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Female receptivity in *Anastrepha ludens* (Diptera: Tephritidae) is not modulated by male accessory gland products

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ABSTRACT

In numerous insects, accessory gland products (AGPs) transferred from males to females during mating are responsible for female sexual inhibition, but these products can be affected by male condition. Here, we investigated the effect of AGPs on female receptivity of the Mexican fruit fly *Anastrepha ludens* (Loew), and the effect of male and female strain, male irradiation, AGP dose and sexual activity period on the effectiveness of these AGPs in inhibiting female remating. Injections of aqueous extracts of male accessory glands into the abdomen of females did not reduce their receptivity either at 0.2 or 0.8 male equivalent. Females injected with AGPs behaved like virgin females and not as mated females. Neither male origin, female origin (wild *versus* mass-reared), nor male irradiation (sterile *versus* fertile males) had an effect in inhibiting female remating. Also, injections of glands obtained during the sexual calling period of males, or obtained during the morning when males are not sexually active had no effect on female remating behavior. Mated mass-reared females were more likely to remate than wild females. We conclude that inhibition of female sexual receptivity of *A. ludens* is mediated by factors other than AGPs, such as the number of sperm stored by females, the stimulus of copulation *per se* or more probably, mediated by a combination of factors. More research is needed to elucidate the role of AGPs in this species.

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1. Introduction

During mating, male insects transfer sperm and male accessory gland products (AGPs). AGPs are composed mostly of proteins (Gillott, 2003). AGPs produce profound changes in the postmating behavior of females, such as: a reduction in sexual receptivity, an increase in oviposition, an increase in female food intake and a decrease in female longevity (Chen et al., 1988; Gillott, 2003; Poiani, 2006). These products have been shown to aid in sperm transport and storage, and can protect the female reproductive tract through antibacterial and antifungal peptides (Avila et al., 2011). Aside from the evolutionary implications of AGPs in sexual conflict theory, their ability to inhibit female remating has practical relevance for pest control.

Some of the effects of AGPs on female postmating behavior have been studied using direct injections of these secretions into virgin females. Among these effects, the most relevant for insect pest species are a decrease in sexual receptivity (Radhakrishnan and Taylor, 2007; Yamane et al., 2008a,b; Shutt et al., 2010; Abraham et al., 2012; Yu et al., 2013; Brent and Hull, 2014), an increase in female sexual refractory period (Himuro and Fujisaki, 2008; Helinski et al., 2012), an induction of oviposition (Yamane and Miyatake, 2010; Yu et al., 2013), inhibition of host-seeking behavior (Fernández and Klowden, 1995), a reduction of longevity (Xu and Wang, 2011; Yu et al., 2013) and a change in the orientation behavior toward host fruit odor (Jang, 1995). On the other hand, there are cases were the injection of AGPs homogenates do not induce refractoriness in females (Klowden, 2001; Lentz et al., 2009).

Understanding the mechanisms that modulate female receptivity may provide useful information that could contribute towards controlling insect pests using environmentally friendly methods such as the sterile insect technique (SIT). This technique is a viable pest management tool for integrated pest management programs, widely used to suppress fruit flies (Diptera: Tephritidae), and other insect pests by effectively inducing sterility in pest populations (Knipling, 1955, 1959). The success of this technique relies on the ability of sterile males to successfully compete against wild

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males, obtaining copulations with wild females to fertilize their eggs and ultimately, inhibit renewal of female receptivity (Hendrichs et al., 2002).

Implementation of the SIT requires both mass-rearing and subsequent irradiation to sterilize males. The loss of natural attributes of fruit fly males, due to unintentional artificial selection during mass-rearing or somatic damage produced by irradiation, is well known (Liimatainen et al., 1997; Taylor et al., 2001; Mossinson and Yuval, 2003; Rull et al., 2005; Gavriel et al., 2009). Mass rearing conditions and the number of generations a strain has been held in colonization can affect male mating performance and related traits (Moreno et al., 1991; Cayol, 2000; Lux et al., 2002b). Irradiation can also affect male mating performance and ability to inhibit female receptivity. For example, in studies of the Mexican fruit fly *Anastrepha ludens*, wild males obtained more matings with wild females than irradiated laboratory males (Rull et al., 2005). High irradiation doses can also reduce male mating performance (Moreno et al., 1991; Calcagno et al., 2002; Lux et al., 2002a). In the South American fruit fly *Anastrepha fraterculus*, AGPs from laboratory males were as efficient as those of wild males in inhibiting female receptivity, while the AGPs of males were negatively affected by irradiation (Abraham et al., 2012). However, there was no difference in the refractory period and remating rate of wild females mated with laboratory sterile males or wild males (Abraham et al., 2013). In contrast, in the Queensland fruit fly, *Bactrocera tryoni*, irradiated males were as efficient as non-irradiated fertile males in inducing sexual inhibition of mass-reared females (Radhakrishnan et al., 2009).

