Timing of Irradiation and Male Mating History Effects on Female Remating in Anastrepha ludens (Diptera: Tephritidae)

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Timing of irradiation and male mating history effects on female remating in *Anastrepha ludens* (Diptera: Tephritidae)

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Abstract

For the sterile insect technique to be successful, sterile males need to mate with wild females, transfer an ejaculate and inhibit females from remating. This study evaluated the capacity of irradiated *Anastrepha ludens* (Loew) (Diptera: Tephritidae) males of the genetic sexing strain Tapachula 7 (Tap-7) for inducing the sexual refractory period (time between the first and second mating) of wild females. The following factors were evaluated: 1) irradiation versus no irradiation of male pupae, 2) the age at which male pupae were irradiated (72, 48, and 24 h before adult emergence), and 3) male sexual experience (virgin, once mated, and twice mated). There was no effect of irradiation or age at irradiation on male ability to inhibit female remating or on sexual refractory period. However, wild females had shorter refractory periods when mated with virgin Tap-7 males, compared with males that previously had mated once or twice. We suggest that because inhibition of remating in *A. ludens* is determined by the full male ejaculate, virgin Tap-7 males are probably delivering smaller ejaculates compared with sexually experienced males. This study increases our knowledge of the reproductive biology of the genetic sexing strain of *A. ludens.*

Key Words: copulation; mass production; SIT; sterility

Resumen

Para que la técnica del insecto estéril sea exitosa, los machos estériles necesitan aparearse con hembras silvestres, transferir un eyaculado e inhibir el re-apareamiento de las hembras. Este estudio evaluó la capacidad de machos irradiados de *Anastrepha ludens* (Loew) (Diptera: Tephritidae) de la cepa sexada genéticamente Tapachula 7 (Tap-7) para inducir el periodo refractorio sexual (tiempo transcurrido entre la primera y segunda cópula) de hembras silvestres de campo. Los siguientes factores fueron evaluados: 1) irradiados versus no irradiados, 2) la edad a la que la pupa fue irradiada (72, 48, y 24 h antes de la emergencia), y 3) la experiencia sexual del macho (virgen, 1 cópula, o 2 cópulas). La irradiación o la edad a la que la pupa fue irradiada no tuvo efecto sobre la habilidad de los machos para inhibir el re-aparamiento de la hembra o sobre su periodo refractorio sexual. No obstante, las hembras de campo presentaron periodos refractorios menores cuando se aparearon con machos vírgenes de la cepa Tap-7, al compararse con machos que previamente se aparearon una o dos veces. Se sugiere que, dado que la inhibición del re-apareamiento en *A. ludens* está determinada por todos los componentes del eyaculado, los machos vírgenes de la cepa Tap-7 probablemente transfieren eyaculados más pequeños que los transmitidos por machos con experiencia sexual. Este estudio aumenta nuestro conocimiento sobre la biología reproductiva de la cepa sexada genéticamente de *A. ludens*.

Palabras Clave: cópula; producción masiva; TIE; esterilidad

The sterile insect technique (SIT) consists of mass-production, sterilization, and release of target insects into affected areas (Knipling 1955). Sterile males must mate with wild females and prevent them from reproducing through the transfer of infertile sperm and the transfer of an adequate ejaculate including accessory gland fluid to ensure temporary or permanent refractoriness (Robinson 2005). Because male sexual performance can be affected by irradiation (Moreno et al. 1991; Calcagno et al. 2002; Lux et al. 2002), careful timing and dosage of sterilization are needed to minimize negative effects. For example, the age at which pupae are sterilized is particularly important as it can affect germ tissue (Robinson 2005). If flies are irradiated earlier in their pupal stage this can have negative effects on mating competitiveness, flight, and pheromone production; older pupae are more resistant to damaging effects than younger pupae (Bakri et al. 2005). For example, in the Queensland fruit fly *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae), female response to male pheromones is lower if male pupae are irradiated at an earlier stage (Fletcher & Giannakakis 1973), and in the pink bollworm *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae), pupae age at the time of irradiation can affect male and female longevity (Henneberry & Clayton 1988). In most tephritids (Diptera: Tephritidae) for which the SIT has been developed, pupae are sterilized by gamma irradiation 48 h before adult emergence, but the optimal age for irradiation can vary, according to species and temperature among other factors (Resilva & Pereira 2014).

