UNIVERSIDADE FEDERAL DE PELOTAS
Programa de Pós-Graduação em Fitossanidade

DISSERTAÇÃO

Avaliação da Técnica do Inseto Estéril para *Anastrepha fraterculus* (Diptera: Tephritidae) e *Drosophila suzukii* (Diptera: Drosophilidae)

Alexandra Peter Krüger

Pelotas, 2018

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“*Toda longa caminhada começa com o primeiro passo.”*
Lao-tsé

Resumo

KRÜGER, Alexandra Peter. **Avaliação da Técnica do Inseto Estéril para *Anastrepha fraterculus* (Diptera: Tephritidae) e *Drosophila suzukii* (Diptera: Drosophilidae).** 2018. 88f. Dissertação (Mestrado) – Programa de Pós-graduação em Fitossanidade, Universidade Federal de Pelotas, Pelotas, 2018.

Anastrepha fraterculus (Wied., 1830) (Diptera: Tephritidae) e *Drosophila suzukii* (Matsumura, 1931) (Diptera: Drosophilidae) são espécies de importância econômica para a fruticultura. Atualmente, estas duas espécies são predominantemente controladas com inseticidas, que apresentam riscos de combinação ambiental e da saúde humana. A Técnica do Inseto Estéril (TIE) é uma forma de supressão e erradicação de insetos, que pode ser utilizada no manejo destas espécies. A TIE consiste na liberação inundativa de insetos esterilizados que competem pela cópula com a população selvagem, reduzindo os níveis populacionais nas gerações subsequentes. Para assegurar o sucesso desta técnica, é necessário testar doses que permitam atingir a esterilidade dos insetos, mas não comprometam a qualidade dos mesmos. Desta forma, o objetivo deste trabalho foi verificar o efeito de diferentes doses de radiação na esterilidade e em parâmetros de controle de qualidade de *A. fraterculus* e *D. suzukii*, assim como o efeito da dose esterilizante sobre o comportamento reprodutivo de *D. suzukii*. Para tanto, foram testadas as doses de 0, 40, 50, 60 e 70 Gy sobre a esterilidade, longevidade sob estresse e habilidade de voo de *A. fraterculus* e as doses de 0, 75, 150 e 200 Gy sobre os mesmos parâmetros para *D. suzukii*. Também foram testados os efeitos da esterilidade de machos e fêmeas de *D. suzukii*, sobre o seu comportamento de cópula e recópula. Os resultados obtidos neste estudo demonstraram que as doses necessárias para induzir esterilidade em *A. fraterculus* e *D. suzukii* são 70 Gy e 200 Gy, respectivamente, e que estas doses não apresentam efeito deletério sobre a habilidade de voo e longevidade dos insetos irradiados. Ainda, a esterilidade de machos de *D. suzukii* não interfere na probabilidade de cópula e recópula, porém machos estéreis apresentaram maior duração de cópula. A esterilidade das fêmeas resultou na menor probabilidade de copular, porém não afeta a probabilidade de recópula.

Palavras-chave: mosca-das-frutas sul-americana, drosófila-da-asa-manchada, radiação, controle autocida.

Abstract

KRÜGER, Alexandra Peter. **Evaluating the Sterile Insect Technique to *Anastrepha fraterculus* (Diptera: Tephritidae) and *Drosophila suzukii* (Diptera: Drosophilidae).** 2018. 88f. Dissertação (Mestrado) – Programa de Pós-Graduação em Fitossanidade. Universidade Federal de Pelotas, Pelotas, 2018.

Anastrepha fraterculus (Wied., 1830) (Diptera: Tephritidae) and *Drosophila suzukii* (Matsumura, 1931) (Diptera: Drosophilidae) are species of economic importance to fruit production. Currently, these two species are predominantly controlled by insecticides, presenting risks to environmental contamination and human health. The Sterile Insect Technique (SIT) is a technique to supress and eradicates insects, which can be used to control these species. SIT consists in the inundative release of sterilized insects that compete with the wild population, reducing infestation levels in subsequent generations. To assure the success of this technique, it is necessary testing doses that allow achieving the insects sterility, but without compromising their quality. Thus, the objective of this study was to verify the effect of different radiation doses on sterility and quality of *A. fraterculus* and *D. suzukii*, as well as the effect of the sterilizing dose on the reproductive behavior of *D. suzukii*. Therefore, we tested the following doses: 0, 40, 50, 60 and 70 Gy on sterility, longevity under stress and flight ability of *A. fraterculus* and 0, 75, 150 and 200 Gy on the same parameters of *D. suzukii*. In addition, we tested the effects of male and female sterility of *D. suzukii* on its mating and remating behavior. The results obtained in this study showed that the necessary doses to induct sterility on *A. fraterculus* and *D. suzukii* are 70 Gy and 200 Gy, respectively, and these doses do not present a deleterious effect on the flight ability and the longevity of the irradiated insects. Yet, male sterility of *D. suzukii* does not interfere on the likelihood to mate and remate, however, sterile males showed a longer copula. The sterility of females resulted in a lower likelihood to mate, although it did not affected the probability of remating.

Keywords: South American fruit fly, Spotted Wing Drosophila, radiation, autocidal control.

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Sumário

1- Introdução

O Brasil é o terceiro maior produtor mundial de frutas, com uma produção anual de 43.112 milhões de toneladas em um total de 2.260 milhões de hectares, porém, o país exporta apenas 2% da produção (AGRIANUAL, 2015). Um dos principais fatores limitantes para a produção e exportação de frutas é a ocorrência de insetos-praga, com destaque para as moscas-das-frutas e, recentemente, a drosófila-da-asa-manchada que causam danos diretos e indiretos à fruticultura. Os danos diretos são causados pela oviposição no interior do fruto e pela alimentação das larvas, enquanto que os danos indiretos são relacionados com as medidas regulatórias que dificultam a exportação das frutas (DUARTE; MALAVASI, 2000; DIAS; GARCIA, 2014).

No Brasil, moscas da família Tephritidae tem destaque dentre as principais pragas de frutíferas, sendo as espécies *Anastrepha fraterculus* (Wiedemann, 1830), *Anastrepha obliqua* (Macquart, 1835), *Anastrepha grandis* (Macquart, 1846), *Bactrocera carambolae* Drew e Hancock, 1994 e *Ceratitis capitata* (Wiedemann, 1824) consideradas de maior importância econômica, e ainda, apresentam restrições quarentenárias em diversos países (ZUCCHI, 2000; MALAVASI; NASCIMENTO, 2003). Destas, *A. fraterculus* é a espécie com maior representatividade no Rio Grande do Sul (SALLES; KOVALESKI, 1990; SALLES, 1995; GARCIA; CORSEUIL, 1998; GARCIA; CAMPOS; CORSEUIL, 2003; GARCIA; LARA, 2006; NUNES et al., 2012). Além destas, em 2013, foi detectada no sul do Brasil a *Drosophila suzukii* (Matsumura, 1931), espécie pertencente à família Drosophilidae capaz de causar danos em frutos de epicarpo delgado (DEPRÁ et al., 2014; SCHLESENER et al., 2014). Ao contrário das demais espécies do gênero *Drosophila* que ovipositam em frutos maduros, *D. suzukii* é capaz de ovipositar em

frutos intactos e em estágio precoce de amadurecimento, devido ao seu ovipositor serreado (WALSH et al., 2011; ANFORA et al., 2012).

A mosca-das-frutas sul-americana, *A. fraterculus*, está associada a mais de 115 plantas hospedeiras em 28 famílias botânicas, e apresenta ampla distribuição geográfica, estando presente entre latitudes 27ºN e 35ºS (ALLINGHI et al., 2007; ZUCCHI, 2017). Os danos gerados por estes insetos podem ser observados desde em frutos verdes até naqueles em estado avançado de maturação (NAVA; BOTTON, 2010). O controle desta praga é realizado principalmente com o auxílio de produtos químicos, em iscas tóxicas ou aplicação em cobertura (KOVALESKI et al., 2000). Por sua vez, inseticidas químicos são frequentemente associados com problemas ambientais e na saúde humana (CARVALHO; NASCIMENTO, 2002; ALLINGHI et al, 2007).

Por outro lado, a drosófila-da-asa-manchada, *D. suzukii*, nativa da Ásia, está amplamente disseminada pela Europa, América do Norte e América do Sul (HAUSER, 2011; TEIXEIRA; REGO, 2011; CINI; IORIATTI; ANFORA, 2012; DEPRÁ et al., 2014; DOS SANTOS et al., 2017). *Drosophila suzukii* é considerada a praga emergente mais importante de frutos de tegumento frágil, sendo capaz de causar danos a uma série de frutos nativos e exóticos (KINJO; KUNIMI; NAKAI, 2014; ANDREAZZA et al., 2017). A forma de controle mais utilizada é o controle químico, sendo os organofosforados, piretroides e espinosinas os inseticidas que apresentam melhores resultados (SCHLESENER et al., 2015; SCHETELIG et al., 2017; SCHLESENER et al., 2017).

Os programas de Manejo Integrado de Pragas (MIP) em fruticultura têm incentivado o uso de vários métodos e táticas de controle, com o intuito de reduzir a densidade populacional de insetos-praga e minimizar os desequilíbrios ecológicos (CARVALHO; NASCIMENTO; MANTRAGOLO, 2000). Desta forma, a pesquisa científica tem buscado alternativas de controle de pragas para estabelecer o MIP em pomares, visando a redução da utilização de agrotóxicos.

A Técnica do Inseto Estéril (TIE) é considerada a estratégia de controle mais sustentável e espécie-especifica disponível para o controle de pragas (HENDRICHS et al., 2002; SCHETELIG et al., 2017). Esta técnica consiste na produção massal, esterilização e liberação inundativa de insetos, os quais competem com a população selvagem no acasalamento, resultando em gerações inviáveis (WALDER, 2000; DIAS; GARCIA, 2014). Ainda, a TIE atende as exigências atuais do mercado

consumidor quanto a necessidade de produtos saudáveis, sem a presença de resíduos (HENDRICHS et al., 2002).

A TIE foi proposta pela primeira vez por Knipling em 1955 e utilizada com sucesso para erradicar *Cochliomyia hominivorax* Coquerel, 1858 (Diptera: Calliphoridae) da América do norte e central (KNIPLING, 1955; KLASSEN; CURTIS, 2005). Atualmente, esta técnica é utilizada em diversos países para a supressão e até mesmo a erradicação de insetos praga. No Brasil, a TIE foi adotada pela primeira vez em 2005, pela Biofábrica Moscamed Brasil, com a finalidade de suprimir a população de *C. capitata* na região frutícola do semi-árido (PARANHOS; NASCIMENTO; WALDER, 2010). O sucesso da aplicação da TIE varia entre a completa erradicação da praga alvo até o abandono de sua utilização, e por isso, apesar de mais de 60 anos de pesquisa, ainda existe muito espaço para melhorias a está técnica (FISHER et al., 1985; McINNIS; LANCE; JACKSON, 1996; BAKRI; MEHTA; LANCE, 2005; RULL; DIAZ-FLEISCHER; ARREDONDO, 2007).

A esterilização dos insetos pode ser alcançada por meios físicos ou meios químicos. Porém, a utilização de esterilizantes químicos é limitada, visto que estes apresentam problemas toxicológicos e oncológicos aos organismos vivos, além do aparecimento de resistência e tolerância por parte de alguns insetos tratados (LaBRECQUE; SMITH, 1968; WALDER, 2000).

