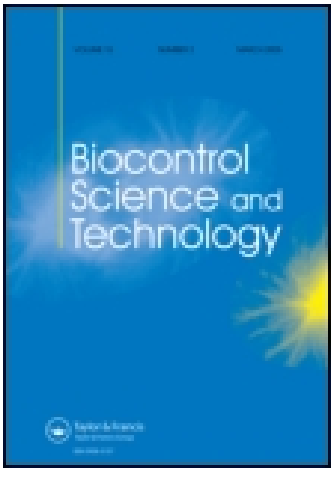

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X-ray doses to safely release the parasitoid Diachasmimorpha longicaudata (Hymenoptera: Braconidae) reared on Anastrepha fraterculus larvae (Diptera: Tephritidae)

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RESEARCH ARTICLE

X-ray doses to safely release the parasitoid *Diachasmimorpha longicaudata* (Hymenoptera: Braconidae) reared on *Anastrepha fraterculus* larvae (Diptera: Tephritidae)

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Diachasmimorpha longicaudata is a koinobiont larval parasitoid that is currently used to control fruit flies of the genera *Anastrepha*, *Ceratitis* and *Bactrocera.* In the rearing process, a fraction of the host larvae that are exposed to parasitoids escape from parasitism and develop into viable and fertile flies. This creates the need to eliminate emerging flies before the parasitoids are shipped for release, increasing costs due to additional handling steps. Exposure of fly eggs or larvae to gamma-irradiation before they are parasitised has been used to reproductively sterilise hosts, or even inhibit their emergence. Our aim was to determine whether X-ray radiation applied to *Anastrepha fraterculus* third instar larvae before they are exposed to parasitoids, inhibits fly emergence in non-parasitised larvae without affecting the performance of the parasitoids that emerge from parasitised larvae. Three X-ray doses: 6250.2 R, 8333.6 R and 10417 R (equivalent to 60, 80 and 100 Gy, respectively) and one γ-ray dose (100 Gy) were tested. Fly emergence decreased with increasing doses of radiation, showing null values for the higher X-ray dose and the dose of 100 Gy. Irradiation showed either no impact or a positive effect on parasitism rate and fecundity. Sex rate was biased towards females in almost every dose. We conclude that the two types of radiation evaluated here were equally effective in suppressing fly emergence with no detrimental effects on the biological quality of the produced parasitoids. X-rays offer an alternative method of irradiation than the conventional radiation source, i.e. γ-rays. These results represent a significant improvement in the development of a biological control programme against *A. fraterculus.*

Keywords: biological control; gamma rays; *Anastrepha fraterculus*; *Diachasmimorpha longicaudata*; fruit fly pests; natural enemies

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Introduction

Fruit fly pests in Argentina

In Argentina there are two fruit fly species of economic importance: the Mediterranean fruit fly, *Ceratitis capitata* (Diptera: Tephritidae) (Wiedemann) and the South American fruit fly, *Anastrepha fraterculus* (Diptera: Tephritidae) (Wiedemann) (Aluja & Liedo, 1986; Ovruski, Cancino, Fidalgo, & Liedo, 1999). *C. capitata* is originally from the tropics of mainland Africa, but was registered in Argentina in the early twentieth century (Vergani, 1952). *A. fraterculus*, is a native species to South America and is mainly distributed in subtropical humid regions (Ovruski, Colin, Soria, Oroño, & Schliserman, 2003; Segura et al., 2006), mostly coexisting with *C. capitata* (Ratkovich & Nasca, 1953). Both species cause direct damage to the fruit during their larval development. Furthermore, the presence of these flies in a given region limits the access of horticultural commodities to potential markets due to the quarantine restrictions imposed by countries that are free of these pests (Malavasi, Rohwer, & Campbell, 1994; Ovruski et al., 1999; SENASA-Perú, 1999). In Argentina, annual losses due to direct damage, in spite of intensive control measures, are estimated between 15% and 20% of the fruit and horticultural production (Alvarado & Ritacco, 1991; Guillén & Sánchez, 2007).