The age at irradiation could also affect male performance in terms of ability to inhibit females from remating. Ideally, released sterile

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males should prevent wild females from remating with wild males (Bertin et al. 2010; Scolari et al. 2014). Mating inhibition in tephritids is achieved through sperm and products of the accessory glands transferred to the female during mating (Jang 1995; Mossinson & Yuval 2003; Radhakrishnan & Taylor 2007; Abraham et al. 2012; 2014), However, irradiation can affect male ability to prevent female remating. For example, in the tephritids *Ceratitis capitata* (Wiedemann) and *Anastrepha serpentina* (Wiedemann), sterile males are less effective in inhibiting females from remating. In contrast, in *Anastrepha fraterculus* (Wiedemann), no differences were found in the sexual refractory period (time between 1 mating and the next) of wild females that had copulated with sterile or wild males (Gavriel et al. 2009; Abraham et al. 2013; Landeta-Escamilla et al. 2016). Similarly, sterile males were effective at inhibiting *B. tryoni* females and standard bisexual strain *Anastrepha ludens* (Loew) females (Radhakrishna et al. 2009; Abraham et al. 2014). However, the timing of irradiation may be particularly important as sterility needs to be achieved by causing dominant lethal mutations, translocations, and other chromosomal aberrations, and not by decreasing sperm numbers or the overall amount of the ejaculate (Bakri et al. 2005; Lance & McInnis 2005).

Nevertheless, gamma irradiation often does decrease sperm numbers (e.g., Harmer et al. 2006; Radhakrishnan et al. 2009), and this decrease may be particularly drastic if males mate repeatedly. Male multiple matings could result in aspermia (Hooper 1989) and possible ejaculate depletion. For example, in boll weevils *Anthonomus grandis* Boheman (Coleoptera: Curculionidae), and the tephritids *Bactrocera cucurbitae* Coquillet and *B. tryoni*, sterile males quickly reach sperm depletion after 3 consecutive matings (Haynes & Mitchell 1977; Kuba & Itô 1993; Radhakrisnan et al. 2009), however, in the bisexual strain of *A. ludens* and wild *Anastrepha obliqua* (Macquart), sperm depletion is not so apparent between 1 and 3 matings (Pérez-Staples & Aluja 2006; Abraham et al. 2016). These results suggest that the interaction between irradiation and multiple male matings could produce a smaller or lower quality ejaculate with consequences on female post-mating behavior such as remating. Thus, here we evaluated the capacity of sterile *A. ludens* males from the genetic sexual strain (GSS) Tapachula-7 (Tap-7) in inducing the refractory period of wild females, by varying pupal age at which irradiation took place, and male sexual experience.

Anastrepha ludens Tap-7 GSS male have a genetic mutation that makes it possible to distinguish males (brown pupae) from females (black pupae) (Zepeda-Cisneros et al. 2014). The time to sexual maturity, compatibility with wild flies, and mating competitiveness of this strain is comparable to the bisexual strain (Orozco et al. 2013), although Tap-7 males have a different chemical pheromone composition than the bisexual strain (Bosa et al. 2016). Also, recent studies found lower emergence, lower amount of fliers, and shorter longevity under irradiation for the Tap-7 strain (Arredondo et al. 2016), but comparable anti-predator response, dispersal, and field survival compared with the bisexual strain (Flores et al. 2015; González-López et al. 2016). Compared with the bisexual strain, Tap-7 males have lower fertility due to their chromosomal arrangement (Zepeda-Cisneros et al. 2014). A dose of 60 gray (Gy) induces 99% sterility in Tap-7 males (Orozco-Dávila et al. 2015). For the bisexual strain of *A. ludens*, the recommended age to irradiate varies from 13 to 45 d after pupation depending on the temperature (28–15 °C, respectively) (Resilva & Pereira 2014). Generally, both strains are irradiated 48 h before emergence (FAO/IAEA/USDA 2014; Orozco-Dávila et al. 2015). Although it is not known how many times a male can mate throughout its lifetime, wild males in field cages will mate up to 4 times in 4 d (Aluja et al. 2001). However, for Tap-7 males, there is no information on how pupal age at irradiation and male mating history can affect female post-copulatory behavior such as remating.

Materials and Methods

STUDY SITE

Evaluations were carried out between May and Aug 2015 at the laboratories of Desarrollo de Métodos, at the MoscaFrut facility (Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación–Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria) in Metapa de Domínguez, Chiapas, Mexico.

