval host weight[a]	RD	4	0.86	0.92	4	6.89	0.14	4	5.53	0.23
	LHA	3	110.07	<0.0001*	3	102.66	<0.0001*	3	108.72	<0.0001*
	RD × LHA	12	20.72	0.05	12	21.65	0.04*	12	10.87	0.53
Larval host mortality[a]	RD	4	4.38	0.35	4	2.04	0.72	4	6.32	0.17
	LHA	3	27.59	<0.001*	3	4.32	0.22	3	83.02	<0.0001*
	RD × LHA	12	16.01	0.19	12	2.81	0.99	12	15.99	0.19
No. of scars on puparium[a]	RD	4	2.81	0.58	4	21.92	0.0002*	4	6.49	0.16
	LHA	3	6.85	0.07	3	53.30	<0.0001*	3	11.47	<0.0094*
	RD × LHA	12	16.97	0.15	12	30.55	0.0023*	12	14.35	0.27
No. of first instar larvae/host[a]	RD	4	4.63	0.32	4	4.9	0.29	4	14.91	0.0049*
	LHA	3	3.10	0.37	3	10.59	0.014*	3	12.84	<0.0050*
	RD × LHA	12	16.97	0.15	12	8.71	0.72	12	17.54	0.12
Melanization on parasitoid larvae[b]	RD	4	46.72	<0.0001*	4	5.85	0.0004*	4	25.31	<0.0001*
	LHA	3	46.66	<0.0001*	3	11.49	<0.0001*	3	3.73	0.0003*
	RD × LHA	12	7.29	<0.0001*	12	1.51	0.15	12	1.46	0.16

*Significant variables.

[a] Generalized linear model and multiple means analysis by Tukey's HSD test ($\alpha = 0.05$).

[b] Non-parametric robust ANOVA and multiple means analysis by minimal difference significant ($P = 0.05$).

Scars, parasitoid first instar, and melanization levels

Tests were carried out to determine the effect of both radiation doses and larval host age on the number of scars in the host's puparia, superparasitism, and the level of melanization in parasitoid larvae. For this, 3–4-day-old pupae coming from 5-, 6-, 7-, and 8-day-old *A. obliqua* larvae exposed to parasitoids were dissected under a stereoscopic microscope (Discovery V8, Carl Zeiss® Gottingen, Germany). Ten pupae from each treatment were dissected on the base of a Petri dish by using dissecting needles. Before dissection, the number of oviposition scars on the surface of the puparium was counted, which represent oviposition attempts or oviposition performed on the host larva (Montoya *et al.*, 2000*a*). After that, each puparium was opened, and the first instar number of the corresponding parasitoid species was recorded. The presence of more than one parasitoid first instar larva within the host's body was considered a superparasitized host (Montoya *et al.*, 2011). The first instar of *D. crawfordi*, *U. anastrephae*, and *D. longicaudata* was determined as described by Miranda *et al.* (2015), Aluja *et al.* (2013), and Ibrahim *et al.* (1994), respectively. The size and shape of both mandibles and cephalic heads were used as basic features to identify the first instar parasitoid. In addition, the degree of melanization was also evaluated for those encapsulated parasitoid larvae. A classification criterion was applied regarding the presence of melanization in parasitoid larvae, ranging from one to ten according to the increasing amount of melanin covering the parasitoid body, a qualitative indicator of host immunological reactions (Suárez *et al.*, 2020). Each treatment was repeated ten times.

Data analysis

A generalized linear model with a normal distribution for the analysis of parameters was used. The analysis was performed under a bifactorial design, where the radiation dose and the larval host age were used as fixed factors. Each species of the parasitoid was analyzed individually. Mean comparisons were analyzed by Tukey's honestly significant difference (HSD) test at $P = 0.05$. The means of emergence and sex ratio in *D. crawfordi* were compared applying the Bonferroni adjustment to avoid statistical problems with zero values. The qualitative data of melanization were analyzed by a non-parametric robust analysis of variance (ANOVA). The comparison of the means was performed with