Biological control against fruit fly pest

The augmentative release of parasitoid wasps has been evaluated in several biological control strategies against fruit fly pests. One of most successful wasp species for controlling tephritid flies is *Diachasmimorpha longicaudata* (Ashmead) (Hymenoptera: Braconidae) (González, Montoya, Pérez-Lachaud, Cancino, & Liedo, 2007; Lawrence, Greany, Nation, & Baranowski, 1978; Malavasi, Nascimento, Paranhos, Costa, & Walder, 2007; Montoya & Cancino, 2004; Wong & Ramadan, 1987). This species is a solitary endoparasitoid of the larval-pupal stages of fruit flies, native to the Indo-Australian region (Wharton & Gilstrap, 1983). It is capable of developing on a large number of tephritid species like *C. capitata* and other species belonging to the *Anastrepha* and *Bactrocera* genera (Carvalho & Nascimento, 2002). This characteristic represents a significant advantage to control fruit flies in areas where *C. capitata* and *A. fraterculus* are sympatric, such as in the citrus regions of northern and central Argentina. In addition, *D. longicaudata* has been effective in both tropical (Cancino, Lopez, & Aguilar, 1995; Sivinski, 1996) and arid environments (Greany, Ashley, Baranowsky, & Chambers, 1976).

D. longicaudata is reared in several countries, e.g. México, Guatemala, Costa Rica, Perú and Brasil (Cancino et al., 1995; Carro, Hernández, Zúñiga, & Cancino, 2001; Carvalho & Nascimento, 2002; Montoya et al., 2000; Sivinski, 1996). Under mass rearing, the parasitism rate rarely exceeds 50%, and the parasitised and non-parasitised pupae have a similar aspect. This implies that it is necessary to separate large numbers of non-parasitised hosts from the biological control agents, which decreases efficiency in large scale mass-rearing (Hendrichs et al., 2009). A solution to this problem, as proposed by Sivinski and Smittle (1990), consists in irradiating the fly larvae with an adequate dose before exposure to the parasitoids. This radiation dose must inhibit fly emergence from the non-parasitised pupae, but allow a normal development of parasitoids from the parasitised pupae, so that their performance as biocontrol agents is unaltered.

Irradiation and its effect on fruit fly larvae

Gamma (γ) radiation based on ^{60}Co is the most widely used method for insect irradiation in pest control programmes, such as the Sterile Insect Technique and biological control (Mastrangelo et al., 2010). However, the transportation of radioactive material is getting much more elaborate and restrictive, and therefore costly (Mehta, 2009). This presents a serious impediment to the use of gamma-radiation for the control of insect pests. X-ray irradiators do not have this limitation because they consist of electron accelerators that generate high-energy X-rays without using radioactive materials: the irradiator is disconnected from the power supply and the radiation is discontinued. Both γ and X-rays, even though different in nature, have similar effects on the biological material and relative biological effectiveness, especially on insects (Bakri, Mehta, & Lance, 2005; Mastrangelo et al., 2010; Parker et al., 2008).

Little information is available about irradiation of *A. fraterculus* (Allinghi, Calcagno, Petit-Marty, et al., 2007; Allinghi, Gramajo, Willink, et al., 2007; IDIDAS, 2002), particularly concerning the radiation dose to be applied to avoid adult emergence. However, data about irradiation used on larvae of other tephritid species could be taken as a reference point (Cancino & Montoya, 2006; Cancino, Ruíz, Gómez, & Toledo, 2002; Menezes et al., 1998). In general, the γ-ray dose to be applied to third instar larvae of *Anastrepha* genus is between 20 and 45 Gy, depending on the tested species (Cancino & Montoya, 2006; Cancino & Ruiz, 2001; Cancino, Ruíz, Sivinski, Gálvez, & Aluja, 2009; Menezes et al., 1998; Sivinski & Smittle, 1990). According to the literature (Cancino & Montoya, 2006; Cancino & Ruiz, 2001) and preliminary studies conducted in our laboratory (Viscarret et al., 2012), the irradiation dose also depends on the volume of larvae to be irradiated and the substrate where the larvae are kept at the moment of irradiation. Irradiator status and the physical condition of larvae also play a role in the effectiveness of radiation to suppress fly emergence (Cancino, Ruíz, Pérez, & Harris, 2009). It is worth mentioning that all these data were reported using γ-rays, and there is no available information on X-ray doses.

In the present work, we aimed at taking a step towards the use of *D. longicaudata* for the biological control of *A. fraterculus*, with particular interest in minimising radiation doses. This step involved determining the X-ray radiation dose to be applied to *A. fraterculus* larvae in order to inhibit fly emergence without affecting the development and subsequent performance of the parasitoid *D. longicaudata* reared on those irradiated larvae.