Sendo assim, a radiação ionizante é a mais utilizada, sendo proveniente de radioisótipos (principalmente ^{60}Co e ^{137}Ce) ou de equipamentos especiais (raio x e elétrons acelerados) (WALDER, 2000; MASTRANGELO et al., 2010). Quando um material biológico é irradiado, são formados radicais livres e ocorrem quebras nas cadeias duplas dos cromossomos das células. Quando os danos ocorrem nas células germinativas, ocorre a indução de mutações letais dominantes nos óvulos e espermatozóides (LaCHANCE; SCHMIDT; BUSHLAND, 1967; CURTIS, 1971; BAKRI MEHTA; LANCE, 2005). Desta forma, após a fecundação, durante a mitose, a fusão dos cromossomos danificados levam a perda de telômeros e a formação de fragmentos de cromossomos dicêntricos (KLASSEN, 2005; ROBINSON, 2005). Ainda, para minimizar os danos sobre as células somáticas, é ideal que a irradiação seja realizada em um período em que a maioria das células somáticas esteja diferenciada, e desta forma, as células germinativas serão atingidas (ROBINSON, 2005).

Para a implementação desta técnica, é necessário o conhecimento da biologia e do ciclo de vida do inseto em estudo. De acordo com Lance; McInnis (2005), o ciclo de vida curto, o desenvolvimento holometábolo e a reprodução sexuada, são pré-requisitos para a esterilização de insetos. Além disso, a dose de radiação utilizada para esterilização não deve afetar a habilidade dos machos de voar, copular e transferir espermatozoides para as fêmeas selvagens (ROBINSON; CAYOL; HENDRICHS, 2002).

Para assegurar a esterilidade dos insetos liberados, é indicado que estes possuam 99,5% de esterilidade em cruzamentos entre machos estéreis e fêmeas férteis (FAO/IAEA/USDA, 2003). Porém, as doses utilizadas para atingir esta porcentagem, frequentemente afetam negativamente a sobrevivência e agressividade dos insetos irradiados, indicando assim a importância estudos que objetivem encontrar doses que associem alta esterilidade e alta qualidade de insetos irradiados (COLLINS et al., 2009; DOMINIAK et al., 2014). Além disso, a ocorrência de *remating* e seu efeito sobre a fertilidade das fêmeas devem ser considerados na adoção da TIE. De acordo com Barclay (2005), a poligamia é compatível com esta técnica, desde que a cópula seja aleatória e os indivíduos estéreis sejam competitivos. Desta forma, o objetivo deste trabalho foi verificar os efeitos de diferentes doses de irradiação gama sobre a esterilidade e qualidade de *A. fraterculus* e *D. suzukii*, além dos efeitos da esterilização sobre o comportamento de cópula e recópula de *D. suzukii*.

Artigo 1 – Entomologia Experimentalis et Applicata

2- Artigo 1 - Impact of gamma radiation dose on sterility and quality parameters of *Anastrepha fraterculus* (Diptera: Tephritidae)

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Short title: Irradiation dose on *A. fraterculus*

Keywords: South American fruit fly, Sterile Insect Technique, insect quality control, pest management

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ABSTRACT

Anastrepha fraterculus (Wiedemann, 1830) (Diptera: Tephritidae), is a major fruit pest, which is currently controlled using organophosphate and pyrethroid insecticides, which represents a risk to beneficial arthropods, human health and food contamination. The sterile insect technique (SIT) is a potential alternative tool for the management of this pest, however, only conflicting data is found regarding the optimal dose to achieve sterility. Thus, this study evaluated the effect of gamma radiation doses (0, 40, 50, 60 and 70 Gy) on male and female reproductive sterility, gonads morphometry, emergence, flight ability, and longevity under nutritional stress of *A. fraterculus*. Full female sterility was achieved at 50 Gy, while full male sterility was achieved at 70 Gy. Both ovarian and testicular sizes were affected by irradiation, while no influence was observed on the quality parameters evaluated. Our results suggest that 70 Gy applied 48 h before adult emergence can be used to sterilize *A. fraterculus* in a SIT programme.

Introduction

The South American fruit fly, *Anastrepha fraterculus* (Wiedemann, 1830) is a highly polyphagous pest, considered a major fruit pest in South America, and is widely distributed throughout the tropical and subtropical regions (Allinghi et al., 2007a; Cladera et al., 2014; Poncio et al., 2016). Traditionally, this species is controlled basically with organophosphate and pyrethroid insecticides applied in full coverage or as toxic baits (Nava & Botton 2010). However, these insecticides are highly deleterious to natural enemies and pollinators, and represent a risk to health of agricultural workers and food contamination (Nava & Botton 2010; Poncio et al., 2016; Garcia et al., 2017).

The Sterile Insect Technique (SIT) is an environmental friendly, species specific insect control method that has been used to supress and eradicate tephritid fruit flies in many regions of the world (Rull et al., 2007; Dias & Garcia, 2014; Dominiak et al., 2014). SIT relies on the release of high-quality, sterilised insects and their ability to mate with the wild population and induce reproductive failure (Knipling, 1955; Collins et al., 2009; Bloomfield et al., 2017).

Irradiation affects the reproductive cells during the pupae development, causing, in most species, dominant lethal mutation in sperm and ovarian atrophy (Klassen, 2005). However, the irradiation dose must be sufficient to achieve an adequate level of sterility but should not impair the sexual abilities of the sterile insects, such as flight and longevity (FAO/IAEA/USDA, 2003; Krüger et al., 2018).

Tephritidae is considered a homogeneous group regarding doses to achieve sterility. The mean dose needed to sterilize tephritid flies is 65 Gy (Bakri & Hendrichs, 2002); however, it is necessary to access the sterilizing dose for each species. Although sterilizing doses were tested for *A. fraterculus*, conflicting data were found. While Allinghi et al. (2007a) determined 70 Gy as the dose needed to sterilize both male and female of *A. fraterculus*, Mastrangelo et al. (2010) suggested, through a PROBIT analysis, 36.3 Gy should be the dose

used to achieve 99% sterility of males and 57.3 Gy should be used to achieve the same amount of sterility in females of this species.

Besides sterility, it is important to verify the effects of the radiation on the quality of the insects. Radiation may impact somatic cells and result in abnormalities, reduction in lifespan and flight ability and even the death of the insect (Bakri et al., 2005). Negative effects of radiation on some tephritids species have been reported, such as a decrease in courtship in *Ceratitis capitata* (Wiedemann, 1824) (Diptera: Tephritidae) (Lux et al., 2002), emergence and flight ability in *Anastrepha obliqua* (Macquart, 1835) (Diptera: Tephritidae) and in *Bactrocera tryoni* (Froggatt, 1897) (Diptera: Tephritidae) (Toledo et al., 2004; Dominiak et al., 2007), and an increase in mating frequency in *Anastrepha ludens* (Loew, 1873) (Diptera: Tephritidae) (Rull et al., 2005) and mortality of irradiated insects in *A. obliqua* (Toledo et al., 2004). The present study aims to examine the effects of gamma radiation on reproductive sterility, gonads morphometry, flight ability and longevity under nutritional stress of *A. fraterculus*.

Material and Methods

Rearing technique

The laboratory colony of *A. fraterculus* was kept in climate-controlled rooms, with temperature of 25±1ºC, 70±10% relative humidity and 12h photophase. Flies were obtained from infested peaches (*Prunus persica* L.)collected in Pelotas, Rio Grande do Sul, Brazil (31.461792 S, 52.524371 W), in the spring of 2016. Adults were kept in plastic cages (570 × 385 × 371mm) (l by w by h) and provided with a solid diet based on sugar (União®, São Paulo, SP, Brazil), wheat germ (Walmon®, São Paulo, SP, Brazil) and brewer's yeast (Bionis® YE MF e NS; Biorigin, Lençóis Paulistas, SP, Brazil) (3:1:1) (Nunes et al., 2013) and a water soaked cotton clump in a Petri dish (55mm) served as water source. Mangoes (*Mangifera indica* L.) fruits were exposed to the flies and served as oviposition substrate and

for larval development, as described by Dias et al. (2017). Pupae used in the experiments were from the 4th to 7th generation of the laboratory rearing.

Irradiation procedure

Approximately 250 pupae (48 hours before emergence) were placed in 50 mm Petri dishes, sealed with plastic film, and irradiated using a cobalt-60 source (Eldorado 78, Atomic Energy of Canada Ltd Chalk-River, Canada). Irradiation was performed at ambient temperature at different target doses (40, 50, 60 and 70 Gy), calibrated following Krüger et al. (2018). In addition, a control Petri dish (0 Gy) was prepared, but it was not exposed to irradiation. A total of four irradiation events occurred between May 2017 and September 2017, and each event was considered as one block during which the following bioassays were performed.

Reproductive sterility

Following irradiation, pupae were placed into plastic cups (700 mL), and allowed to freely emerge at 25$\pm$1ºC, 70$\pm$10% relative humidity and 12h photophase. Within 2 days after emergence, 8 males and 8 females of each dose treatment were placed into 5L plastic cages. For each dose treatment, three cages were set up for both combinations: irradiated males and unirradiated females and unirradiated males and irradiated females. All of the unirradiated adults were sourced from plastic cups that stayed in the laboratory. The cages were provided with water-soaked cotton and the solid food described above. When the adults were 15 days old, a mango fruit was placed inside each cage, and the flies were allowed to oviposit for three days. After the period of exposition, the mangoes were kept in a 2L plastic container, on a layer of vermiculite, for larval development. The plastic container was covered with voil. After 15 days, the number of larvae and pupae were assessed to estimate the sterility.

Ovary and testes morphometry

After the mango exposition for reproductive sterility assessment, 15 males and 15 females from each dose and each block were killed in 70% ethanol. The reproductive system was

extracted from the abdomen under a stereomicroscope, following the dissection procedure indicated by Chou et al. (2012). The following biometric parameters were recorded for ovaries: length, from the anterior end of the germarium to the calyx area and width, taken from the anterior end of the vitellarium. In addition, we calculated the ovarian index, by multiplying ovary length by ovary width, as suggested by Chou et al. (2012). Similarly, the testicular biometric parameters recorded were: length, from the apical region to the vas deferens, and width, taken from the spermatid region. We also calculated the testicular index, by multiplying testes length by testes width.

Flight ability

Three subsamples of 30 pupae from each dose treatment and the control were placed over moist black cloth on 90mm Petri dishes. Black 100mm tall tubes (94mm inner diameter) with a fine coat of unscented talcum powder in the interior (to prevent flies from walking out) were placed over the Petri dishes. A 15mm width of talcum powder was wiped off the base of each tube to provide newly emerged flies an additional surface to rest. The three tubes containing the subsamples of each treatment were placed into mesh cages (350 × 280 × 280mm). In the top of each cage, six yellow stick cards (90 × 100mm) were hung to trap fliers and prevent flies returning into the tubes. Once emergence was completed, individual flies were classified following the FAO/IAEA/USDA (2003) manual, as: 1) fliers if they successfully escape the tube, 2) not emerged if still inside an unopen pupal case, 3) partly emerged if they failed to emerge completely from the pupal case, 4) deformed if they had completely shed the pupal case but had damaged wings, and 5) not fliers if they had completely shed the pupal cases, and had morphologically normal wings, but failed to escape the tube. Calculations from Collins et al. (2008) were used to assess percentage of emergence, percentage of fliers and rate of fliers (the percentage of fliers corrected by emergence). All flies that emerged (fliers and not fliers) were sexed to identify any effects of irradiation treatment on sex ratio.