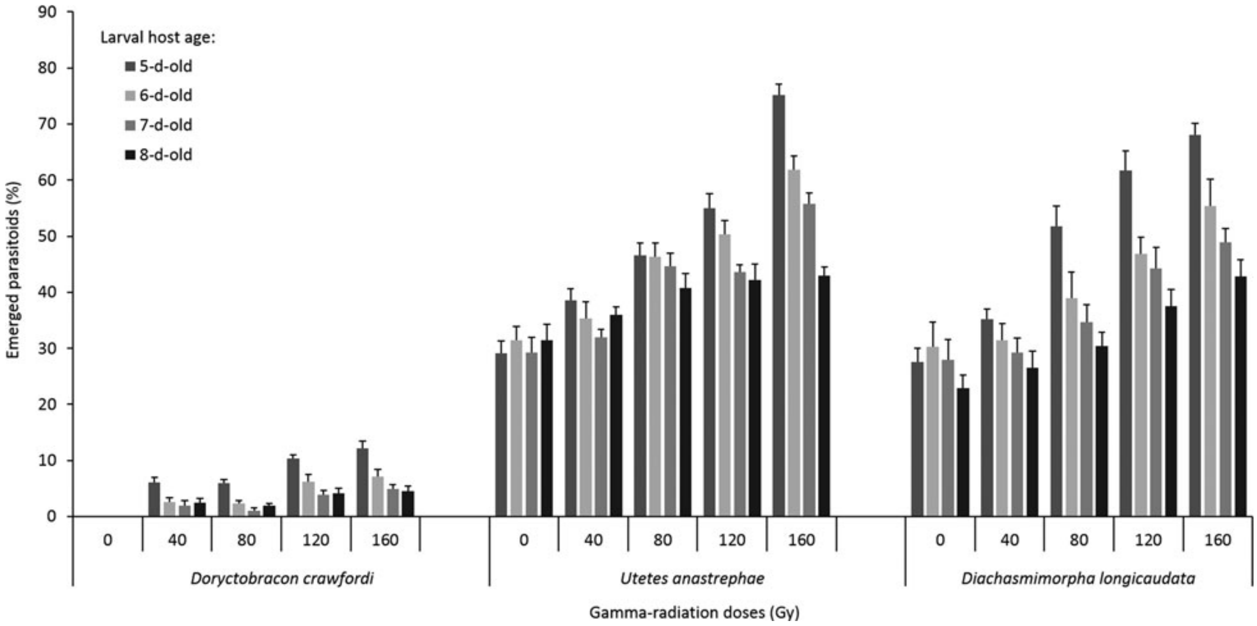

Figure 1. Percentages of parasitoid emergence (±SE) in 5-, 6-, 7-, and 8-day-old *A. obliqua* host larva irradiated at different doses and parasitized by *D. crawfordi*, *U. anastrephae*, and *D. longicaudata*.

the least significant difference test. JMP software, version 11 (SAS Institute, 2013) (JMP®SAS Institute, Inc.) and R version 3.6.1 (R Core Team, 2020) were used for the different analyses.

Results

Parasitoid emergence and parasitoid offspring sex ratio

Both radiation doses and larval host age, and their interaction had a significant effect on the parasitoid emergence in the three parasitoid species (table 1). The younger host larvae (5-, 6-, and 7-day-old) irradiated at higher doses (120–160 Gy) produced significantly the highest percentages of parasitoid emergence (fig. 1; table 2). There was a significant effect of radiation doses on the sex ratio in *D. crawfordi*, due to considerable difference with the control (0 Gy) (table 1), since the parasitoid emergence only occurred from irradiated host larvae (table 2). The highest proportion of *D. crawfordi* female offspring was generated in irradiated youngest larvae (5-day-old) (table 2). The sex ratio in both *U. anastrephae* and *D. longicaudata* was not significantly affected by the variation in radiation dose, but larval host age had a significant effect (table 1). The highest proportion of *U. anastrephae* female offspring was produced in 6-day-old larvae, while in *D. longicaudata* was slightly higher in older larvae (table 2). However, the interaction between both radiation doses and larval host age on sex ratio was not significant in the three parasitoid species (table 1).

Effect of radiation doses on A. obliqua *larvae*

Significant effects and interaction of radiation doses with age were found on the weight of larval host exposed to *U. anastrephae* (table 1). In both *D. crawfordi* and *D. longicaudata*, there was no interaction between dose and age in the average weight and mortality of exposed larvae. However, a significant weight difference in larval host age was found in the three evaluated parasitoid species (table 1). Host weight increased significantly in host larvae aged 6–8-day-old (table 3). Interestingly, a significant effect of larval host age on host mortality was found in both *D. crawfordi* and *D. longicaudata*, but not in *U. anastrephae* (table 1). Mortality was significantly higher in younger host larvae (table 3).

Scars, parasitoid first instar, and melanization levels

There were no *D. crawfordi* adults that emerged from non-irradiated *A. obliqua* larvae, although these host larvae were parasitized. This was checked by the scars in the host's puparia and by the parasitoid's first instar larvae inside hosts. There was no significant relationship between radiation dose, larval host's age, and their interaction as a function of both the number of scars and *D. crawfordi* first instar (table 1). Superparasitism in *A. obliqua* larvae by *D. crawfordi* was infrequent. The means did not exceed 1.3 first instar parasitoid larvae per dissected host puparium (table 4). Significant effects of radiation doses, larval host's age, and their interaction were found on the number of scars in the host's puparia parasitized by *U. anastrephae* (table 1). A significantly higher number of scars was found with low radiation doses (0–80 Gy) in host puparia from 8-day-old larvae (table 4). There was no significant effect of increasing radiation doses and their interaction with the larval host's age on the presence of parasitoid first instars larvae per host larvae (table 1). However, the number of parasitoid first instar was significantly influenced by the larval host's age (table 1). The highest superparasitism level was recorded in 7-day-old *A. obliqua* larvae, while the lowest superparasitism value was recorded in 5-day-old host larvae (table 4). The superparasitism caused by *U. anastrephae* was quite low (table 4). Similarly, increasing radiation doses did not significantly influence the number of scars caused by *D. longicaudata* (table 1). Nevertheless, a significant decrease in the number of scars was found in 8-day-old host larvae compared to 5-day-old larvae (table 4). Significant effects of radiation dose and larval host age were found on the number of the parasitoid in the first instar, but the interaction between both fixed factors was not significant (table 1). An increase in the first instar per host puparium occurred at the highest radiation dose evaluated (160 Gy).