INSECTS

Anastrepha ludens males from the GSS Tap-7 and mass-reared standard bisexual females were obtained from the MoscaFrut facility. Both bisexual and Tap-7 strains were reared for more than 70 generations in the facility, thus flies were well adapted to laboratory crowding conditions as adults and larvae. Females oviposit into an artificial medium and their larvae develop in an artificial diet made of corn cob fractions, corn flour, sodium benzoate, methylparaben, citric acid, guar gum, and purified water, and are maintained under controlled temperature and humidity (Domínguez et al. 2010; Zepeda-Cisneros 2014). For the Tap-7 strain, pupation occurs without a pupation substrate. Male pupae were obtained 72 h before emerging as adults and were dyed with fluorescent colorant AX-18-N Signal Green (Day Glo, Color Corp., Cleveland, Ohio), with 2 g of colorant per 1 kg of pupae. Females used from the bisexual strain were collected as pupae 48 h before emerging and were not dyed. Wild male and female *A. ludens* individuals were obtained from infested fruit, cultivar 'Matasano' (*Casimiroa edulis* Llave & Lex.; Rutaceae), collected at Las Margaritas municipality in Chiapas, Mexico.

Anastrepha ludens pupae from the various strains and treatments were placed separately in wooden mesh cages measuring 30 × 30 × 30 cm. On the day of emergence, adults were separated by categories (males from the Tap-7 strain, females from the bisexual strain, wild males, and wild females) and confined in similar cages, each 1 containing 100 individuals. Adults were fed sugar with hydrolyzed protein (MP Biomedicals, LLC, Santa Ana, California) at a ratio of 24:1 (Liedo et al. 2013). Flies were kept under laboratory conditions at 24.0 °C ± 1.5, 70.5 ± 10.0% relative humidity with fluorescent light for 12 hr (850 ± 110 lux, 7 AM to 7 PM) followed by 12 hr of darkness.

EXPERIMENTAL DESIGN

A balanced bifactorial design was used. The following factors were evaluated: 1) the effect of irradiation (irradiation versus no irradiation), 2) the age at which pupae were irradiated (72, 48, and 24 h before adult emergence), and 3) the sexual experience of males (virgin, once mated, and twice mated).

MALE TREATMENTS

Three samples of 250 g of pupae (1 sample for each of the above mentioned ages) were obtained from the Tap-7 GSS. Samples were irradiated at 80 Gy under hypoxia conditions in a Cobalt-60 irradiator (model GB-127, Nordion International Inc., Ottawa, Canada). A non-irradiated sample of pupae was retained as a control.

Males with varying sexual experience were obtained as follows. On day 13 after emergence, 1 adult male was placed with 2 adult females from the bisexual strain (age 12 ± 1 d) in a mesh cage (15 × 15 × 15 cm). Observations were carried out continuously from 3 PM to 7 PM during the time of day of sexual activity (Aluja et al., 2000). When mating commenced, the non-mating female was carefully removed from the cage to avoid interference. Once mating ended, the mated female was removed leaving the male in the cage with food and water. To obtain twice mated males, the procedure was repeated the next day. Thus, twice mated males were obtained during 2 consecutive days before the evaluation, and once mated males 1 day before. Additionally, a number of males were retained as virgin until the day of evaluation. Different individuals of the same range of ages were used for each independent group of males (virgin, once mated, and twice mated).

Each treatment level comprised of 4 male irradiation groups (72, 48, 24 h before adult emergence or no irradiation) and 3 groups of males with varying sexual experience (virgin, once mated, and twice mated), Thirty five females per treatment were evaluated and experiments were replicated 3 times with different insect batches every time (N = 35 × 4 × 3 × 3 = 1,260 females).

FEMALE REMATING

On the day of evaluation, males from each treatment were individually placed with two 16 d-old wild virgin females. Once copulation ended, the male was removed from the cage leaving the female behind, thus we obtained females having mated with virgin, once mated, and twice mated males. Female sexual refractory period was tested at 2, 4, 6 and 8 d after mating, at which time the same females were exposed to two 15- to 21-day-old wild virgin males. On each day, copulating females were recorded and then removed. Non-mating females were used again and given the opportunity to remate. Observations were carried out from 3 PM to 7 PM.

DATA ANALYSES

The effect of male irradiation (a pool of the 3 irradiation age groups) versus no irradiation (control) on female likelihood to remate and sexual refractory period (number of days between 1 mating and the next) was analyzed by a logistic regression with irradiation, male sexual experience, and the interaction of irradiation and male sexual experience as predictors. Repetition was not significant and was excluded from the model.

The effect of timing of irradiation (at 72, 48 or 24 h before adult emergence) and male sexual experience on female likelihood to remate and the sexual refractory period was analyzed by an ordinal logistic regression with male irradiation age, male sexual experience, and the interaction between them as predictors. Repetition was not significant and was excluded from the models.

Contingency tables were used to compare the sexual refractory period across 4 d of evaluation induced by males with varying sexual experiences. Pearson squared Chi test at α = 0.05 was used to test the null hypothesis of no difference in the proportions of remating females across days according to male sexual experience. To further understand the effect of male sexual experience on female sexual refractory period, the proportions of remating females within days also were tested for differences with a Chi squared test at α = 0.05.