143 Longevity under Nutritional Stress.

144 From each treatment group, 24 pupae were placed into separate wells of 24-well microplates,
145 covered and allowed to emerge. No food or water was provided. The microplates were
146 checked for mortality three times each day (0900, 1300, 1700 hours) as indicated by Collins et
147 al. (2009). Date and time of emergence and death of each adult in each cell was recorded
148 Statistical Analysis.

149 Hartley and Shapiro-Wilk tests were applied, respectively, in order to verify the assumptions
150 of homoscedasticity and normality of residues for flight ability, longevity, ovary and testicles
151 morphometry data. Data from testicles morphometry and male sterility were root-squared
152 transformed. Posteriorly, all the data was submitted to analysis of variance. If significant
153 ($P \leq 0.05$) differences were detected, results were analysed using exponential or polynomial
154 function, where "y" is the observed variable, "y0" correspond to the maximum or minimum
155 level of the observed variable, "a" is the maximum estimated value for the observed variable,
156 "b" is the slope and "x" is the irradiation dose. For female sterility, absence of larvae
157 collected in several irradiated groups restricted the possible statistical approaches. As the
158 main concern of a SIT program is achieving sterility above 99.5% (FAO/IAEA/USDA, 2003),
159 we combined all results across blocks for each treatment.

160 Results

161 Reproductive sterility

162 Irradiation dose affected male sterility ($F_{4,52}=291.04$; $P<0.0001$). The number of larvae
163 obtained in mango fruits presented a decrease in function of dose increase ($F=1095.45$; $df=4$;
164 $P<0.0001$; Figure 1). From the 0 Gy male × unirradiated female (male control) treatment, we
165 collected 1219 larvae. From the 40 Gy irradiated male × unirradiated female treatment, 46
166 larvae were collected. From the 50Gy irradiated male × unirradiated female treatment, 42
167 larvae. From the 60 Gy irradiated male × unirradiated females treatment we collected 13

168 larvae, and from the treatment where 70 Gy irradiated male were coupled with unirradiated
169 females, we did not collected any larvae.
170 Irradiation dose also affected female sterility ($F_{4,52}$=163,63; P<0.0001). From the unirradiated
171 male × 0 Gy female (female control) treatment, we collected 1074 larvae. From the
172 unirradiated male × 40 Gy irradiated female treatment 3 larvae were collected, from one
173 single mango. Females irradiated at 50, 60 and 70 Gy did not lay any eggs.
174 Ovary and testes morphometry
175 Irradiation dose affected all the parameters evaluated for ovaries (length: $F_{4,551}$=156.88;
176 P<0.0001; width: $F_{4,551}$=61.22; P<0.0001and index: $F_{4,551}$=432.01; P<0.0001). These
177 parameters presented an exponential decay in function of irradiation dose increase (length: F=
178 3070.62; df= 4; P<0.0001; width: F= 3953.93; df= 4; P<0.0001 and index: F= 4491.29; df= 4;
179 P<0.0001; Figure 2).
180 Irradiation also had an effect on the testicular parameters (length: $F_{4,566}$=41.76; P≤0.0001;
181 width: $F_{4,566}$=213.25; P≤0.0001 and index: $F_{4,566}$=198.18; P≤0.0001). The parameters decrease
182 in function of irradiation dose increase (length: F= 42.01; df= 4; P=0.0233; width: F= 172.37;
183 df= 4; P=0.0058 and index: F= 114.48; df= 4; P=0.0087) (Figure 3).
184 Flight ability
185 There was no evidence of irradiation dose effect on percentage of emergence ($F_{4,52}$=0.61;
186 P=0.6544; average(±sd) =95.72±3.90), percentage of fliers ($F_{4,52}$=0.77; P=0.55; average(±sd)
187 =60.61±19.11) or rate of fliers ($F_{4,52}$=0.58; P=0.6744; average(±sd) =63.40±20.14). There was
188 also no evidence that the treatments influenced the sex ratio ($F_{4,12}$=0.51; P=0.7317).
189 Longevity under nutritional stress
190 Longevity in hours, as a continuous outcome, was not affected by irradiation dose
191 ($F_{4,444}$=1.88; P=0.1125). The average (±sd) longevity of flies was 104.39±23.81.

192 Discussion

193 A sharp decrease in larval recovery was observed as male irradiation dose increase, and while
194 there was a reduction of approximately 96,23% of larvae recovery from the 40 Gy irradiated
195 males x unirradiated females treatments, no larvae were recovered at 70 Gy, showing
196 complete male sterility. Allinghi et al. (2007a) observed similar results when testing effects of
197 irradiation dose on an Argentinean laboratory population of *A. fraterculus*, and suggested 70
198 Gy as the dose to be used in SIT programmes. In fact, other species of this genus are also
199 sterilized using less than 100 Gy. The dose used to sterilize *Anastrepha serpentina*
200 (Wiedemann, 1830) (Diptera: Tephritidae) and *A. ludens* males is 80 Gy (Rull et al., 1996;
201 Landeta-escamilla et al., 2015), for *Anastrepha suspensa* (Loew, 1862) (Diptera: Tephritidae)
202 males the dose needed is 50 Gy (Walder & Calkins, 1993) and for *A. obliqua*, 40 Gy is
203 sufficient to result in 99.5% male sterility (Toledo et al., 2004).
204 Difference in radiosensitivity between males and females is expected due to the stage of
205 development of the gametes when pupae are irradiated (Carpenter et al., 2005). In our
206 experiments females were more sensitive to irradiation than males, becoming fully sterile at
207 50 Gy. Larvae were recovered from one single repetition from 40 Gy irradiated females,
208 showing that although sterility is high, it is not complete at this dose. Allinghi et al. (2007b)
209 also observed a reduced number of fertile eggs laid by females irradiated at 40 Gy. For other
210 species of *Anastrepha*, lower doses are needed to completely sterilize females when compared
211 to males. The dose used to sterilize *A. ludens* females is 40 Gy (Rull et al., 2007), for *A.*
212 *obliqua* females is 20 Gy (Toledo et al., 2004), and for *A. suspensa* females is 25 Gy (Walder
213 & Calkins, 1993). It is extremely important to completely sterilize a female before releasing
214 them in the ambient, since residual fertility can contribute in progeny to the next generation of
215 the target population (Robinson, 2002).

In males, gamma irradiation is capable of causing damage on spermatogenesis, leading to dominant lethal mutations in spermatids and spermatozoids, resulting in sterility, and sometimes, smaller testicles. Testicles of irradiated males were smaller in about 13.52% in length and 28.02% in width, when compared to testicles of unirradiated males. Our results differ from those found by Bartolucci et al. (2006), where no differences on length and width were observed between testicles of irradiated and unirradiated *A. fraterculus*. However, reduction on biometric parameters of male gonads of irradiated insects were already reported in *C. capitata* (Abdel-Malek et al., 1975) and *Bactrocera zonata* (Saunders, 1842) (Diptera: Tephritidae) (Shehata et al., 2006).

Female sterility is caused by the ovarian atrophy. The reduction is size were observed for *A. fraterculus* in all doses applied, where the ovaries dissected from irradiated flies were, on average, 60.84% smaller in length and 70.24% in width. The atrophy is caused by the interference of the radiation on cell division in the female reproductive system during its development in the pupal phase (Walder & Calkins, 1992). Ovarian atrophy results in the lack of egg production, which was reported in other irradiated tephritid females (Walder & Calkins, 1992; Toledo et al., 2004; Allinghi et al., 2007a; Bartolucci et al., 2008; Collins et al., 2009/ Rull et al., 2014). The inability of an irradiated female to lay eggs is favorable do SIT implementation, since released females would not oviposit into the fruits (Allinghi et al., 2007a).

Radiation has mutagenic properties that can cause somatic damage to sterile flies, thus, quantifying the negative effects of dose on quality of irradiated insects is essential to determine the optimal irradiation dose. In our study, the quality parameters evaluated were not affected by irradiation dose. Our results are similar with data observed for *B. tryoni* (Collins et al., 2009; Bloomfield et al., 2017), but in contrast to *A. obliqua* (Toledo et al., 2004), *A. ludens* (Rull et al., 2005; Rull et al., 2007), *B. zonata* (Mahmoud & Barta, 2011) and *C.*

241 *capitata* (Lux et al., 2002; Guerfali et al., 2011). In both this study and that of Collins et al.
242 (2009), pupae and flies from all treatments were kept under identical conditions, separating
243 treatments and control only during the irradiation, aiming to consider specifically the effects
244 of irradiation on quality parameters.
245 The late pupae stage presents a smaller number of mitotic cells, resulting in less somatic
246 damage due to radiation, and better sterile insect quality (Allinghi et al., 2007a; Paithankar et
247 al., 2017). Thus, the lack of detrimental effects of irradiation on quality control shows that
248 irradiation applied to *A. fraterculus* mature pupae is adequate, since metamorphosis is almost
249 complete. The results obtained in this study support the use of SIT as a control strategy for *A.*
250 *fraterculus*. A dose of 70 Gy applied 48 h before emergence not only induced male and
251 female sterility but also did not impair sterile insect's quality.
252

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259

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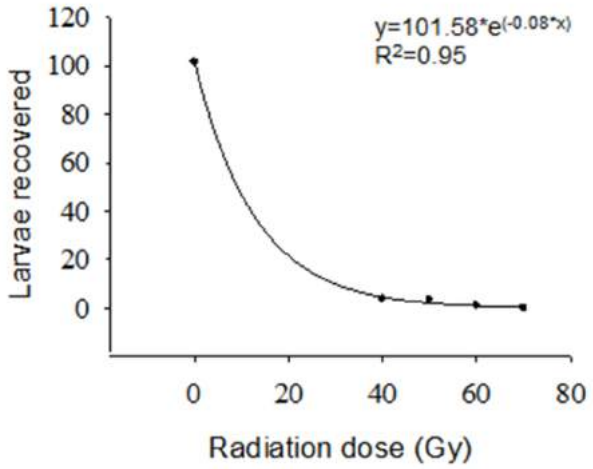
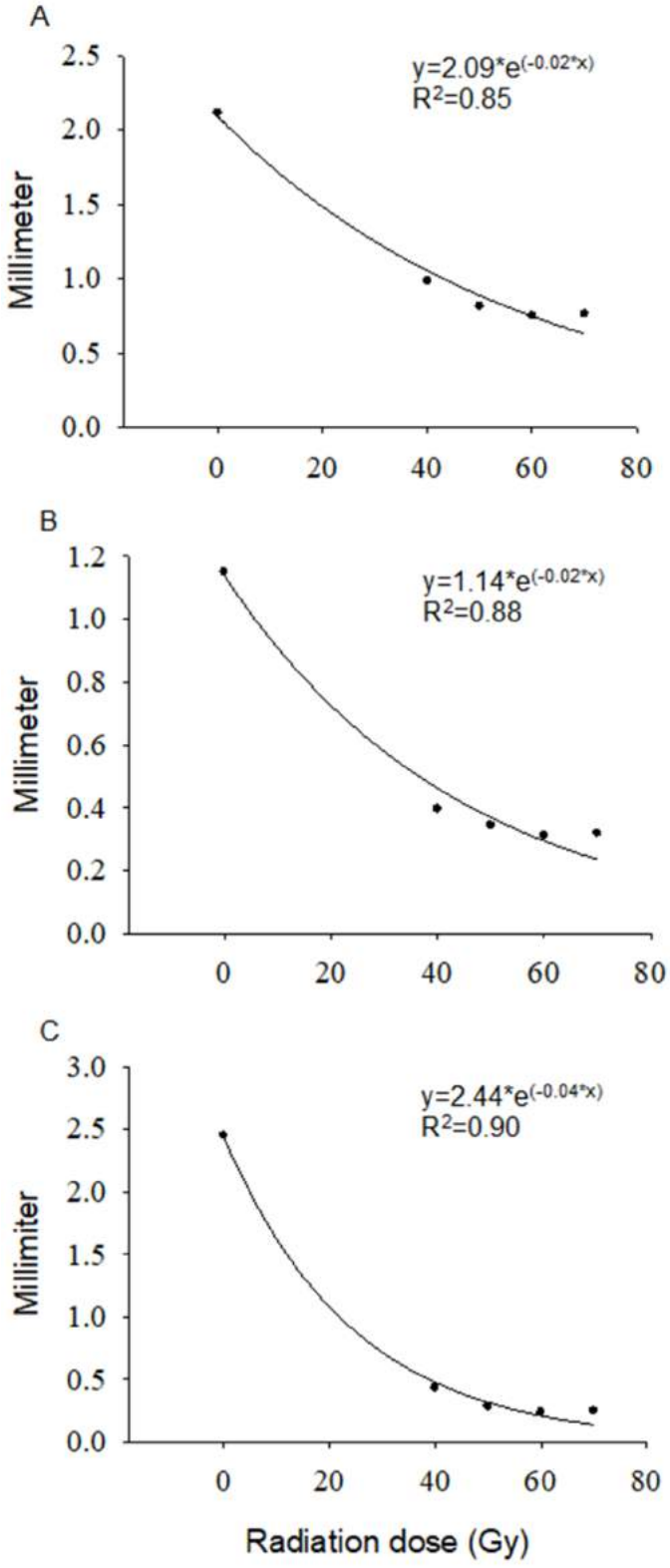