Material and methods

Biological materials

All the parasitoids and fruit flies used in this study were reared at the Laboratorio de Insectos de Importancia Económica, Instituto de Genética 'Ewald A. Favret' (IGEAF, INTA Castelar). *A. fraterculus* larvae were reared using an artificial diet based on yeast, wheat germ, sugar, and agar (Vera, Abraham, Oviedo, & Willink, 2007). The adults were provided with water and a mixture of sugar and hydrolyzed yeast (4:1 rate; Jaldo, Gramajo, & Willink, 2001). *D. longicaudata* were reared using larvae of a laboratory strain of *C. capitata* as hosts and were provided with water and honey during the adult stage. *C. capitata* larvae were reared using an artificial

diet based on carrot [modified from Terán (1977)], and the adults were provided with water and a mixture of sugar and yeast (3:1 rate).

Irradiators

Radiation treatments were carried out using a X-irradiator Philips MG 160 Constant Potential X-rays System – Minus H:T. Generator Type 160 kV/4 kW (IGEAF, INTA Castelar). And a γ-irradiator PISI (Planta de irradiación semiindustrial): flat irradiator (1.50 x 1.00 mts), nominal activity: 1.000.000 Curies, activity in March, 2009: 604.368 Curies, placed in 'Centro Nacional de Energía Atómica' (CNEA), Ezeiza, Provincia de Buenos Aires.

Doses and methods

Third instar *A. fraterculus* larvae in artificial diet were irradiated with one of the following doses of X-rays: 6250.2 R, 8333.6 R and 10417.0 R (equivalent to 60, 80 and 100 Gy, respectively). A fourth group of larvae was irradiated with γ-rays at a dose of 100 Gy. All the irradiation treatments were carried out in separate assays; therefore a non-irradiated set of larvae was used/set for each dose as control. Irradiation was performed under normal atmospheric conditions (free oxygen). In each experiment, 0.3 ml of *A. fraterculus* eggs (ca., 4800 eggs) were placed on top of 22 × 18 × 2 cm trays filled with 0.5 kg of diet (see Biological materials for details on rearing protocols). After irradiation, approximately 150 larvae were placed in small Petri dishes (diameter × height: 3 cm × 0.5 cm) and wrapped in voile fabric (oviposition units). Twenty oviposition units were made for each dose: half of them were offered to parasitoids and half were not exposed. Exposure to parasitoids consisted of placing each oviposition unit inside a 3L glass container with 15 males and 15 females of *D. longicaudata* (8 to 12-day-old) for 4 h. Females had one previous oviposition experience (Segura, Viscarret, Ovruski, & Cladera, 2005). Right after exposure, larvae were kept in a container with fresh artificial diet and vermiculite as pupation substrate.

From each oviposition unit offered to the parasitoids (hereafter, replicate) we estimated the parasitism rate [number of emerged parasitoids / (number of emerged parasitoid + number of emerged flies + number of non-emerged pupae)] and the proportion of female descendants (number of female parasitoids / number of emerged parasitoids). Five female and five male parasitoids (less than 48 hours old) were recovered from each replicate, placed in a 3L glass container and provided water and honey. A total of 10 containers (replicates) were arranged for each dose. One oviposition unit containing third instar *C. capitata* larvae (*ad libitum*) was offered to each one of these groups of parasitoids, for four hours. This procedure was repeated every other day, for two weeks. The number of female and male parasitoids alive was recorded before each larval exposure. One week after each larval exposure, pupae were recovered and conditioned to allow the emergence of the offspring. The number of male and female parasitoids, flies and non-emerged pupae was recorded. These data were used to estimate the mean total fecundity during the two-week assay of the parasitoids developed in irradiated larvae, and the proportion of females in their offspring. The experimental conditions were 24 ± 2°C and RH: 70 ± 20%.