A. ludens (Diptera: Tepritidae), is considered one of the most devastating pests of citrus throughout its distribution range, which encompasses southern Texas to Central America (Thomas, 2003; Birke et al., 2006) and is an important pest of mango grown at more than 800 m of elevation in Mexico (Aluja et al., 1996). Currently, this species is controlled with SIT in the northern citrus-producing regions of Mexico (Reyes et al., 2000). *A. ludens* males may call alone or form leks on the underside of leaves, emitting a pheromone and wing fanning during dusk (Aluja et al., 2000). Females arrive at these sites and males attempt to mate. Males carry out elaborate courtship displays, but only a few males obtain copulations (Aluja et al., 2000). Copulations last an average of 58–78 min varying with male age and sexual experience (Pérez-Staples et al., 2010). While males can potentially mate once per day (Aluja et al., 2001, 2008), only 30% of wild females remate throughout their entire life and are inhibited from remating for 12 d on average after their initial mating (Aluja et al., 2009).

Here, we aimed to assess whether *A. ludens* male AGPs had an effect on female receptivity, mating latency and copulation duration. In particular, we sought to determine how male and female strain and male irradiation affected the ability of AGPs to inhibit female receptivity. Due to the decreased ability of sterile mass-reared males to inhibit female receptivity in *A. fraterculus*, we predicted that *A. ludens* wild males would be more able to inhibit female receptivity than mass-reared males, while fertile males would be better able to inhibit female receptivity compared to irradiated males. In addition, we also measured whether AGPs produced during the sexual activity period had an effect on female sexual inhibition compared to AGPs produced when males were not courting.

2. Materials and methods

2.1. Insects

Mass-reared *A. ludens* adults were obtained from the Moscafrut facility in Metapa de Domínguez, Chiapas, Mexico. Flies were obtained from pupae sent by air transportation to Xalapa, Veracruz. Sterile males were irradiated at a dose of 80 Gy in a Gammacel irradiator (Gammacell 220 with cobalt 60 as the source; Atomic Energy of Canada Ltd., Ottawa, Canada) 48 h before emergence. Wild flies were recovered from infested oranges collected at Tuzamapan, and the surroundings of Xalapa. Fruits were taken to the laboratory and placed in 30 × 50 × 15 cm plastic trays with soil. Larvae migrated from the fruit to the soil where they pupated. After 7–10 days, the soil was sieved and recovered pupae were placed in 30 × 30 × 30 cm cages at 26 ± 2 °C and 80 ± 10 RH until adult emergence. Artificial light was provided under a 12:12 photoperiod, with half the lights going off or on at 6:00 and the other half at 6:30 to simulate dawn or dusk. Natural light was also provided from a window. Experiments were carried out at the Instituto de Biotecnología y Ecología Aplicada, Universidad Veracruzana, Xalapa, Veracruz, Mexico.

On the day of emergence, flies were sorted by sex and transferred to 30 × 30 × 30 cm cages in groups of 200–300 adults. Both sexes were provided with water and a diet consisting of sugar and hydrolyzed yeast (Yeast Hydrolyzated Enzymatic, ICN Biomedicals®) in a 3:1 ratio.

2.2. Preparation of male accessory gland homogenates

General methodology followed Jang (1995), Radhakrishnan and Taylor (2007), and Abraham et al. (2012). Accessory glands were dissected from live *A. ludens* males immersed in saline solution (NaCl 0.9%, PISA®). Dissections were done in the morning, except in trial 3 where the dissections were done from 17:00 h to coincide with the window of sexual receptivity in this species. Glands were transferred into a 30 × 6 mm glass micro homogenizer with saline and gently crushed to release the accessory glands products. This aqueous extract was placed in an Eppendorf tube (1.5 ml) and centrifuged at 12,000 rpm at 4 °C for 1 min (Hermle Z 300 K centrifuge). The resulting supernatant was placed in crushed ice and used for injections on the same day it was extracted. The dose of AGPs to be injected was 0.2 male equivalent, defined as the amount of fluid obtained by crushing the accessory gland of a single male in 1 μl of saline (Jang, 1995; Radhakrishnan and Taylor, 2007; Abraham et al., 2012). Here, 20 *A. ludens* males of each treatment were dissected and the glands were homogenized in 100 μl of saline to obtain a 0.2 male equivalent concentration in 1 μl of solution, except in trial 2 where the dose used was 0.8 male equivalent and therefore 80 accessory glands of each treatment were homogenized in 100 μl of saline. Injections were done immediately after extractions to avoid protein degradation.