Table 2. Parasitoid emergence and offspring sex ratio (mean ± SE) recorded from 5-, 6-, 7-, and 8-day-old larvae of *A. obliqua* irradiated at different gamma-radiation doses and exposed to *D. crawfordi*, *U. anastrephae*, and *D. longicaudata* females

		Larval host age (days)			
Parameters	Doses (Gy)	5	6	7	8
D. crawfordi					
Parasitoid emergence	0	0.00 ± 0.00G	0.00 ± 0.00G	0.00 ± 0.00G	0.00 ± 0.00G
	40	6.10 ± 0.94CD	2.6 0 ± 0.81DEFG	2.00 ± 0.82EFG	2.50 ± 0.71DEFG
	80	6.00 ± 066CDE	2.30 ± 0.59DEFG	1.00 ± 0.51FG	1.90 ± 0.43FG
	120	10.30 ± 0.76AB	6.20 ± 1.28CD	3.90 ± 0.75CDEFG	4.20 ± 0.86CDEF
	160	12.20 ± 1.32A	7.1 ± 1.30BC	4.90 ± 0.87CDEF	4.50 ± 6.66CDEF
Sex ratio	0	0.00 ± 0.00Ba	0.00 ± 0.00Bab	0.00 ± 0.00Bb	0.00 ± 0.00Bb
	40	1.86 ± 0.72Aa	0.92 ± 0.42Aab	0.75 ± 0.42Ab	0.57 ± 0.15Ab
	80	1.23 ± 0.35Aa	0.87 ± 0.35Aab	0.45 ± 0.21Ab	0.75 ± 0.24Ab
	120	1.30 ± 0.28Aa	1.73 ± 0.35Aab	0.84 ± 0.2Ab	1.00 ± 0.33Ab
	160	1.57 ± 0.29Aa	1.05 ± 0.15Aab	1.00 ± 0.21Ab	0.91 ± 0.21Ab
U. anastrephae					
Parasitoid emergence	0	27.60 ± 2.46HI	30.30 ± 4.41FGHI	28.00 ± 3.54HI	22.90 ± 2.31I
	40	35.20 ± 1.81EFGH	31.50 ± 2.98EFGHI	29.20 ± 2.62GHI	26.50 ± 3.01I
	80	51.80 ± 3.61BCD	39.00 ± 4.62DEFG	34.7 ± 3.14EFGHI	30.40 ± 2.52FGHI
	120	61.70 ± 3.50B	46.80 ± 2.99CDEF	44.20 ± 3.85CDEF	37.50 ± 2.98EFGH
	160	68.00 ± 2.10A	55.40 ± 4.76BC	48.90 ± 2.50BCDE	42.80 ± 2.96CDEF
Sex ratio	0	1.19 ± 0.12Ab	1.55 ± 0.11Aa	1.24 ± 0.11Aab	1.61 ± 0.19Aab
	40	1.57 ± 0.16Ab	1.95 ± 0.24Aa	1.50 ± 0.17Aab	1.41 ± 0.08Aab
	80	1.35 ± 0.17Ab	1.71 ± 0.25Aa	1.44 ± 0.17Aab	1.60 ± 0.21Aab
	120	1.41 ± 0.10Ab	1.71 ± 0.14Aa	1.50 ± 0.17Aab	1.80 ± 0.20Aab
	160	1.18 ± 0.06Ab	1.58 ± 0.12Aa	1.30 ± 0.12Aab	1.60 ± 0.17Aab
D. longicaudata					
Parasitoid emergence	0	29.10 ± 2.21I	31.50 ± 2.40GHI	29.2 ± 2.70HI	31.40 ± 2.92I HI
	40	38.60 ± 2.09FGHI	35.30 ± 2.96GHI	32.00 ± 1.34GHI	36.00 ± 1.42FGHI
	80	46.60 ± 2.23BCDE	46.30 ± 2.46CDEFG	44.70 ± 2.24CDEFH	40.70 ± 2.62EFGHI
	120	55.00 ± 2.57ABCD	50.30 ± 2.51ABCD	43.60 ± 1.30CDEFGHI	42.20 ± 2.86DEFGHI
	160	75.20 ± 1.92A	61.80 ± 2.54AB	55.80 ± 1.90ABC	42.90 ± 1.55DEFGHI
Sex ratio	0	1.21 ± 0.09Ab	1.24 ± 0.20Ab	2.45 ± 0.50Aa	1.29 ± 0.28Aab
	40	1.11 ± 0.18Ab	1.41 ± 0.13Ab	1.94 ± 0.64Aa	2.35 ± 0.82Aab
	80	1.17 ± 0.24Ab	1.31 ± 0.39Ab	1.26 ± 0.23Aa	1.08 ± 0.16Aab
	120	0.84 ± 0.13Ab	0.87 ± 0.20Ab	1.57 ± 0.41Aa	1.40 ± 0.21Aab