A second set of 10 oviposition units were maintained under similar environmental conditions, but were not exposed to parasitoids. These oviposition units were used to record the number of emerged flies and non-emerged pupae, and to estimate

the proportion of emerged flies as [number of emerged flies / (number of emerged flies + number of non-emerged pupae)]. To determine the fertility of the emerged flies, five virgin females and five virgin males were separated per replicate and dose. These flies were mated with virgin counterparts obtained from the laboratory rearing of *A. fraterculus* and provided with food and water. All the flies used in this study (obtained either from the experiment or from the laboratory rearing) were sexually mature (20-day-old, Segura et al., 2009, 2013). A cylindrical plastic container (3 cm tall × 2 cm in diameter) filled with red coloured water and covered with Parafilm M (Pechiney Plastic Packaging, Chicago, IL, USA) was offered to female flies to collect eggs. After 48 h, the eggs were transferred to a piece of black filter paper, which was placed inside a Petri dish on top of a moist cloth. Petri dishes were placed in a growth chamber under the following environmental conditions 25° ± 1°C and RH: 80%. After 72 h, the proportion of hatching was estimated as [number of hatched eggs / (number of hatched eggs + number of non-hatched eggs)].

The percentage of emerged flies, the proportion of hatched eggs, the parasitism rate, and the female proportion were processed as a binary response and analysed by a logistic regression, with the dose as an independent variable. Model fit was checked by Deviance and the significance of model parameters by Wald statistics (Agresti, 1996). The mean fecundity was compared between each dose and its control by means of a one-way analysis of variance.

Results

Fly emergence and fertility of irradiated larvae

The proportion of emerged flies for the doses of 6250.2 R and 8333.6 R was significantly lower than their controls ($x^2_{(1)} = 270.092$, $p < 0.001$, Wald = 249.307; $p < 0.001$ and $x^2_{(1)} = 3293.438$, $p < 0.001$; Wald = 855.727, $p < 0.001$, respectively). In the case of 10417 R and 100 Gy, fly emergence was completely suppressed (Table 1).

The proportion of hatched eggs was similar for male and female flies emerged from larvae irradiated with 6250.2 R with respect to the control dose ($x^2_{(1)} = 3.735$, $p = 0.053$, Wald = 3.717; $p = 0.054$ and $x^2_{(1)} = 3.335$, $p = 0.068$; Wald = 3.333, $p = 0.068$, respectively). No females emerged from the larvae irradiated with 8333.6 R, and the emerged males presented a significantly reduced fertility when compared to the control males ($x^2_{(1)} = 6.095$, $p = 0.014$; Wald = 5.330, $p = 0.021$) (Table 1).

Parasitism rate, proportion of emerged female parasitoid and parasitoid fecundity in irradiated hosts

A higher proportion of parasitoids emerged from larvae irradiated at 6250.2 R than from non-irradiated larvae ($x^2_{(1)} = 16.609$, $p < 0.001$; Wald = 16.228, $p < 0.001$). The proportion of female parasitoids that emerged from larvae irradiated at this dose was significantly lower than that of the control ($x^2_{(1)} = 11.132$, $p < 0.001$; Wald = 10.557, $p = 0.001$). For the intermediate dose (8333.6 R) both the parasitism rate and the proportion of females were similar in parasitoids developing from irradiated and non-irradiated larvae ($x^2_{(1)} = 0.277$, $p = 0.599$; Wald = 0.277, $p = 0.595$ and $x^2_{(1)} = 1.828$, $p = 0.176$; Wald = 1.827, $p = 0.177$, respectively). In contrast, for the dose of 10417 R the parasitism rate was similar to the control ($x^2_{(1)} = 0.395$, $p = 0.530$; Wald = 0.395, $p = 0.530$), but the female proportion was higher in parasitoids from

Table 1. Effect of irradiation of *A. fraterculus* larvae on fly emergence and fertility, for each tested dose and the corresponding control (non-irradiated larvae).

		Fly fertility	
Radiation dose	Fly emergence	Females	Males
6250.2 R	0.12 ± 0.03*	0.47 ± 0.08	0.63 ± 0.09
Control	0.39 ± 0.04	0.42 ± 0.06	0.49 ± 0.09
8333.6 R	0.02 ± 0.00*	No emergence	0.33 ± 0.33*
Control	0.89 ± 0.01	–	0.79 ± 0.06
10417 R	No emergence	–	–
Control	0.84 ± 0.05	–	–
100 Gy	No emergence	–	–
Control	0.41 ± 0.04	–	–

Fly emergence was measured as proportion of emerged pupae. Fly fertility was measured as proportion of hatched eggs. Mean values are followed by the standard error of the mean. Asterisks indicate significant differences between dose and control. Statistics and *p*-values are shown in the text.

irradiated larvae ($x^2_{(1)}$ = 38.337, $p < 0.001$; Wald = 37.963, $p < 0.001$). When γ radiation was tested, both the parasitism rate and the proportion of females were higher for the parasitoids developing from irradiated larvae than for its control ($x^2_{(1)}$= 32.484, $p < 0.001$; Wald = 32.770, $p < 0.001$ and $x^2_{(1)}$ = 4.109, p = 0.043; Wald = 4.091, p = 0.043, respectively) (Table 2).