414

415 Figure 1. Number of larvae recovered from fertile female and irradiated males crosses of *A.*

416 *fraterculus* at different gamma radiation doses.

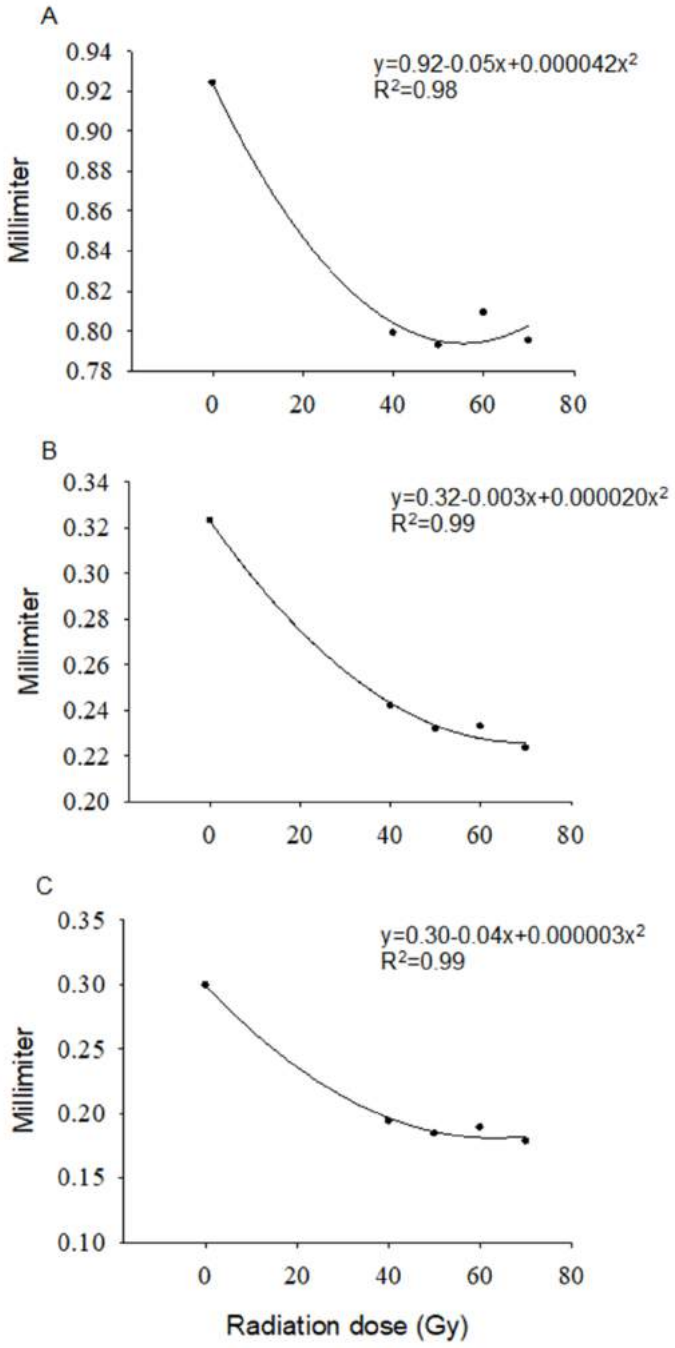
417



418

419 Figure 2. Ovary length (A), ovary width (B) and ovary index (C) of females of *A. fraterculus*

420 irradiated at different doses.



421

422 Figure 3. Testicles length (A), testicles width (B) and testicles index (C) of males of *A.*

423 *fraterculus* irradiated at different doses.

424

425 Table 1. Percentage of emergence, percentage of fliers, rate of fliers (%) and longevity (h) of
426 *A. fraterculus* irradiated at different doses.

Dose (Gy) [a]	Percentage of emergence[ns]	Percentage of fliers[ns]	Rate of fliers (%)[ns]	Longevity (h) [ns]
0	96.00 ± 4.00	63.00 ± 16.00	65.00 ± 17.00	103.78 ± 25.61
40	96.00 ± 5.00	60.00 ± 16.00	63.00 ± 18.00	110.78 ± 21.35
50	96.00 ± 3.00	64.00 ± 17.00	67.00 ± 18.00	105.39 ± 17.63
60	94.00 ± 4.00	55.00 ± 25.00	58.00 ± 27.00	99.78 ± 26.81
70	96.00 ± 3.00	61.00 ± 23.00	63.00 ± 23.00	101.91 ± 25.71

427 [a]Values represent the mean ± SD.
428 [ns]Not significant

Artigo 2 - Journal of Economic Entomology (*in press*)

3- Artigo 2 - Effects of Irradiation Dose on Sterility Induction and Quality Parameters of *Drosophila suzukii* (Diptera: Drosophilidae)

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ABSTRACT

Drosophila suzukii (Matsumura, 1931) (Diptera: Drosophilidae) is a widely distributed pest of soft-skinned and stone fruits that is controlled mainly with pesticides. An alternative to the chemical control is the Sterile Insect Technique (SIT), an ecologically friendly method of pest management that could be used against *D. suzukii*. The objective of the present study was to evaluate the effects of gamma radiation on reproductive sterility, ovarian morphometry and quality parameters of *D. suzukii*. Full female sterility was achieved at 75 Gy, while an adequate level of male sterility (99.67%) was obtained at 200 Gy. The ovarian size showed an exponential decay in function of irradiation dose increase. There was no significant influence of irradiation dose on the quality parameters evaluated. Our data suggest that gamma radiation can be recommended to be used in a SIT program for *D. suzukii*.

KEYWORDS: Spotted Wing Drosophila, gamma radiation, Sterile Insect Technique, quality control

Introduction

The spotted wing drosophila (SWD), *Drosophila suzukii* (Matsumura, 1931) (Diptera: Drosophilidae), is a widely distributed pest associated with the cultivation of soft-skinned and stone fruits, found in Asia, Europe (Calabria et al. 2012, Cini et al. 2012), and North and South America (Bolda et al. 2010, Walsh et al. 2011, Deprá et al. 2014). Recent studies have revealed that the invasion of different continents comes from different pathways, but the presence of *D. suzukii* in these areas may contribute to the establishment of populations in different locations, where the insect has not been detected yet (dos Santos et al. 2017, Fraimout et al. 2017). The rapid capacity to spread and become a pest is attributed to the female's serrated ovipositor that allows them to lay eggs in ripe and ripening fruits (Lee et al. 2011), its large host range (Stacconi et al. 2015) and short life cycle (Emiljanowicz et al. 2014, Tochen et al. 2014). In addition, the oviposition scar and developing larvae in fruit tissue result in unmarketable fruit and may accelerate decomposition (Walsh et al. 2011, Schlesener et al. 2015).

Currently, *D. suzukii* management relies mainly on the use of chemicals (Burrack et al. 2015). However, the short generation time of SWD and limited residual control afforded by insecticides forces producers to use frequent applications to maintain low pest levels (Renkema et al. 2016), endangering human health and the environment. The Sterile Insect Technique (SIT) is an environmental friendly, species specific method of pest management, and has been used to control tephritid fruit flies in many regions of the world (Collins et al. 2009, Dias and Garcia 2014, Dominiak et al. 2014).

SIT depends on the ability to release sterile insects to mate with wild ones and induce reproductive failure, reducing infestation levels in subsequent generations (Knipling 1955). Gamma radiation is the usual method used to sterilize tephritid fruit flies released in SIT

programs (Bakri et al. 2005, Collins et al. 2008). Irradiation affects the reproductive cells during the pupae development through a breaking in chromosomes. In most species, irradiation in males induces dominant lethal mutation in sperm, whereas in females it leads to the inability to lay eggs, due to the ovarian atrophy (Klassen 2005).

In general, tephritids require <100 Gy to achieve high levels of reproductive sterility (Bakri and Hendrichs 2002). However, the dose necessary to achieve an adequate level of sterility is species specific; hence, it is important to evaluate irradiation doses capable of inducing sterility to the target pest. In fact, early studies suggest that smaller insects are more radioresistant. For instance, *Drosophila melanogaster* Meigen, 1830 (Diptera: Drosophilidae) requires a dose of at least 160Gy in pupal stage to result in male sterility higher than 99% (Bakri et al. 2005, Henneberry and McGovern 1963). Therefore, it is expected that *D. suzukii* requires higher doses when compared to tephritids.

In an attempt to evaluate a potential sterilizing dose for *D. suzukii*, Lanouette et al. (2017) suggested 120 Gy, the highest dose tested, as the potential dose to be used on SWD pupae. However, this dose prevented the hatch of 96% of the eggs, while the sterility recommended for an SIT program is >99.5% (FAO/IAEA/USDA 2003).

Furthermore, the sterilizing dose should not impair the sexual abilities of the sterile insects, such as flight, longevity and mating. Deleterious effects of irradiation on insects have been reported for some tephritids species, including *Ceratitis capitata* (Wied., 1824) (Diptera: Tephritidae) (Lux et al. 2002), *Anastrepha obliqua* (Macquart, 1835) (Diptera: Tephritidae) (Toledo et al. 2004), *Anastrepha ludens* (Loew, 1873) (Diptera: Tephritidae) (Rull et al. 2005, Rull et al. 2007) and *Bactrocera tryoni* (Froggatt, 1897) (Diptera: Tephritidae) (Dominiak et al. 2007). The present study aims to examine the effects of gamma radiation on reproductive sterility, ovarian morphometry, flight ability and longevity of *D. suzukii*.

Moreover, we aim to obtain effective values of radiation doses that may be applied to sterilize SWD in an SIT program.

Materials and Methods

General procedures.