The same lowercase letters indicate no significant differences among columns and the same uppercase letters indicate no significant differences among rows (Tukey's HSD test, $P = 0.05$). Only different uppercase letters indicate significant differences among columns and rows by factor interactions. The means of parasitoid emergence and sex ratio in *D. crawfordi* were compared using the Bonferroni adjustment (α/n).

A greater number of first instar parasitoid larvae was found in host puparia from 5- and 7-day-old larvae (table 3). Unlike the other two Neotropical species, superparasitism was more frequent in *D. longicaudata* (table 4).

The melanization decreased in the younger larvae at higher doses of radiation when the larva was parasitized by *D. crawfordi*. The age of larva and doses of radiation had a similar influence on the percentage of melanization obtained in parasitized larvae by both *U. anastrephae* and *D. longicaudata*, although there was no interaction (table 1). Non-irradiated and the oldest larva had the highest percentages of melanization. The decrease of melanization was not clear in older larvae when it was parasitized by *U. anastrephae* (fig. 2).

Discussion

An optimal host radiation dose is essential to improve the standard operational capacity of the fruit fly parasitoid mass production in

Table 3. Larval host weight and host mortality (mean ± SE) tested in 5-, 6-, 7-, and 8-day-old larvae of *A. obliqua* irradiated at different gamma-radiation doses exposed to *D. crawfordi*, *U. anastrephae*, and *D. longicaudata* females

		Larval host age (days)			
Parameters	Doses (Gy)	5	6	7	8
D. crawfordi					
Larval host weight	0	16.24 ± 0.61Ab	18.50 ± 0.40Aa	19.03 ± 0.42Aa	18.74 ± 0.19Aa
	40	15.85 ± 0.42Ab	18.76 ± 0.57Aa	18.89 ± 0.20Aa	18.70 ± 0.54Aa
	80	16.37 ± 0.27Ab	18.68 ± 0.17Aa	19.05 ± 0.24Aa	18.16 ± 0.32Aa
	120	16.83 ± 0.15Ab	18.03 ± 0.46Aa	18.56 ± 0.33Aa	19.61 ± 0.18Aa
	160	17.06 ± 0.07Ab	17.99 ± 0.44Aa	19.02 ± 0.30Aa	18.29 ± 0.55Aa
Larval host mortality	0	13.10 ± 2.35Aa	9.70 ± 2.21Aa	7.10 ± 1.36Ab	10.00 ± 1.54Ab
	40	8.70 ± 1.12Aa	9.90 ± 1.71Aa	6.80 ± 2.06Ab	6.20 ± 1.57Ab
	80	13.20 ± 2.13Aa	13.40 ± 1.39Aa	6.60 ± 1.46Ab	7.70 ± 2.30Ab
	120	10.10 ± 2.01Aa	12.60 ± 2.14Aa	10.5 ± 2.51Ab	5.20 ± 1.51Ab
	160	11.90 ± 1.76Aa	13.30 ± 1.76Aa	10.40 ± 2.30Ab	4.00 ± 1.76Ab
U. anastrephae					
Larval host weight	0	17.69 ± 0.26DEF	19.58 ± 0.39ABC	19.28 ± 0.34ABCD	18.32 ± 0.27BCDEF
	40	16.82 ± 0.18F	19.99 ± 0.53A	19.56 ± 0.28ABC	18.46 ± 0.19ABCDE
	80	17.98 ± 0.34CDEF	19.65 ± 0.42AB	18.91 ± 0.23ABCD	18.76 ± 0.21ABCD
	120	18.39 ± 0.22ABCDEF	19.85 ± 0.61AB	19.11 ± 0.28ABCD	18.93 ± 0.18ABCD
	160	16.84 ± 0.09EF	19.35 ± 0.29ABC	18.95 ± 0.36ABCD	18.93 ± 0.18ABCD
Larval host mortality	0	7.90 ± 1.85Aa	6.40 ± 1.96Aa	7.10 ± 1.94Aa	4.50 ± 1.67Aa
	40	5.00 ± 1.49Aa	5.60 ± 1.44Aa	5.40 ± 1.97Aa	4.30 ± 1.60Aa
	80	5.00 ± 0.82Aa	7.00 ± 1.62Aa	6.30 ± 2.07Aa	4.00 ± 1.20Aa
	120	5.10 ± 1.58Aa	6.60 ± 1.70Aa	5.60 ± 1.02Aa	4.70 ± 1.43Aa
	160	5.20 ± 1.28Aa	6.30 ± 1.39Aa	4.80 ± 1.01Aa	4.80 ± 1.76Aa
D. longicaudata					
Larval host weight	0	17.51 ± 0.38Ac	18.39 ± 0.30Ab	18.76 ± 0.24Aab	18.81 ± 0.36Aa
	40	16.76 ± 0.27Ac	18.51 ± 0.32Ab	18.55 ± 0.30Aab	18.96 ± 0.11Aa
	80	16.80 ± 0.16Ac	17.95 ± 0.18Ab	18.72 ± 0.16Aab	18.50 ± 0.44Aa
	120	16.72 ± 0.25Ac	18.53 ± 0.13Ab	18.54 ± 0.32Aab	18.91 ± 0.18Aa
	160	16.77 ± 0.18Ac	17.62 ± 0.29Ab	18.53 ± 0.11Aab	18.95 ± 0.58Aa
Larval host mortality	0	8.30 ± 1.35Aa	8.90 ± 1.30Ab	3.70 ± 0.94Ac	3.10 ± 0.43Ac
	40	7.80 ± 1.12Aa	3.70 ± 0.59Ab	3.10 ± 0.82Ac	3.00 ± 0.53Ac
	80	8.80 ± 1.54Aa	5.80 ± 1.16Ab	3.10 ± 0.90Ac	4.30 ± 1.38Ac
	120	8.60 ± 1.14Aa	7.20 ± 1.43Ab	2.50 ± 0.52Ac	2.00 ± 0.85Ac
	160	8.60 ± 0.96Aa	6.70 ± 0.65Ab	5.10 ± 0.93Ac	2.40 ± 1.04Ac