Parasitoids emerging from larvae irradiated with 6250.2 R and 10417 R showed a significantly higher fecundity than parasitoids emerging from non-irradiated control larvae ($F_{1,18}$ = 10.96, $p < 0.004$ and $F_{1,18}$ = 11.24, p = 0.004, respectively; Table 3). On the other hand, the female parasitoids emerging from the larvae irradiated with 8333.6 R and 100 Gy presented a similar mean fecundity than that of their controls ($F_{1,18}$ = 0.38, p = 0.548 and $F1_{,16}$ = 0.03, p = 0.874, respectively).

The proportion of females from the progeny of parasitoids reared on larvae irradiated with 6250.2 R and 10147 R was higher than those reared on non-irradiated larvae ($x^2_{(1)}$ = 39.607, $p < 0.001$; Wald = 39.198, $p < 0.001$ and $x^2_{(1)}$= 4.427, p = 0.035; Wald = 4.428, p = 0.035, respectively). In the case of parasitoids

Table 2. Effect of radiation of A. *fraterculus* larvae on parasitism rate and proportion of *D. longicaudata* females in the progeny, for each tested dose and the corresponding control (non-irradiated larvae).

Radiation dose	Parasitism rate	Proportion of females
6250.2 R	0.15 ± 0.02*	0.57 ± 0.04*
Control	0.10 ± 0.03	0.78 ± 0.04
8333.6 R	0.62 ± 0.03	0.57 ± 0.03
Control	0.64 ± 0.03	0.52 ± 0.02
10417 R	0.52 ± 0.04	0.50 ± 0.03*
Control	0.50 ± 0.04	0.34 ± 0.02
100 Gy	0.40 ± 0.03*	0.58 ± 0.05*
Control	0.23 ± 0.04	0.41 ± 0.06

Mean values are followed by the standard error of the mean. Asterisks indicate significant differences between dose and control. Statistics and *p*-values are shown in the text.

Table 3. Effect of radiation of *A. fraterculus* larvae on fecundity and proportion of females in the progeny, of parasitoids developed in irradiated larvae, for each tested dose and the corresponding control (non-irradiated larvae).

Radiation dose	Parasitoids fecundity	Female proportion
6250.2 R	103.4 ± 6.72*	0.59 ± 0.02*
Control	76.6 ± 4.52	0.42 ± 0.08
8333.6 R	76.6 ± 7.57	0.42 ± 0.03
Control	82.5 ± 5.94	0.42 ± 0.02
10417 R	139.8 ± 6.50*	0.64 ± 0.01*
Control	112.3 ± 4.99	0.6 ± 0.02
100 Gy	31.8 ± 3.77	0.46 ± 0.05
Control	30.8 ± 4.27	0.54 ± 0.03

Parasitoids fecundity was measured as the mean number of descendants per female parasitoid emerged from irradiated larvae during the two-week assay. Mean values are followed by the standard error of the mean. Asterisks indicate significant differences between dose and control. Statistics and *p*-values are shown in the text.

that developed from larvae irradiated with 8333.6 R and 100 Gy no differences were observed between the doses and their controls ($x^2_{(1)} = 0.024$, $p = 0.878$; Wald = 0.024, $p = 0.878$ and $x^2_{(1)} = 2.213$, $p = 0.137$; Wald = 2.209, $p = 0.137$, respectively) (Table 3).

Discussion

In the present work the irradiation of third instar *A. fraterculus* larvae was explored as a way to inhibit the emergence of adult flies without affecting the quality of those larvae as a rearing substrate for the parasitoid *D. longicaudata*. The doses of 10417 R and 100 Gy were appropriate to the objectives of our work, because fly emergence was completely suppressed and the biological parameters evaluated on *D. longicaudata* were not negatively affected. The doses of 6250.2 R and 8333.6 R were not suitable because emergence of flies was detected. Furthermore, emerged flies were fertile, although at 8333.6 R only fertile males were detected. It is important to stress that in both cases the parasitoid variables evaluated were not detrimentally affected by the radiation.