2.3. Injections

Sexually mature virgin females (10–20 d-old) were gently placed into a small mesh bag, secured with the ventral side up and placed on an inverted Petri dish. The bag was secured with a plastic band. Secured females were placed under a dissecting microscope (Leica S8AP0) with a 40× lens. Females were then injected with 1 μl of solution containing the AGPs through the intersegmental membrane near the third abdominal sclerite. A Hamilton 1 μl microsyringe (Hamilton Co., Reno, Nevada. Microliter™ #7001) was used to inject females. To rule out the effect of the injection treatment on the sexual receptivity of the female, the same amount of saline was injected into control females ("saline females"). After injections, females were transferred to 750 mL plastic containers in groups of 20–30 individuals with adult diet and water.

2.4. Mated females

On the day of the injections, one 10–20 d-old virgin female and one male of each treatment according to the experiment were placed in 500 mL plastic containers at 16:00 h. The mating period started approximately at 16:30 h. Containers were checked for copulating pairs continuously for 6 h after releasing flies. The number of copulating pairs and copulation duration was recorded. After the end of copulation, females were transferred to 750 mL plastic containers in groups of ca. 20–30 individuals with adult diet and water. Matings were conducted in a dark room lit with a dim red lamp to allow observation.

2.5. Treatments

Forty-eight hours after the injection or copulation of females, we evaluated the receptivity of females from all treatments. In all experiments, we used virgin females as controls, and females injected only with saline (‘‘saline females’’). As an additional control, in trial 1 we also used punctured females, i.e., females that were punctured with the Hamilton syringe but were not injected any substance.

2.5.1. Effect of male strain in wild and mass-reared females

We evaluated the receptivity of wild females injected with AGPs from mass-reared (15 d-old) or wild (15–20 d-old) males, and the receptivity of females mated with these males. In a separate experiment we evaluated the receptivity of mass-reared females injected with AGPs from mass-reared (15–23 d-old) or wild (18–21 d-old) males, and the receptivity of females mated with these males.

2.5.2. Effect of irradiation

We evaluated the receptivity of mass-reared females injected with AGPs from mass-reared irradiated (12 d-old) or fertile (10 d-old) males. Here, we increased the dose to 0.8 male equivalent. We also evaluated the receptivity of females mated with irradiated or fertile males.

2.5.3. Effect of time of dissection

To evaluate whether the effect of AGPs could be seen during the sexually active period, we dissected and injected glands starting at 17:00 h. (during the ‘‘calling’’ and sexual activity period of this species). We evaluated if injections of glands dissected during the evening had the same effect in inhibiting receptivity compared to previously mated females. We tested this separately for 17 d-old mass-reared flies and for 18–23 d-old wild flies.

2.6. Evaluation of female receptivity

Two days after either injections or matings, one female from each treatment, and one mature virgin male of the same condition as the female (i.e., wild females with wild males and mass-reared females with mass-reared males) were placed in 500 mL plastic containers at 16:00 h in the laboratory at 24 °C. Containers were checked for copulating pairs continuously for 6 h after releasing flies. Mating latency, copulation duration and the number of copulating pairs were recorded. Matings were conducted in a dark room lit with a dim red lamp to allow observation.

2.7. Statistics

To analyze mating latency and copulation duration we applied a one-way ANOVA with female category as class variable and the time between fly release and the start of copulation was recorded as mating latency. The time the pair spent in copula was calculated as copulation duration and were used as dependent variables. Tukey HSD tests were used for post hoc comparisons. Non-parametric Kruskal–Wallis was applied when ANOVA assumptions were not met and multiple comparisons were carried out by means of Dunn's test. The number of copulation pairs was analyzed with χ^2-tests of Homogeneity. For a comparison among more than two treatments, the sequential Bonferroni method (Rice, 1989) was applied after χ^2-tests. In the experiments we compared: (1) receptivity among categories of control females, (2) receptivity between the two categories of injected females and, (3) receptivity between the two categories of mated females. If no significant differences were found, the data were pooled and three new categories were analyzed: control virgin females, injected females and mated females. Then we compared: (4) control virgin females *versus* injected females and (5) injected females *versus* mated females. When no apparent *a priori* differences in female receptivity were observed, all females were compared in a single analysis (trial 1 and 3 for mass-reared females).

3. Results

3.1. Effect of fly strain

3.1.1. Wild females

There were no significant differences in the mating latency or copula duration of wild females from any treatment (ANOVA, $F = 1.79$, df = 6, 110, $P = 0.127$ and $F = 0.42$, df = 6, 106, $P = 0.863$, respectively) (Tables 1 and 2). There were no differences in the receptivity of control females (i.e., virgin females, punctured females, saline females) (χ^2 test, $\chi^2 = 0.557$, df = 2, $P > 0.05$), between females injected with AGPs from either wild or mass-reared males ($\chi^2 = 0.043$, df = 1, $P > 0.05$), nor between females mated with wild or mass-reared males ($\chi^2 = 0.713$, df = 1, $P > 0.05$). There were no significant differences in the mating probability of injected or virgin females ($\chi^2 = 2.735$, df = 1, $P > 0.05$), yet there was a significant difference between injected and previously mated females ($\chi^2 = 35.288$, df = 1, $P < 0.001$). Injected females were more likely to mate (Fig. 1).