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augmentative biological control programs. In this regard, the use of well-implemented radiation provides multiple practical benefits, such as fly emergence inhibitions, good quality of host larvae used for parasitoid rearing, and an increased parasitoid adult emergence (Cancino *et al.*, 2012). The trials of this study showed that younger *A. obliqua* larvae (5–6-day-old) irradiated at higher radiation doses (120 and 160 Gy) are mainly suitable to produce *D. crawfordi*, *U. anastrephae*, and *D. longicaudata* under mass-rearing conditions. Predictably, high gamma-radiation doses did not affect the host larva quality or health. Only the weight of host larvae used for *U. anastrephae* had an interaction between the age and the radiation dose, which could be part of the close coevolutive relationship between the host–parasitoid (Marsaro Júnior *et al.*, 2011; Jesus-Barros *et al.*, 2012; Murillo *et al.*, 2015). However, in general, the relevant quality parameters in radiated *A. obliqua* larvae, such as larval host weight and host mortality (Orozco-Dávila

Table 4. Scars on host puparia and first instar larvae of parasitoid (mean ± SE) found in host puparia coming from 5-, 6-, 7-, and 8-day-old *A. obliqua* larvae irradiated at different gamma-radiation doses and exposed to *D. crawfordi*, *U. anastrephae*, and *D. longicaudata* females