A laboratory colony of *D. suzukii* was established with flies from infested blackberries collected in Pelotas, Rio Grande do Sul, Brazil (31°38'20''S and 52°30'43''W), in the summer of 2016. Flies were reared on artificial diet, at a temperature of 23± 1ºC and 12:12 (L:D) h photoperiod. The diet recipe consisted of agar (8g), yeast (40g), cornmeal (80g), glucose (100g), propionic acid (3ml), methyl paraben (0.8g dissolved in 8ml of 90% ethanol), and water (1000ml). To obtain experimental pupae, sexual mature SWD adults were placed into glass tubes (85mm high × 25mm in diameter) containing approximately one-third of their volume of artificial diet for oviposition. After 24 h, adults were removed and the tubes were placed in a B.O.D. (Biochemical Oxygen Demand), at same abiotic conditions as the colony, for 10 d, until pupae were ~24 h before emergence.

Pupae were placed in 50mm Petri dishes containing wet cotton to avoid dehydration, sealed with plastic film, and transported (~20 min) to Centro de Radiação Multipropósito (CIMP) in Pelotas at the Federal University of Pelotas, RS. Irradiation was performed using an Eldorado 78 (Atomic Energy of Canada Ltd, Chalk-River, Canada, cobalt-60 source) at ambient temperature. Each Petri dish was irradiated separately at different target doses (75, 150 and 200 Gy), calibrated by the method of substitution, as indicated by Cagnotti et al. (2012). In addition, the control Petri dish (0 Gy) was also transported to CIMP but was kept aside. In total, four irradiation events occurred between September 2016 and January 2017, and each event was considered as one block during which the following bioassays were performed.

Reproductive Sterility.

Following irradiation, SWD pupae from all treatments were placed into plastic cups (700 ml) marked with each treatment and allowed to freely emerge in a chamber at 23± 1ºC and 12:12 (L:D) h photoperiod. Within hours after emergence, adults were separated by sex to prevent potential matings. To assess reproductive sterility of male and female flies irradiated at different doses, for each of the four dose treatments (75, 150, 200 Gy and the control 0 Gy), we set 10 couples for both combinations: irradiated male × unirradiated female and unirradiated male × irradiated female. All of the unirradiated adults were sourced from glass tubes that stayed in the laboratory. Each couple was placed into a plastic cup (200 ml) with a hole covered with voile cloth, and arranged into a B.O.D. at 23+1ºC, 12:12 (L:D) h. The plastic cup was provided with an oviposition substrate consisting of a cylindrical slice (25 mm diameter, 10 mm thick) of agar (19g), raspberry jelly (10g), methyl paraben (0.8g dissolved in 8ml of 90% ethanol) and water (850ml). Food (hydrated mixture of sugar, wheat germ and hydrolyzed yeast in a 3:1:1 ratio) was provided into microcentrifuge lids and were changed twice a week.

Oviposition substrates were checked daily. When the first eggs were laid, pre-oviposition period were recorded and eggs were eliminated. Eggs were carefully collected with a scalpel blade and lined over a moist filter paper in a Petri dish and counted, from the second egg laying. All eggs were incubated at 23±1ºC for 3 d to determine egg hatch. Eggs with a broken and empty chorion were recorded as hatched, whereas those that remained turgid were recorded as unviable. Fecundity and fertility were assessed from second to seventh egg laying.

Ovary morphometry.

To assess the ovary development, we used 12-d-old females from each treatment group. Flies were killed in 70% ethanol prior to examination. The reproductive system was extracted from

the abdomen under a stereomicroscope, and the dissection was conducted as indicated by Chou et al. (2012). Ovaries were removed and the following biometric parameters were recorded: length, from the anterior end of the germarium to the calyx area, width, taken from the anterior end of the vitellarium. We calculated the ovarian index, by multiplying ovary length by ovary width, as suggested by Chou et al. (2012).

Flight Ability bioassay.

After irradiation, three subsamples of 30 pupae from each treatment were placed over moist black cloth on 90mm Petri dishes. Black 100mm tall tubes (94mm inner diameter) with a fine coat of unscented talcum powder in the interior (to prevent flies from walking out) were placed over the Petri dishes. A 15mm width of talcum powder was wiped off the base of each tube to provide a surface to newly emerged flies rest. The three tubes containing the subsamples of each treatment were placed into mesh cages (350 × 280 × 280mm) and allowed to emerge. In the top of each cage, six yellow stick cards (90 × 100mm) were hung to trap fliers and prevent flies returning into the tubes. Once emergence was completed, individual flies were classified following the FAO/IAEA/USDA (2003) manual, as follows: 1) fliers if they successfully escape the tube, 2) not emerged if still inside an unopen pupal case, 3) partly emerged if they failed to emerge completely from the pupal case, 4) deformed if they had completely shed the pupal case but had damaged wings, and 5) not fliers if they had completely shed the pupal cases, and had morphologically normal wings, but failed to escape the tube. Calculations from Collins et al. (2008) were used to assess percentage of emergence and percentage of fliers and rate of fliers (the percentage of fliers corrected by emergence). All flies that emerged (fliers and not fliers) were sexed to identify any effects of irradiation treatment on sex ratio.

Longevity under Nutritional Stress.

From each treatment group, 30 pupae were removed and placed into individual wells of 96-well microplates. No food was provided, but a strip of moist filter paper (1.0 × 0.5 cm) was added to each well to provide humidity to the pupae. The microplates were checked for mortality three times each day (0900, 1300 and 1700 h) as indicated by Collins et al. (2009). Date and time of emergence and death of each adult in each cell were recorded, as well as its sex. Percentage of flies still alive at 48h was calculated following Collins et al. (2009).

Statistical Analysis.

Hartley and Shapiro-Wilk tests were applied, respectively, in order to verify the assumptions of homoscedasticity and normality of residues for flight ability, longevity, ovary morphometric and male sterility data. Ovary morphometric data had to be log-transformed to satisfy conditions of normality. Posteriorly, all the data were submitted to analysis of variance. If significant ($P \leq 0.05$) differences were detected, results were analysed using exponential or polynomial function, where y is the observed variable, y0 correspond to the maximum or minimum level of the observed variable, a is the maximum estimated value for the observed variable, b is the slope and x is the irradiation dose. For female sterility, absence of egg laying for several irradiated groups restricted the possible statistical approaches. However, as the main concern of an SIT program is achieving sterility above 99.5% (FAO/IAEA/USDA 2003), we combined all results across blocks for each treatment.

Results

Reproductive Sterility.

Irradiation dose had an effect on male sterility ($F_{3,145}=410.85$; $P \leq 0.0001$) but not in preoviposition period of females coupled with irradiated males ($F_{3,145}=0.94$; $P=0.4219$). The egg hatchability decreased in function of dose increase ($F= 887.65$, $df=3$, $P \leq 0.0001$; Fig. 1). From the 0 Gy male × unirradiated female (male control) treatment, we collected 3834 eggs,

178 from which 3452 hatched (90.04% fertility). From the 75 Gy irradiated male × unirradiated
179 female treatment, 4114 eggs in total were collected, of which 669 hatched (16.26% fertility).
180 From the 150 Gy irradiated male × unirradiated female treatment, 3419 eggs were collected,
181 from which 89 hatched (2.60% fertility). From the unirradiated females copulated with 200
182 Gy irradiated males, we collected 3575 eggs, from which 12 hatched (0.33% fertility). From
183 the unirradiated male × 0 Gy female (female control) treatment, we collected 2569 eggs, of
184 which 2256 hatched (87.82% fertility). Females irradiated at 75, 150 and 200 Gy did not lay
185 any eggs.

186 **Ovary morphometry.**

187 Irradiation dose affected both ovarian length ($F_{3,153}$=1,102.06; P≤0.0001) and ovarian width
188 ($F_{3,153}$=1170.68; P≤0.0001). Given the influence of the treatment on both measures, it was
189 expected the significant effect in ovarian index ($F_{3,153}$=1,630.57; P≤0.0001). All biometric
190 ovarian parameters presented an exponential decay in function of irradiation dose increase
191 (ovarian length: F= 1,698.15, df=3, P≤0.0001; ovarian width: F= 1965.61, df=3, P≤0.0001
192 and ovarian index: F= 1,315.54, df=3, P≤0.0001; Fig. 2).

193 **Emergence, Flight Ability and Sex Ratio.**

194 There was no evidence that percentage of emergence from flight ability test was significantly
195 influenced by irradiation dose ($F_{3,41}$=0.21; P=0.8881; Fig. 3). There was also no evidence that
196 percentage of fliers was influenced by irradiation dose ($F_{3,41}$=1.99; P=0.1864; Fig. 3). Given
197 that neither proportion of emergence nor proportion of fliers were influenced by the
198 irradiation dose, it was not surprising that the rate of fliers was also not influenced by
199 irradiation dose ($F_{3,41}$=3.63; P=0.0577; Fig. 3). Irradiation dose also did not affect sex ratio
200 ($F_{3,41}$=1.00; P=0.4362; Fig. 3).

Longevity under Nutritional Stress.

The percentage of flies alive at 48h did not vary among the treatments ($F_{3,376}$=0.62; P=0.6223) (Fig. 3). Longevity in hours, as a continuous outcome, was also not influenced by irradiation dose ($F_{3,376}$=1.86; P0.1364), nor sex ($F_{3,376}$=0.66; P=0.4182).

Discussion

Irradiation dose affected the induction of sterility in *D. suzukii*, although from the tested doses, only one dose (200 Gy) satisfactorily meets the mean sterility of >99.5%, as recommended by FAO/IAEA/USDA (2003). Furthermore, the sterilizing dose for SWD is much larger than the mean dose indicated to induce sterility in Tephritidae (around 65 Gy) (Bakri and Hendrichs 2002), which reflects a higher resistance to radiation compared to other fruit fly species.

Full sterility of female was achieved at 75 Gy, similarly as found by Lanouette et al. (2017). At this dose, males were about 83.74% sterile. Carpenter et al. (2005) suggested that the difference in radiosensitivity between males and females is related to the stage of development of the gametes at the time of irradiation, since female reproductive cells are in a higher mitotic rate in late-stage pupae. Thus, irradiation, at a sufficient dose, leads to the atrophy of the germinal cell structures, as we can see through the ovarian morphometry. At the dose of 75 Gy, the ovarian development was greatly compromised (Fig. 4).

Ovarian atrophy caused by irradiation was observed by Walder and Calkins (1992) in *Anastrepha suspensa* (Loew, 1862) (Diptera: Tephritidae) at a dose as low as 25 Gy. In the same study, they report that no females showed signs of ovarian regeneration, which indicates that the damage caused by irradiation is permanent.Other authors also report a lack of egg production due to ovarian atrophy in irradiated tephritid females (Toledo et al. 2004, Allinghi et al. 2007, Bartolucci et al. 2008, Collins et al. 2009, Rull et al. 2014). Since irradiated

females are not able to lay eggs is favorable to SIT implementation, released females would not oviposit into the fruits, eliminating a potential damage (Allinghi et al. 2007).

The variation in ovary size allows the differentiation between irradiated and unirradiated females, and the identification of sterile insects in the field is important in the SIT implementation. When an SIT program is implemented, population levels of the target pest is monitored with traps. Irradiated flies usually are dyed with fluorescent powder before release, in order to allow the differentiation between wild and sterile insects when trapped (Bartolucci et al. 2008). Nevertheless, when a fly lacks dye, it is necessary to use other tools to identify the origin of the fly, such as the observation of the gonads. Thus, in *D. suzukii*, the observation of ovaries could be used to distinguish wild and sterile trapped females.