Results

IRRADIATION AND MALE MATING HISTORY

Of the 1,260 wild females evaluated, 754 (60%) remated within 8 d. There was no effect of male irradiation (χ^2 = 0.22, df = 1, *P* = 0.63, *N* = 1,260), male sexual experience (χ^2 = 2.13, df = 2, *P* = 0.34), or the interaction (χ^2 = 0.56, df = 2, *P* = 0.75) on the likelihood of females remating.

Conversely, taking into account only the females that remated, the analysis on female sexual refractory period (number of days between 1 mating and the next) revealed that whereas male irradiation did not affect the refractory period (χ^2 = 0.01, df = 1, *P* = 0.92, *N* = 754), there was an effect of the male previous sexual experience (χ^2 = 7.75, df = 2, *P* = 0.02). There was no effect of the interaction (χ^2 = 2.86, df = 2, *P* = 0.24) (Table 1).

An analysis of male sexual experience indicated that the refractory period induced by virgin males was shorter from that induced by once mated males (χ^2 = 10.1, df = 3, *P* = 0.01, *N* = 497), or twice mated males (χ^2 = 12.42, df = 3, *P* = 0.006, *N* = 496). However, no differences were observed in the refractory period of females mating with males previously mated once or twice (χ^2 = 2.38, df = 3, *P* = 0.49, *N* = 515). Further comparisons of sexual refractory period by day revealed a higher proportion of females remating at day 2 (χ^2 = 5.6, df = 1, *P* = 0.01, *N* = 496 and χ^2 = 2.7, df = 1, *P* = 0.09, *N* = 497) for virgin versus twice mated, and virgin versus once mated males, respectively. By day 6, a lower proportion of females remated after mating with a virgin versus once mated males (χ^2 = 7.8, df = 1, *P* = 0.005, *N* = 497) and for females mating with a virgin versus twice mated male (χ^2 = 10.4, df = 1, *P* = 0.001, *N* = 496) (Table 1)

TIMING OF IRRADIATION AND MALE MATING HISTORY

There was no effect of male pupal age at irradiation (χ^2 = 2.31, df = 2, *P* = 0.31, *N* = 945), or male sexual experience (χ^2 = 1.56, df = 2, *P* = 0.45) on female likelihood to remate. There was a marginal effect of the interaction between age at irradiation and male mating history on female likelihood to remate (χ^2 = 9.32, df = 4, *P* = 0.053) (Fig. 1). Taking into account only the females that remated, there was no effect of male age at irradiation (χ^2 = 0.89, df = 2, *P* = 0.64, *N* = 565), male mating history (χ^2 = 4.1, df = 2, *P* = 0.12) or the interaction (χ^2 = 7.35, df = 4, *P* = 0.12) on female sexual refractory period.

Table 1. Sexual refractory period of wild *Anastrepha ludens* females remating with wild males after mating with the GSS strain of *A. ludens* (Tapachula-7) males irradiated, or not irradiated and with varying sexual experiences (virgin, once mated, or twice mated).

			Remating in females		Proportion of females remating by sexual refractory period				
Factor	Male treatment	*N*	No	Yes	2 d	4 d	6 d	8 d	Effect
Irradiation	Irradiated	315	123	192	0.323	0.391	0.198	0.089	ns
	Not irradiated	945	383	562	0.338	0.381	0.176	0.105	ns
Sexual experience	Virgin	420	181	239	0.393 a	0.381 a	0.113 a	0.113 a	A
	Once mated	420	162	258	0.322 b	0.395 a	0.205 b	0.078 a	B
	Twice mated	420	163	257	0.292 b	0.373 a	0.222 b	0.113 a	B

Capital letters indicate significant differences between male treatments (rows) (Pearson chi test, *P* < 0.05); lowercase letters indicate significant differences between days (columns) (Pearson chi test, *P* < 0.05); ns = not significant.