		Larval host age (days)			
Parameters	Doses (Gy)	5	6	7	8
D. crawfordi					
No. of scars	0	1.60 ± 0.25Aa	1.76 ± 0.15Aa	2.48 ± 0.49Aa	2.08 ± 0.44Aa
	40	2.68 ± 0.24Aa	2.16 ± 0.19Aa	2.36 ± 0.63Aa	2.12 ± 0.29Aa
	80	2.28 ± 0.23Aa	1.92 ± 0.11Aa	1.92 ± 0.39Aa	2.24 ± 0.29Aa
	120	2.32 ± 0.23Aa	1.60 ± 0.11Aa	1.80 ± 0.38Aa	3.44 ± 0.64Aa
	160	2.30 ± 0.53Aa	1.76 ± 0.13Aa	2.16 ± 0.48Aa	2.16 ± 0.31Aa
No. of first instar	0	0.96 ± 0.04Aa	1.00 ± 0.00Aa	0.92 ± 0.08Aa	0.84 ± 0.09Aa
	40	1.28 ± 0.11Aa	0.88 ± 0.08Aa	0.96 ± 0.12Aa	0.96 ± 0.07Aa
	80	1.08 ± 0.08Aa	1.00 ± 0.00Aa	1.04 ± 0.10Aa	1.04 ± 0.09Aa
	120	1.00 ± 0.00Aa	0.96 ± 0.09Aa	0.80 ± 0.11Aa	1.12 ± 0.12Aa
	160	1.00 ± 0.21Aa	1.12 ± 0.06Aa	1.04 ± 0.19Aa	1.08 ± 0.11Aa
U. anastrephae					
No. of scars	0	1.50 ± 0.42CD	2.53 ± 0.45BCD	2.66 ± 0.58BCD	3.70 ± 0.73ABC
	40	1.50 ± 0.30CD	1.80 ± 0.31CD	3.26 ± 0.65ABCD	5.20 ± 0.62A
	80	1.30 ± 0.30CD	1.60 ± 0.27CD	2.20 ± 0.40CD	4.90 ± 1.15AB
	120	1.00 ± 0.25CD	2.00 ± 0.33CD	2.86 ± 0.36ABCD	2.30 ± 0.51BCD
	160	0.80 ± 0.24D	1.40 ± 0.13CD	2.40 ± 0.43CD	1.40 ± 0.33CD
No. of first instar	0	0.90 ± 0.10Ab	0.86 ± 0.09Aab	0.93 ± 0.06Aa	0.90 ± 0.10Aab
	40	0.90 ± 0.10Ab	0.93 ± 0.06Aab	1.06 ± 0.11Aa	1.10 ± 0.11Aab
	80	0.80 ± 0.13Ab	0.93 ± 0.06Aab	1.06 ± 0.11Aa	1.30 ± 0.33Aab
	120	0.70 ± 0.15Ab	0.93 ± 0.22Aab	1.06 ± 0.06Aa	1.00 ± 0.14Aab
	160	0.60 ± 0.16Ab	1.00 ± 0.00Aab	1.00 ± 0.13Aa	0.80 ± 0.13Aab
D. longicaudata					
No. of scars	0	2.36 ± 0.36Aa	2.32 ± 0.31Aab	2.80 ± 0.28Aab	1.66 ± 0.33Ab
	40	2.24 ± 0.35Aa	2.40 ± 0.30Aab	2.92 ± 0.42Aab	3.06 ± 0.58Ab
	80	3.28 ± 0.32Aa	2.40 ± 0.31Aab	2.64 ± 0.37Aab	2.60 ± 0.50Ab
	120	2.64 ± 0.37Aa	2.72 ± 0.29Aab	3.04 ± 0.47Aab	1.86 ± 0.35Ab
	160	3.92 ± 0.50Aa	2.48 ± 0.23Aab	3.20 ± 0.37Aab	2.13 ± 0.46Ab
No. of first instar	0	0.80 ± 0.08Ba	1.08 ± 0.09Bab	1.24 ± 0.10Ba	0.80 ± 0.10Bb
	40	1.24 ± 0.13Aba	1.20 ± 0.08ABab	1.20 ± 0.19ABa	1.13 ± 0.13ABb
	80	1.48 ± 0.15Aba	1.24 ± 0.08ABab	1.28 ± 0.15ABa	1.00 ± 0.16ABb
	120	1.28 ± 1.15Aba	1.28 ± 0.09ABab	1.40 ± 0.21ABa	1.00 ± 0.13ABb
	160	1.76 ± 0.17Aa	1.20 ± 0.08Aab	1.36 ± 0.11Aa	1.06 ± 0.18Ab

The same lowercase letters indicate no significant differences among columns and the same uppercase letters indicate no significant differences among rows. Only different uppercase letters indicate significant differences among columns and rows by factor interactions (Tukey's HSD test, $P = 0.05$).

et al., 2014), were not affected regarding the non-irradiated larvae. Similarly, the normal development of the parasitoid within the host was not affected by the use of higher radiation doses.

Increased parasitoid emergence could be achieved with radiation doses higher than 160 Gy. However, it is advisable to assess the effects of radiation exposure time and crowding effects on the host larvae. Particularly, parasitoid emergences in the three evaluated parasitoid species were enhanced and the sex ratio was slightly female-biased. The sex ratio was related to the host age in a different way for each species. More female offspring were recovered from young host larvae in the two native braconid species (5-day-old for *D. crawfordi* and 6-day-old for *U. anastrephae*). Koinobiont parasitoids usually deposit female eggs on young hosts for two reasons: to avoid a stronger antagonistic reaction from older hosts and to provide suitable nutrition to parasitoid larval instars inside the host. This could be considered an