Previous studies relating irradiation and *D. suzukii* estimated X-ray doses to be used as postharvest treatment for quarantine control of this pest, using an electron linear accelerator. Kim et al. (2016) mated irradiated males with unirradiated females, and concluded that 150 Gy was the X-ray dose to be used in to induce SWD sterility. According to Mastrangelo et al. (2010), electron linear accelerator are expected to be more biologically effective per unit absorbed, and this could explain the difference between the result obtained in the previous study and our research.

The parameters of quality control such as emergence, flight ability and survival of *D. suzukii* were not significantly affected by irradiation dose, and this is consistent with *B. tryoni* (Collins et al. 2009), but in contrast to *A. obliqua* (Toledo et al. 2004), *A. ludens* (Rull et al. 2005, Rull et al. 2007) and *C. capitata* (Lux et al. 2002, Guerfali et al. 2011). In our experiment, similarly to Collins et al. (2009), the design was specifically chosen to consider only the effects of irradiation; hence, we carefully kept irradiated and unirradiated pupae and flies under close to identical conditions. In fact, the only period when treatments and control were separated was during irradiation.

Quality control tests are mainly applied to verify the quality of the mass-reared insects, yet they can be also used to verify deleterious effects caused by radiation in sterile flies and the mutagenic properties of radiation. The percentage of emergence is directly related to the number of adults that can be released. The flight ability of sterile flies is essential, since those flies that are not able to fly to shelter, food or to a partner are lost to the SIT program. The longevity test under nutritional stress is an indicative of the amount of nutritional reserves present when adults emerge (Calkins and Parker, 2005).

The lack of effects of irradiation on quality control parameters indicates that the age of the pupae and the amount of radiation were adequate. The late pupae stage is more radiotolerant as observed by Paithankar et al. (2017) for *D. melanogaster*, probably due to the smaller number of rapidly dividing cells, making a less susceptible stage to radiation damage. In fact, according to Allinghi et al. (2007), if radiation is applied to mature pupae, the metamorphosis is almost complete and the detrimental effects of radiation on organs with low metabolic rate are minimized. However, there was a numerical difference in the percentage of fliers in our study, representing a decrease of nearly 14% in 200Gy treated flies when compared to control, indicating a deleterious effect of the radiation, which should be further studied.

The implementation of an SIT program against *D. suzukii* has potential benefits in terms of providing a new alternative pest control strategy. In addition, the results obtained in the present study support the use of a dose of 200 Gy applied 24h before adult emergence to induce sterility in SWD. Nonetheless, before SIT implementation, it is crucial to perform more complementary tests, such as the effects of sterility on mating behavior, since *D. suzukii* is a polyandric species, and tests in field cages or greenhouses to obtaining a more accurate data, in order to determine sterile insect competitiveness.

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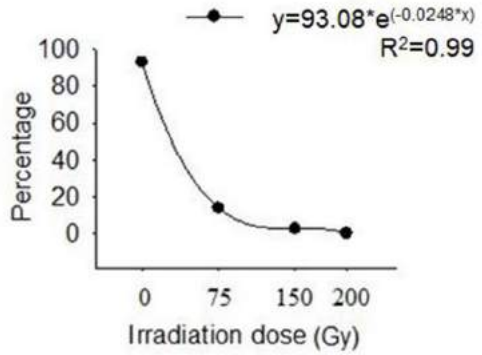

465

466 **Figure 1**. Egg hatch from fertile female and irradiated male crosses at different gamma

467 radiation doses.

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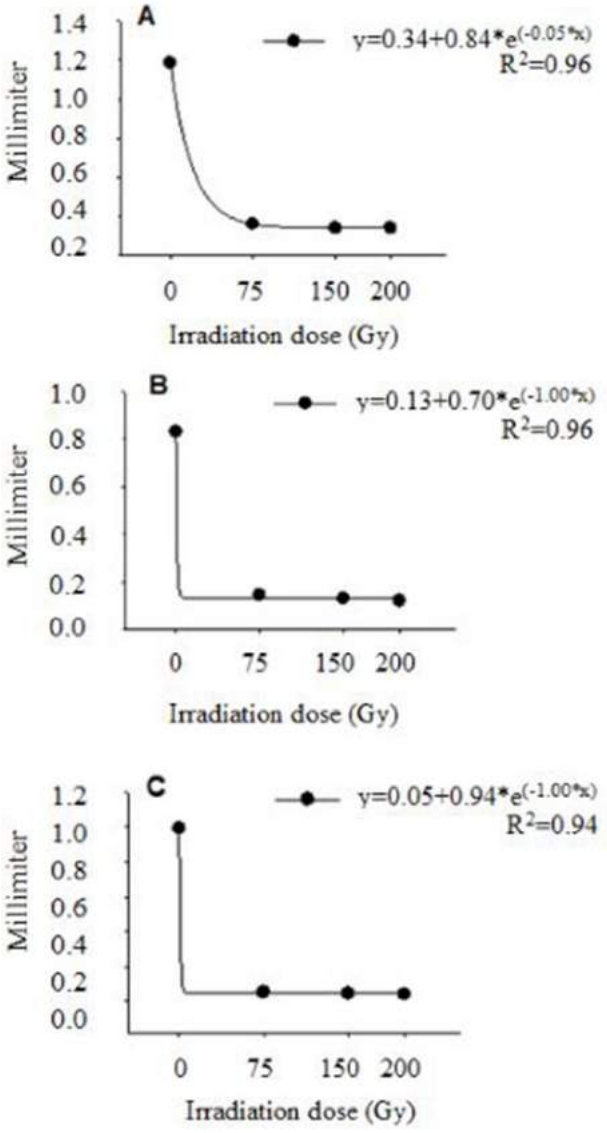

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470 **Figure 2**. Ovary length (A), ovary width (B) and ovary index (C) of females irradiated at

471 different doses.

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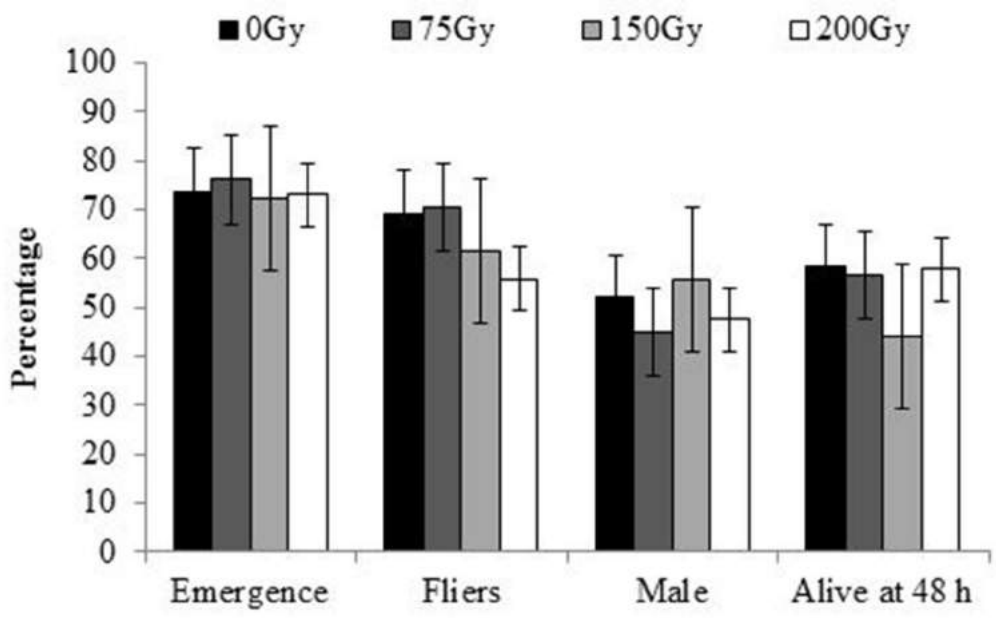

473
474 **Figure 3**. Effects of irradiation dose on percentage of emergence, percentage of fliers,

475 percentage of males (sex ratio) and percentage of flies alive at 48h.

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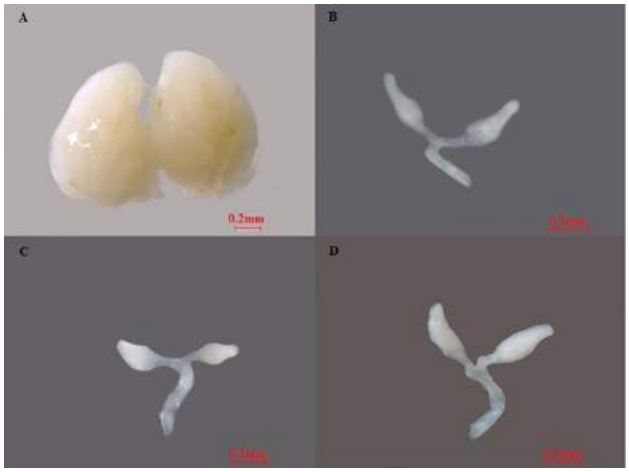

Figure 4. Ovaries from control flies (0 Gy) (A), flies irradiated at 75 Gy (B), 150 Gy (C) and 200 Gy (D).

Artigo 3 - Journal of Insect Physiology

4- Artigo 3 - Radiation effects on *Drosophila suzukii* (Diptera: Drosophilidae) reproductive behavior

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Abstract

Female remating is a widespread behavior, reported in several insects species. This behavior can affect the efficiency of Sterile Insect Technique (SIT), however, little is known about the postcopulatory behavior of some pest species considered as candidates to be controlled by this technique, such as *Drosophila suzukii* (Matsumura, 1931) (Diptera: Drosophilidae). In this study, we investigated the effects of male and female sterilization on mating and remating behavior of *D. suzukii*. First, we tested the occurrence of multiple mating in different combinations between sterile and fertile males and females. Then, we tested the effects of male and female sterility on female propensity to mate and remate. We found an overall low remating rate by *D. suzukii* females. Male sterility did not influence mating and remating likelihood; however, copula duration of sterile males was shorter compared to fertile males. On the other hand, sterile females were less likely to mate. Our findings encourage further research regarding the use of SIT to control *D. suzukii*.

Keywords: Spotted wing Drosophila, mating, female remating, sexual receptivity, sterile insect technique

1. Introduction

Polyandry is a mating behavior where females mate with multiple males. This behavior is a widespread phenomenon and is considered a major component of mating systems (Arnqvist and Nilsson, 2000; Denis et al., 2017). However, the precise reasons for a female multiple mating is still unknown. The most obvious benefit associated to polyandry is the increase diversity of genetic constituents of offspring and overall lifetime reproductive success (Arnqvist and Nilsson, 2000; Zeh and Zeh, 2001). However, fitness costs have also been associated to mating, such as decreasing female longevity and increasing of female death rate (Chapman et al., 1995; Rice, 2000).

The level of polyandry seems to result from the balance between costs and benefits, and ranges from monogamy to high promiscuity (Torres-Vila et al., 2004). Nonetheless, several factors have been reported to affect female remating frequency, such as first copula duration (Farias et al., 1972, Saul et al., 1988), nutritional status (Blay and Yuval, 1997), strain (Vera et al., 2002), and male sterilization (Katiyar and Ramirez, 1970; Gavriel et al., 2009; Abraham et al., 2012).