There is no available information about the use of irradiation on *A. fraterculus* larvae to suppress fly emergence, but several authors have worked on other *Anastrepha* species using γ-rays. Sivinski and Smittle (1990) recorded an inhibition of *A. suspensa* emergence after larvae were irradiated without the rearing medium (naked larvae) with a dose of 40 Gy. Previous studies evaluated different doses of γ radiation to suppress fly emergence in *Anastrepha ludens* (Loew), *Anastrepha obliqua* (Macquart) and *Anastrepha serpetina* (Wiedemann). These doses depended on the species, the volume, the number and the physical conditions of the larvae during the irradiation (Cancino, Ruíz, Viscarret, Sivinski, & Hendrichs, 2012). For example, when small lots of larvae were used, the doses required to inhibit fly emergence in *A. ludens*, *A. obliqua* and *A. serpentina* were 20 Gy, 30 Gy and 20 Gy, respectively. However, when 1 L of larvae was irradiated, the doses had to be raised to 40 Gy, 40 Gy and 30 Gy, respectively, in order to suppress fly emergence (Cancino, Ruíz, López, et al., 2009; Cancino, Ruíz, Sivinski, et al., 2009; Cancino et al., 2012).

The 10417 R dose for X-rays (equivalent to 100 Gy in γ radiation), and 100 Gy for γ-rays, are relatively high when compared to the values obtained by other authors for other fly species (Cancino et al., 2012). The differences may stem from the fact that we studied another *Anastrepha* species; but the type of radiation and irradiator could also have influenced our results (Mehta, 2009). Conditions during the irradiation were very different from above, since in our study the larvae were not naked during the irradiation process, but immersed in the artificial diet, and we used unaltered atmospheric conditions during the irradiation. As a result, we needed relatively higher values of radiation, in agreement with the studies of Cancino, Ruíz, Sivinski, et al. (2009), Cancino, Ruíz, López, et al. (2009), (Cancino et al., 2012). We deliberately focused on the effect of radiation on larvae immersed in diet, because under our scenario of mass rearing and release of natural enemies (using an agar-based larval rearing medium from which is difficult to obtain naked hosts), the amount of work required to separate the larvae from the medium would make the rearing process less operative, and inefficient. As a result, in the case of *A. fraterculus* and other rearing systems in which the separation of larvae from the artificial rearing medium is difficult, irradiation protocols require higher doses, but the handling time is reduced.

The values obtained for the estimated variables on *D. longicaudata* were similar to those reported by other authors for this parasitoid species reared in *Anastrepha* spp. (Cancino, Ruíz, Pérez, et al., 2009; Cancino et al., 2002; Sivinski & Smittle, 1990), and are consistent with those of other braconid parasitoid species (Cancino, Ruíz, Sivinski, et al., 2009), even when the doses we used were higher. Thus, it is likely that the irradiation procedure and protocols applied in this work may actually be transferred to a higher scale.

In general terms, the fecundity of female parasitoids which emerged from hosts treated with X-rays during the experiments was higher than that of control females. The radiation could have affected the immune system of the fly larvae, thus facilitating the parasitoid development and, in rearing terms, increasing their quality (Bai, Chen, Cheng, Fu, & He, 2003; Vey & Causse, 1979). This phenomenon can be considered advantageous, since irradiating the hosts before the parasitism event not only prevents the emergence of the fly but also results in higher production yields (if the irradiation procedure is applied during the maintenance of the colony) and greater fecundity of the released female parasitoids.

Although X-rays and γ-rays are not equivalent due to the physical differences between them, we used the two radiation types for the same purpose and found comparable results. We found that the 10417 R dose of X-rays is as effective as its equivalent dose in γ-rays (100 Gy), in agreement with the results obtained by Bakri et al. (2005), Mastrangelo et al. (2010) and Parker et al. (2008), who argue that both types of rays have similar effects on irradiated insects and present similar relative biological effectiveness. This finding should lead to a novel X-ray use in pest control programmes, and particularly in programmes for controlling *A. fraterculus* and *C. capitata* in Argentina. This work allowed us to describe particular characteristics of *A. fraterculus* as host for *D. longicaudata*, previously unreported. The results obtained here represent a significant step towards the development of rearing and shipping protocols of *D. longicaudata* on *A. fraterculus*, and therefore contribute to the use of this parasitoid in biological control programmes against fruit flies in Argentina, offering an alternative source of radiation to the traditional γ-rays.

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Disclosure statement

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