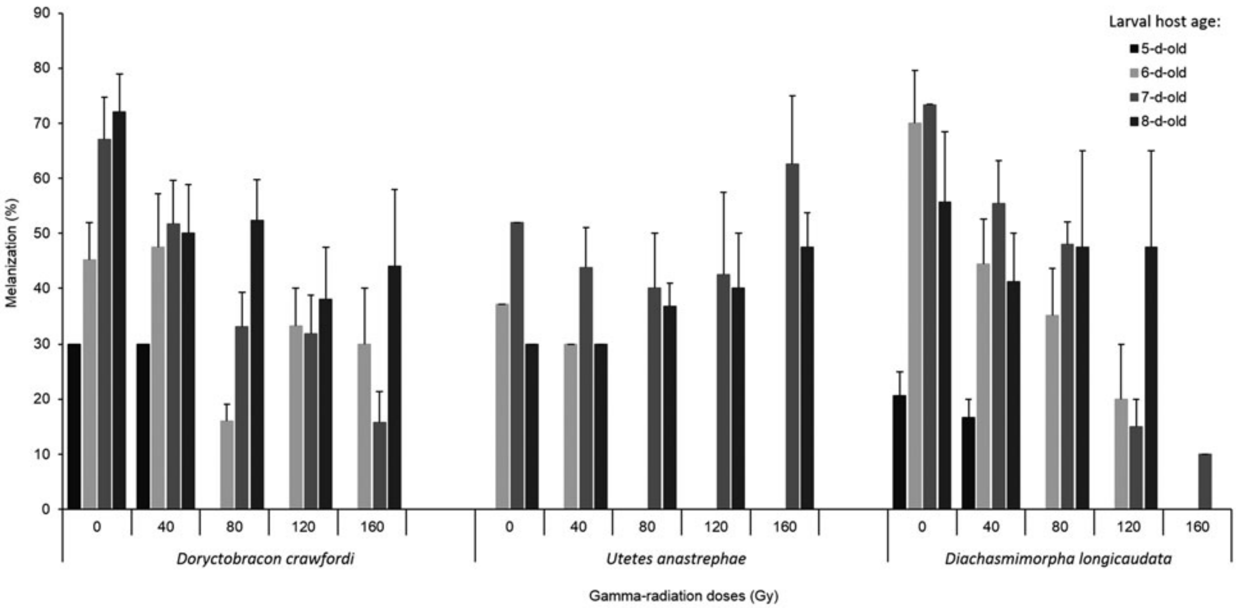

Figure 2. Percentages of melanization (±SE) in 5-, 6-, 7-, and 8-day-old *A. obliqua* host larva irradiated at different doses and parasitized by *D. crawfordi*, *U. anastrephae*, and *D. longicaudata*.

adaptive strategy (Vinson and Iwantsch, 1980; Hu and Vinson, 2000; Kaeslin *et al.*, 2010), displayed by both Neotropical braconids. Even though *D. longicaudata* is a kind of koinobiont, it could have another strategy, taking into account its status as an exotic species that established a new trophic association with *A. obliqua*. Therefore, *D. longicaudata* could attack older larvae by avoiding host immune reactions through oviposition of eggs with entomopoxvirus (Lawrence, 2005).

Interestingly, the results showed that *D. crawfordi* has been able to survive and develop successfully inside irradiated *A. obliqua* larvae, even at 40 Gy. Lower radiation doses (<20 Gy) were not effective. However, this Neotropical parasitoid was unable to develop into non-irradiated *A. obliqua* larvae even though they have been parasitized. Parasitoid emergence and biased female sex ratio were linked to radiation doses, which could be used as indicators of a decrease in antagonistic reactions of *A. obliqua* larvae (Strand and Pech, 1995; Reed *et al.*, 2007; Xu *et al.*, 2016). In that sense, it is important to note that the host's immune system is weakened by radiation (Hendrichs *et al.*, 2009), which reduces its ability to suppress parasitoid development (Al khalaf and Abdel Baki, 2013; Sang *et al.*, 2016). This fact was verified with *D. crawfordi* adult emergence from irradiated *A. obliqua* larvae since as expressed by Poncio *et al.* (2016) *A. obliqua* is not a suitable host for *D. crawfordi* in nature despite the sympatric coexistence between them. Furthermore, the larval host's age is another critical factor, which adversely affects parasitoid emergence. The host's immune system usually strengthens as the larvae grow older, providing greater protection against parasitoid development (Hegazi and Khafagi, 2008; Beckage, 2009) and in turn, a remarkable reduction in host mortality in *Anastrepha* species mass rearing (Orozco-Dávila *et al.*, 2017). Younger host larvae are usually more sensitive to handling under rearing conditions, and also are more vulnerable to the parasitoid attack due to the early stage of development of the host's immune system (Sisterson and Averill, 2003; Ideo *et al.*, 2008). Data from trials of the present study verified this assertion, being that both the mortality and the parasitism percentages were appreciably higher in the youngest host larvae compared to the older larvae.

Superparasitism in *A. obliqua* larvae in both *D. crawfordi* and *U. anastrephae* was scarce; apparently, superparasitism is not a very usual strategy in Neotropical *Anastrepha* parasitoid species (Ayala *et al.*, 2018). Although the number of oviposition scars recorded in the host puparia parasitized by *U. anastrephae* was higher at low doses in 7–8-day-old larvae, there was no consistency with the parasitoid first instar number. The first instar larva recorded was often in 7-day-old host larva. There is not sufficient information about the foraging and host selection in *U. anastrephae*. The low superparasitism level in the native *U. anastrephae* may indicate that it is not necessarily a survival mechanism, as is the case of introduced parasitoid species (Vinson and Iwantsch, 1980; Kraaijeveld *et al.*, 2011). *Utetes anastrephae* has a sympatric co-evolutionary relationship with *A. obliqua*, which would justify the low superparasitism in larvae of this tephritid. In contrast, superparasitism in *D. longicaudata* was a lot more frequent and constant than in both Neotropical parasitoid species, but with a greater preference in *A. obliqua* larvae irradiated at high doses. This introduced braconid parasitoid has adapted to *Anastrepha* spp. larvae in nature (Montoya *et al.*, 2017), and optimizes their resources (host larvae) by using superparasitism as an effective survival mechanism (Montoya *et al.*, 2000*b*, 2012; González *et al.*, 2007). Superparasitize host larvae increased the probability of survival of one *D. longicaudata* individual per host after high intraspecific competitive activity in the first instar (Montoya *et al.*, 2011).