Polyandry is common in many species of *Drosophila*, although a considerable variation in remating frequency occurs among the members of this genus (Singh et al., 2002). While some *Drosophila* species can achieve up to 96% of remating frequency, *Drosophila subobscura* Collin, 1836 (Diptera: Drosophilidae) rarely remate a second time (Maynard-Smith, 1956; Singh et al., 2002). Experiments regarding multiple mating were performed on a series of drosophilids, for certain species, we have a lack of knowledge on weather females are monadrous or polyandrous, such is the case of the Spotted Wing Drosophila (SWD), *Drosophila suzukii* (Matsumura, 1931) (Diptera: Drosophilidae).

SWD is an widely distributed pest species, originally from Asia, and currently found in Europe (Calabria et al., 2012, Cini et al., 2012), North and South America (Bolda et al., 2010,

Walsh et al., 2011, Deprá et al., 2014), and able to invade new areas, where it has not been detected yet (dos Santos et al., 2017). Recent studies reported the possibility of adopting the Sterile Insect Technique (SIT) as a strategy to suppress *D. suzukii* populations, by itself or integrated to other techniques, such as biological control and *Wolbachia* (Garcia et al., 2017; Lanouette et al., 2017; Nikolouli et al., 2017; Schetelig et al., 2017; Krüger et al., 2018). However, it is crucial to understand remating behavior of this species, to better apply SIT to control SWD. SIT depends on the ability of mass-reared and sterilized insects to mate with wild ones and induce reproductive failure, reducing infestation levels in subsequent generations (Knipling, 1955). Although desirable, monogamy is not a mandatory feature for a species to be eligible for SIT, since polygamy is considered compatible with the SIT, as long as the mating is random (Barclay, 2005).

Irradiation can affect the quality of sterile males, including its ability to inhibit females from remating (Cayol et al., 2000; Landeta-Escamilla et al., 2016). Previous studies reported that irradiated males of *Anastrepha fraterculus* (Wiedemann, 1830) (Diptera: Tephritidae), *Anastrepha serpentina* (Wiedemann, 1830) (Diptera: Tephritidae) and *Ceratitis capitata* (Wiedemann, 1824) (Diptera: Tephritidae) were less likely to supress female remating (Abraham et al., 2012; Landeta-Escamilla et al., 2016; Gavriel et al., 2009; Mossinson and Yuval, 2003). In contrast, no evidence of irradiation effects on inhibition of female remating was observed in *Bactrocera cucurbitae* (Coquillett, 1899) (Diptera: Tephritidae) and *Bactrocera tryoni* (Froggatt, 1897) (Diptera: Tephritidae) (Kuba and Itô, 1993; Radhakrishnan et al., 2009; Collins et al., 2012; Haq et al., 2013).

If sterile males fails in suppress female remating, mated females could remate with wild males, decreasing the efficiency of SIT (Landeta-Escamilla et al., 2016). As females can store sperm from different matings, they can use viable sperm from wild male instead sterile sperm from irradiated male, and produce progeny (Bertin et al., 2010; Scolari et al., 2014).

Since there is no information on female remating behavior of *D. suzukii*, we aim to evaluate if females remated, and if so, how long was the sexual refractory period. We also sought to determine the effects of male and female sterility on the remating behavior of SWD.

2. Materials and methods

Flies were obtained from a colony established in the Laboratório de Ecologia de Insetos, in the Universidade Federal de Pelotas. The laboratory rearing originated from infested blackberries collected in January 2016, in Pelotas, Rio Grande do Sul, Brazil (31°38'20"S and 52°30'43"W). Flies were reared on artificial diet, following Schlesener et al. (2017), at a temperature of 23 ± 2ºC, 70 ± 10% relative humidity (RH) and 12:12h (L:D) photoperiod.

2.1 General procedure

Sterile insects were obtained by irradiating pupae 24 hours before emergence at 200 Gy using an Eldorado 78 (Atomic Energy of Canada Ltd, cobalt-60 source), following conditions and procedure proposed by Krüger et al. (2018). Unirradiated pupae were retained as control. Following irradiation, SWD pupae were placed into plastic cups (700 ml) and allowed to freely emerge in a chamber at 23 ± 2ºC and 12:12h (L:D) photoperiod. Newly emerged flies were separated by sex to prevent potential matings, and placed into plastic cups with water and a hydrated mixture of sugar (União®, São Paulo, SP, Brazil), wheat germ (Walmon®, São Paulo, SP, Brazil) and hydrolyzed yeast (Bionis YE NS and Bionis YE MF, Biorigen®, Lençóis Paulistas, SP, Brazil) in the proportion of 3:1:1 (adapted from Nunes et al., 2013).

To conduct the mating experiments, mating chambers were constructed from modified centrifuge tubes (50 ml) (Synth®, Diadema, SP, Brazil). A retangled hole was cut in the side of the tube (2 × 4 mm) and covered with voile fabric allow aeration. An orifice (10 mm diameter) was cut on the top, where a drilled microcentrifuge tube (1.5 ml) (Eppendorf, São Paulo, SP, Brazil) was inserted to provide hydrated food (as described above). To avoid fermentation and contamination, the food was changed twice a week. The mating chamber

was provided with an oviposition substrate consisting of a squared slice (10×10×10 mm) of agar (19g) (Vetec®, Duque de Caxias, RJ, Brazil), raspberry jelly (10g) (Neilar®, Rio do Sul, SC, Brazil), methyl paraben (Synth®, Diadema, SP, Brazil) [0.8g dissolved in 8mL of 90% ethanol (Synth®, Diadema, SP, Brazil)] and distilled water (850mL) (adapted from Salles et al., 1992). Besides providing a place to lay eggs, the oviposition substrate provided humidity, and it was changed every two days. Observations began at the onset of the lights (08:00 am) and ended at 11:00 am, according to the period of higher mating activity (i.e.: first 3 hours of light) (Revadi et al., 2015). All the bioassays were performed under temperature of 23 ± 2ºC, 70 ± 10% relative humidity (RH) and 12:12h (L:D) photoperiod. The experimental design was completely randomized, and each female was considered as a repetition. In the bioassays performed to evaluate the effect of male and female sterility on reproductive behavior, mating activity was evaluated when insects were four-days-old, as indicated by Revadi et al. (2015).

2.2 Multiple mating of females

When one day old, fertile and sterile female flies were singly housed in mating chambers. In the next morning, two males, either fertile or sterile, were placed in each mating chamber. A total of 120 females were observed for each mating combination: fertile female × fertile males (F♀×F♂), fertile female × sterile males (F♀×S♂), sterile female × fertile males (S♀×F♂) and sterile female × sterile males (S♀×S♂). Flies were observed continually to register the occurrence of copula. Unsuccessful males were removed from the chambers to prevent disturbance of the copulating pairs. At the end of the copulation, the successful males were removed and discarded. If no copulation had started during the observation period, both males were removed. This procedure of offering two virgin males to females was repeated every 2 days for 16 days. The males offered to females were from the same treatment (i.e., sterile or fertile) through all the mating opportunities. The readiness to mate (number of days from adult emergence until the first copula) and remating frequency were observed.

2.3 Effect of male sterility on female remating behavior

A total of 250 fertile female flies were placed individually in mating chambers. Then, 24 h later, two males, either fertile or sterile, were assigned to each female. Once a copula occurred, time of initiation and cessation were recorded for determination of its duration, and the unsuccessful male was removed. Successful males and females that did not copulate were discarded. Following the initial mating, every two days, two virgin males, either a fertile or a sterile, were housed with each mated female, and copula observation occurred as described above. A total of 7 opportunities of remating were given to each mated female. The latency period (time taken for copulation to commence in minutes), the sexual refractory period (number of days since first mating in days) and copula duration (in minutes) were evaluated.

2.4 Effect of female sterility on female remating behavior

We placed 120 fertile and 120 sterile females individually in mating chambers. For each female, two males, either fertile or sterile, were offered (i.e. fertile male × fertile female - F♂×F♀, fertile male × sterile female- F♂×S♀, sterile male × sterile female - S♂×S♀, sterile male × fertile female - S♂×F♀). Copulations were observed as described above. After mating, males were removed and females were kept in the mating chambers. Every two days, during 14 days, two virgin fertile males were placed in each mating chamber. The latency period, the sexual refractory period and copula duration were observed.

2.5 Statistical analysis

The effect of male and female irradiation on probability of mating and remating were analyzed by Chi-square likelihood ratio tests. The readiness to mate, latency period, the sexual refractory period and copula duration were submitted for analysis of variance (ANOVA) through the F test ($P \leq 0.05$). All the analysis were conducted using R Program (R Development Core Team 2011).

3. Results

3.1 Multiple mating of females

Irradiation did not affect the readiness to mate ($F_{3,73}$=2.51, *P* =0.0655). Fertile females when coupled with fertile males were ready to mate 4.87±1.89 days after emergence, and when coupled with sterile males, first mating occurred 4.31±2.29 days after emergence. Sterile females when coupled with fertile males start to mate at 6.38±2.88 days and sterile females when coupled with sterile males were ready to mate 5.60±2.85 days after emergence.

For the fertile female × fertile males treatment, from the 30 females analyzed, 21 mated once and two mated twice, resulting in 8.69% remating females. For the fertile female × sterile males treatment, from 30 females analyzed, 13 mated once, and none remated. From the 30 females analyzed of the sterile female × fertile males, 17 mated once, seven mated twice, one mated thrice and one mated five times, resulting in 34.61% remating females. From the 30 females of the sterile female × sterile males treatment, 17 mated once and only one mated twice yielding 5.55% remating females.

3.2 Effect of male sterility on female remating behavior

There is no evidence that male sterility have an effect on female likelihood to mate (χ^2=1.07, df=1, *P* =0.3004) nor on inhibition on female remating (χ^2=1.72, df=1, *P* =0.1903). In fact, only 7.29% of all the females remated. Sterility of males also did not affect the likelihood to be rejected in a second mate (χ^2=3.03, df=1, *P* =0.08).

Male condition (i.e.: fertile or sterile) had no effect on latency ($F_{1,126}$=0.09, *P* =0.7548), and the average time taken to a copula to initiate was 98.98 min. Also, although sterile males had longer copula durations than fertile males ($F_{1,126}$=5.34, *P* =0.0225) (Fig 1), copula duration did not affect female likelihood to remate (χ^2=1.94, df=1, *P* =0.1640).

3.3 Effect of female sterility on female remating behavior

The combination of male and female condition affected the mating probability, as well as female sterility itself, however, male condition *per se* had no effect (Combination: χ^2=25.76, df=3, *P* <0.0001; Female: χ^2=15.83, df=1, *P* <0.0001; Male: χ^2=0.28, df=1, *P* =0.5981) (Fig 2). While 72.5% of fertile females mated, only 47.5% of sterile females mated. However, neither male nor female sterility had an effect on female remating (Combination: χ^2=1.51, df=3, *P* =0.6794; Female: χ^2=1.04, df=1, *P* =0.3074; Male: χ^2=0.0, df=1, *P* =1.0000). As a matter of fact, only 17.5% of females remated.

Female sterility had an effect on the time taken to a copula to commence ($F_{1,142}$=45.90, *P* <0.0001) (Fig 3). However, female condition did not affect copula duration ($F_{1,142}$=1.57, *P* =0.2116) (Table 1), refractory period ($F_{1,40}$=0.06, *P* =0.8033) (Table 1), nor remating duration ($F_{1,40}$=1.56, *P* =0.2191) (Table 1). Average values (±sd) observed for copula duration was 23.76 ± 6.88 min, for refractory period, when occurred, was 7.95 ± 3.86 days, and for remating duration was 26.5 ± 11.82 min.