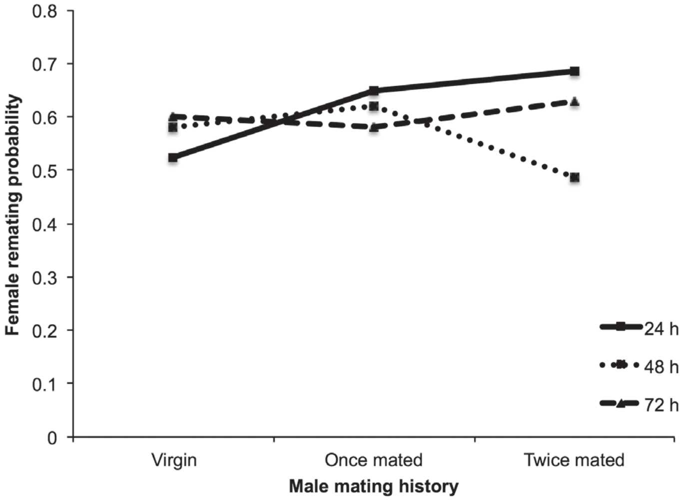

Fig. 1. Interaction between age of pupal irradiation (24, 48, or 72 h before emergence) and male previous sexual experience (virgin, once mated, or twice mated) of the GSS strain of *A. ludens* (Tapachula-7) males on wild female likelihood to remate (*N* = 945).

Discussion

For *A. ludens* GSS Tap-7 males, irradiation or the timing of irradiation did not affect female sexual refractory period, yet male mating history did. More wild females mating with virgin Tap-7 males remated 2 d after their initial mating compared with females that had mated with once or twice mated males. At 6 d after their initial mating, this effect reversed, as more females remated if they had mated with a sexually experienced male. However, in the context of continuous weekly releases for the GSS strain of *A. ludens,* increased wild female remating only 2 d after first mating with a virgin Tap-7 male is more relevant.

A males' previous sexual experience can have important consequences for both pre- and post-copulatory behavior. For example, wild *A. ludens* females prefer to mate with sexually experienced older males, and in the Caribbean fruit fly, *Anastrepha suspensa* (Loew)*,* males with sexual experience increase their pheromone production, mate faster, and have higher juvenile hormone levels in their hemolymph compared with virgin males (Teal et al. 2000; Pérez-Staples et al. 2010). In the bisexual strain of *A. ludens,* sexually experienced males transfer more sperm compared with virgin males, and this may also be the case for Tap-7 males (Abraham et al. 2016). All these results suggest that sexual experience can modify male behavior including the ejaculate, and consequently may be relevant in the context of SIT for the GSS strain of *A. ludens,* as it impacts how quickly wild females remate.

For certain species, irradiation can affect male ability to inhibit female remating. For example, *C. capitata* females and mass-reared *A. serpentina* females are more likely to remate when mated with a sterile male (Gavriel et al. 2009; Landeta-Escamilla et al. 2016). Here, wild females mated to sterile Tap-7 males were just as likely to remate as females mated to fertile males, and there was no effect of male irradiation on female sexual refractory period. Likewise, for the bisexual strain of *A. ludens* there was no effect of male irradiation or male mating history on the probability of female remating (Abraham et al. 2016).

Pupal age at irradiation is an important factor that can potentially reduce male mating competitiveness, as irradiation of young pupae can produce somatic damage with consequences on male quality and fertility (Bakri et al. 2005). For example, in the melon fly *B. cucurbitae*, egg hatch increased when females mated with a male irradiated 48 h before eclosion in comparison with females that had mated with males irradiated at 72 h (Sokei & Kuba, 2002). During irradiation, reproductive cells are exposed resulting in dominant lethal mutations (Hooper 1989), thus the timing of irradiation is important so that it does not hinder spermatogenesis. Irradiation at young pupal ages could affect the early spermatogonial cells, as cells in spermatogenesis are more radiosensitive (easily killed) than mature sperm (Hooper 1989). We lack knowledge on how irradiation at varied pupal ages could affect other components of the ejaculate, which together with sperm induce mating inhibition in *A. ludens* (Abraham et al. 2016). Here, however, for Tap-7 males there was no clear effect of pupal age at irradiation on their ability to inhibit female remating.

In the South American fruit fly *A. fraterculus,* pupal age at irradiation had no effect on male fertility (egg hatch), and no effect of radiation was found on sperm transfer (Allinghi et al. 2007). Similarly, in this study irradiating pupae at an earlier age than the usual 48 h before emergence had no effect on female sexual refractory period. At these ages, metamorphosis is almost complete and pupae may be mature enough for irradiation with minimal detrimental effects (Hooper 1989; Allinghi et al. 2007).

In conclusion, the age at which pupae are irradiated can have important effects on the quality of males; however, irradiation or the timing of irradiation had no effect on the ability of Tap-7 males to inhibit female remating. This is important for SIT as irradiating male pupae even at 72 h before emergence may not impact their future ability to reduce female receptivity. Male mating history seems to be an important factor that affects female sexual refractory period, as males with sexual experience were able to inhibit wild females for longer than virgin males. Future studies on the ejaculate composition of Tap-7 males are necessary, which may help us understand physiological changes in male flies that could affect female mating responses.

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