The presence of melanin in parasitized host larvae may be a practical qualitative indicator of the host immune reactions (Nappi and Vass, 1993; Suárez *et al.*, 2020). Melanin is the result of enzymatic activity from phenoloxidase as the host's cellular and humoral reaction to parasitization (Boman and Hultmark, 1987;

Nappi and Ottaviani, 2000; Liu *et al.*, 2007). The gradual melanin level depletion is a relevant indicator of the adverse effect caused by increased radiation. Radiation may induce physiological changes involving reduced phenoloxidase action in tephritid larvae. This was reported in larvae of *Anastrepha suspensa* (Loew) (Nation *et al.*, 1995), *C. capitata* (Mansour and Franz, 1996), and *Bactrocera dorsalis* (Hendel) (Chang *et al.*, 2016). Nevertheless, the qualitative determination of melanization is not feasible in all *Anastrepha* species, because observations in *A. ludens* larvae did not provide evidence of melanin in both *D. crawfordi* and *U. anastrephae* eggs and larvae as a signal of the host's immunological reaction (Cancino *et al.*, 2020). The immunological reactions of *A. obliqua* larvae could be related to a more forceful way to parasitism, considering that larval development occurs mainly inside small host fruits, which have soft pulp, thin skin, and large seeds, such as native *Spondias* (Anacardiaceae) species. These host fruit species have highly favorable conditions for parasitoids to find and oviposit *A. obliqua* larvae (Ovruski *et al.*, 2000; Sivinski *et al.*, 2000). Finally, the host's immune response capacity may decline due to increased radiation, which is of considerable significance to be applied as an alternative rearing procedure in different parasitoid–host relationships that are not viable under lab conditions (Hoffmann *et al.*, 2001; Muhammad *et al.*, 2013; Hasan *et al.*, 2019). Similarly, lab-reared parasitoid production levels are low due to the immunological action of the artificially reared host, which could be avoided using radiation (Consoli *et al.*, 2000; Hasan *et al.*, 2019).

In summary, these findings identified both suitable radiation doses and host's ages of *A. obliqua* larvae under mass-rearing conditions, which enable its subsequent use in *D. longicaudata*, *U. anastrephae*, and *D. crawfordi* rearing to substantially improve parasitoid mass production. In this regard, it is noteworthy that the main objective is focused on augmentative biological control through parasitoid mass releases. However, and from a practical viewpoint related to open-field parasitoid releases against *A. obliqua* wild populations, only two parasitoid species of the three species studied, *U. anastrephae* and *D. longicaudata*, may be massively released in marginal areas surrounding commercial orchards, such as backyard orchards and wild vegetation areas. This is because *D. crawfordi* has a very low natural emergence rate from *A. obliqua* puparia (Sivinski *et al.*, 1997). Authors such as Córdova-García (2008) and Poncio *et al.* (2016) have reported that *D. crawfordi* larva development is harshly affected by the immunological defenses of *A. obliqua* larva under natural and lab conditions. *Doryctobracon crawfordi* would be more associated with *A. ludens* in nature (Miranda *et al.*, 2015; Montoya *et al.*, 2017). In contrast, *U. anastrephae* is closely associated with *A. obliqua* in the Neotropics (Sivinski *et al.*, 1997, 2000; López *et al.*, 1999), and it has a coevolutionary process since its short ovipositor is adapted to parasitize *A. obliqua* larvae in small fruits (Sivinski *et al.*, 2001). As mentioned by Aluja *et al.* (2009, 2014), native vegetation can be managed to conserve and multiply native *Anastrepha* parasitoids in rural areas where farmers cannot apply expensive pest control and management procedures. Thus, massive releases of species such as *U. anastrephae* may facilitate such measures. On the other hand, the exotic *D. longicaudata* is considered a new successful association with *Anastrepha* (Schiner) in the Neotropics (Ovruski *et al.*, 2000; Cancino *et al.*, 2002; Montoya *et al.*, 2017; Garcia *et al.*, 2020). It has previously been used against *A. obliqua* in mango orchards in Chiapas, Mexico, through augmentative releases, and their permanent establishment in this region was confirmed (Montoya *et al.*, 2000*b*). Consequently, it is a valuable parasitoid species for use in *Anastrepha* biological control (Montoya *et al.*, 2017).

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