4. Discussion

There is little information about reproductive behavior of *D. suzukii* in literature. Our study revealed novel aspects of mating and remating behavior of this species, as well as the influence of sterility on these aspects. Although mating can be observed in SWD as young as one-day-old (Revadi et al., 2015), in our study flies were ready to mate, on average, around four days after emergence. Comparing to tephritid fruit flies, frequently controlled using SIT, SWD presents a much shorter timespan to be sexual mature. To avoid field mortality, several tephritid SIT programmes keep sterile flies within the facility during sexual maturation period, resulting in higher costs of maintenance (Bachmann et al., 2017). Thus, a short period for sexual maturation represents an asset for SWD in SIT programmes.

For bisexual strains, the release of sterile females can decrease SIT efficiency, since they will compete with wild females for matings (Orozco et al., 2013). However, sterile females of *D. suzukii* were less likely to mate than fertile females, representing another positive aspect for SIT. Similarly, Landeta-Escamilla et al. (2016) also reported that sterile females of *A. serpentina* were less inclined to mate, but the reason remains unknown. Additionally, in our study, male irradiation did not affect female likelihood to mate; this is consistent with *B. cucurbitae* and *B. tryoni* (Collins et al., 2012; Haq et al., 2013), but contrasting to results reported for *A. serpentina* (Landeta-Escamilla et al., 2016).

Understanding female post-copulatory behavior is crucial when SIT is considered to control a pest. Although monogamy is not a requirement of the SIT, a differential rate of remating by females first mated with sterile males or a wild male will compromise the technique (Calkins and Parker, 2005; Radhakrishnan and Taylor, 2008). Species from the genus *Drosophila* are known for their extreme reproductive phenotypes, showing enormous variation in their mating and remating behavior (Bundgaard and Barker, 2000). Despite many species of *Drosophila* display polyandry (Singh et al., 2002); most of the tested SWD females were monandrous. It is possible that if observations for remating were carried over a larger period, a higher number of remated females would be observed. However, due to high levels of natural mortality added to a long refractory period, probably a small proportion of females would survive long enough to have the opportunity to remate (Abraham et al., 2011).

After mating, females experience a series of physiological and behavioral changes that result in a shift on female sexual receptivity (Avila et al., 2011). This receptivity is affected by short and long term factors. The short term effect, known as copulation effect in *Drosophila*, is the decrease in receptivity due to seminal fluids components transferred by males during mating (Neubaum and Wolfner, 1999; Singh et al., 2002). Long term effect is indirectly linked to sperm load, and is called the sperm effect (Manning, 1962; Singh et al., 2002). The lack of

influence of male sterility on the female receptivity to remate, reported in this study, suggests that irradiation do not affect those factors, however, further studies should be developed to confirm this. The effects of male sterility on female remating vary among fruit fly species. Sterile males of *A. serpentina* and *C. capitata* are less likely to inhibit female remating (Gavriel et al., 2009; Landeta-Escamilla et al., 2016), while no difference was found for *A. fraterculus*, *Anastrepha ludens* (Loew, 1873), *B. cucurbitae* and *B. tryoni* (Radhakrishnan et al., 2009; Abraham et al., 2013, Haq et al., 2013, Abraham et al., 2016, Arredondo et al., 2017).

Our data showed that male condition did not influence mating latency period; however, sterile females presented a longer mating latency compared to fertile females. Effects of irradiation on latency were previously reported in males of *B. cucurbitae*, *B. tryoni* and *C. capitata*, but not in females (Radhakrishnan et al., 2009; Haq et al., 2013; Virginio et al., 2017). According to Cayol et al. (1999), mating latency is controlled by females and not by males. In our study, SWD fertile females showed the same receptivity to mate to either sterile or fertile males, while sterile females were less eager to mate. The absence of effects of *D. suzukii* male sterility on latency have significance to SIT, as both sterile and fertile males will initiate courtship at same time, competing fairly for females.

Female condition of *D. suzukii* did not have an effect on mating duration, but sterile males differ from fertile males in the duration of copula. Shorter copulas when sterile males are involved were already reported for *A. serpentina*, *A. fraterculus* and *C. capitata* (Cayol et al., 1999; Allinghi et al., 2007; Landeta-Escamilla et al., 2016; Virginio et al., 2017). Nonetheless, the importance of this effect on the efficiency of the SIT is not clear, since there is no relationship between copulation duration and the ability of males to transfer sperm (Allinghi et al., 2007; Harmer et al., 2006; Collins et al., 2012). Collins et al. (2012) suggest that factors associated to copula duration, others than sperm abundance, play an important

role for remating inhibition by tephritid flies, such as the components of the ejaculate. However, in our study, copula duration did not affect the probability of female remating. Findings reported in this study encourage further research regarding the use of SIT to control *D. suzukii*. Previous studies reported that sterilization do not affect quality of *D. suzukii* (Krüger et al., 2018). In addition, it seems that radiation do not influence the ability of males to mate and inhibit remating in SWD females. Although most of the tested females did not remate, it is important to verify the effects of remating in fecundity and fertility. Some *Drosophila* species are known to use sperm from some male partners (Davis et al., 2016). If this is the case of *D. suzukii*, females previously mated with sterile male, could recover fertility after remating with a fertile male, and jeopardize the success of a SIT programme. Thus, it is necessary to verify the effects of female remating on the fertility recovery of *D. suzukii*.

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Highlights

- First study about effects of sterility on *Drosophila suzukii* mating and remating behavior
- Male sterility did not affect mating or remating likelihood, but affected copula duration
- Sterile females were less likely to mate but no effect was found on remating likelihood
- A low rate of female remating was reported for all treatments

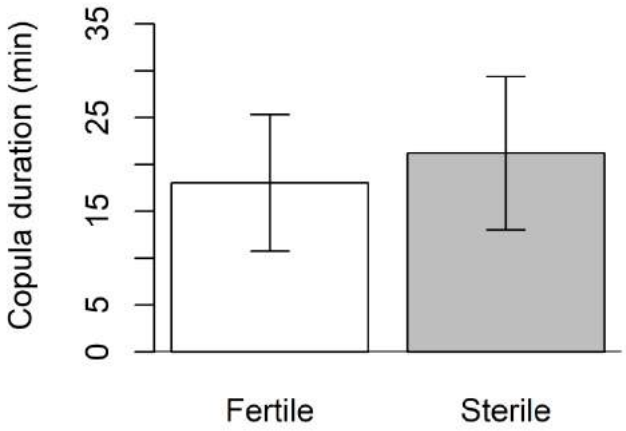

Figure 1. Average (± s.d.)copula duration (min) of fertile or sterile *Drosophila suzukii* males

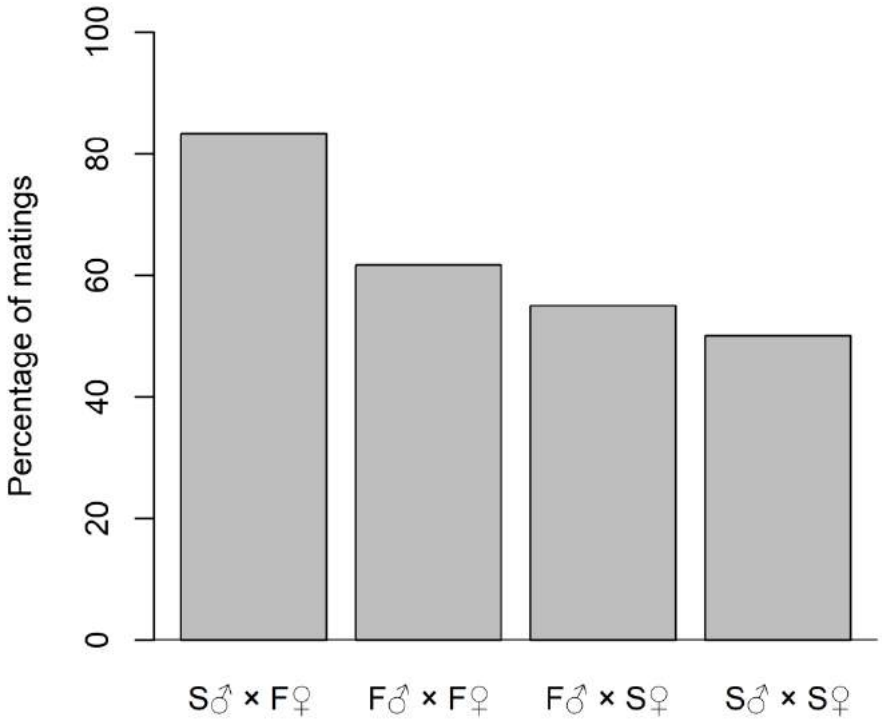

Figure 2. Absolute percentage of mating by sterile males × fertile females, fertile males × fertile females, fertile males × sterile females and sterile males × sterile females of *Drosophila suzukii*.

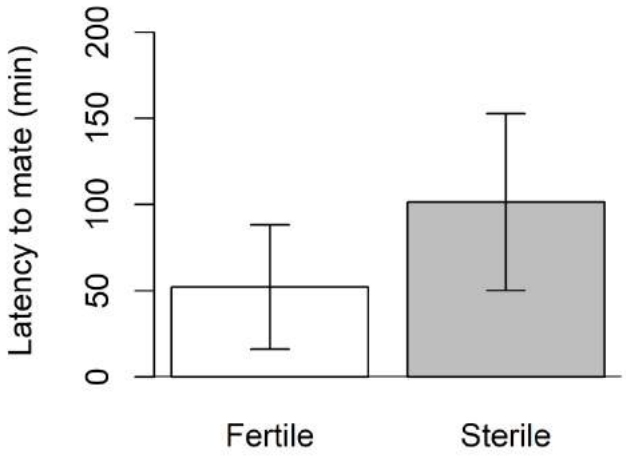

Figure 3. Average (± s.d.) latency period (min) of fertile or sterile *Drosophila suzukii* females

Table 1. Average values (±sd) observed for copula duration (min), refractory period (days), and for remating duration (min) of *D. suzukii*.

Treatment	Copula duration[ns]	Refractory period[ns]	Remating duration[ns]
F♂×F♀	23.49 ± 7.21	8.25 ± 4.83	29.62 ± 13.30
F♂×S♀	22.67 ± 7.33	7.85 ± 3.78	28.15 ± 8.05
S♂×S♀	23.17 ± 7.78	8.36 ± 3.77	20.27 ± 10.06
S♂×F♀	24.98 ± 5.82	7.40 ± 3.78	28.7 ± 15.31

[ns]Not significant

5. Conclusões

- A dose necessária para esterilizar machos de *A. fraterculus* é 70 Gy, enquanto que fêmeas desta espécie possuem ovários completamente atrofiados a partir de 50 Gy.
- As doses aplicadas em pupas de *A. fraterculus* não afetar sua habilidade de voo nem sua longevidade quando expostas a estresse nutricional.
- São necessários 200 Gy para esterilizar machos de *D. suzukii*, enquanto que fêmeas irradiadas com doses a partir de 75 Gy apresentam completa atrofiação dos ovários.
- As doses de irradiação testadas para *D. suzukii* não afetaram negativamente sua habilidade de voo e longevidade sob estresse nutricional.
- Esterilização de machos de *D. suzukii* não afeta a probabilidade de cópula e recópula de fêmeas da espécie.
- Machos de *D. suzukii* estéreis apresentam maior tempo de duração de cópula.
- Fêmeas de *D. suzukii* estéreis apresentam menor probabilidade de cópula.

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