3.1.2. Mass-reared females

There was no significant difference in the mating latency between females of different treatments (ANOVA, $F = 1.01$, df = 6, 211, $P = 0.410$) (Table 1). However, copulation duration was longer in mated females compared with control and injected females (Table 2) (Kruskal–Wallis, $H = 21.80$, df = 6, 211, $P = 0.0013$). AGP injections of fertile or sterile males had no effect on the probability of female mating, no other treatment had an effect (χ^2 test, $\chi^2 = 6.163$, df = 6, $P > 0.405$) (Fig. 2).

3.2. Effect of male irradiation

Mating latency was affected by female treatment (ANOVA, $F = 6.25$, df = 5, 102, $P < 0.001$). Mating latency was shorter in control and injected females compared with mated females (Table 1). Female treatment had no effect on copula duration (ANOVA, $F = 1.22$, df = 5, 102, $P = 0.309$) (Table 2). There were no significant differences in female receptivity between the two categories of control females (χ^2 test, $\chi^2 = 0.007$, df = 1, $P > 0.05$), between the two categories of injected females ($\chi^2 = 0.242$, df = 1, $P > 0.05$), nor between the two categories of mated females ($\chi^2 = 0.247$, df = 1, $P > 0.05$). There were no significant differences between injected or virgin females ($\chi^2 = 0.007$, df = 1, $P > 0.05$). However, there was a significant difference between injected and mated females ($\chi^2 = 31.883$, df = 1, $P < 0.001$), injected females were more likely to mate than previously mated females (Fig. 3).

Table 1
Mating latency (min) of females 48 h after either the injection or mating [mean ± se (N) or Q1-median-Q3 (N)].

Experiment	1. Effect of fly strain		2. Effect of male irradiation	3. Effect of time of dissection	
Female category	Wild females	Mass-reared females	Mass-reared females	Wild females	Mass-reared females
Virgin	186 ± 6 (31)a	127 ± 11 (36)a	31± 3 (25)a	113 ± 15 (11)a	2-3-9 (29)a
Punctured	193 ± 7 (16)a	137 ± 10 (22)a	–	–	–
Saline	180 ± 10 (14)a	136 ± 17 (20) a	43 ± 8 (14)a	113 ± 13 (14)a	8-15-42 (32)b
Injected with AGPs of ♂A	181 ± 7 (20)a	128 ± 8 (29)a	39 ± 3 (26)a	134 ± 11 (7)a	–
Injected with AGPs of ♂B	205 ± 7 (21)a	111 ± 9 (23)a	37 ± 3 (30)a	–	13-15-34 (17)b
Mated with ♂A	202 ± 13 (6)a	139 ± 10 (39)a	69 ± 10 (5)b	174 ± 45 (3)a	–
Mated with ♂B	195 ± 11 (9)a	143 ± 7 (49)a	67 ± 15 (8)b	–	9-28-55 (26)b

Experiment 1. ♂A: wild males. ♂B: mass-reared males.
Experiment 2. ♂A: irradiated males. ♂B: fertile males.
Experiment 3. ♂A: wild males. ♂B: mass-reared males.

Table 2
Copulation duration (min) of females 48 h after either the injection or mating [mean ± se (N) or Q1-median-Q3 (N)].

Experiment	1. Effect of fly strain		2. Effect of male irradiation	3. Effect of time of dissection	
Female category	Wild females	Mass-reared females	Mass-reared females	Wild females	Mass-reared females
Virgin	48 ± 5 (30)a	59-101-132 (36)a	72 ± 6 (25)a	74 ± 7 (11)a	111 ± 14 (11)a
Punctured	41 ± 7 (16)a	98-121-145 (22)ab	–	–	–
Saline	51 ± 5 (12)a	95-107-146 (20)ab	65 ± 10 (14)a	68 ± 8 (14)a	103 ± 11 (19)a
Injected with AGPs of ♂A	51 ± 6 (20)a	51-82-134 (29)a	75 ± 6 (26)a	64 ± 7 (7)a	–
Injected with AGPs of ♂B	49 ± 3 (20)a	73-104-125 (23)a	71 ± 5 (30)a	–	74 ± 14 (9)a
Mated with ♂A	52 ± 11 (6)a	90-139-194 (39)b	89 ± 12 (5)a	47 ± 5 (3)a	–
Mated with ♂B	55 ± 7 (9)a	103-145-183 (49)b	93 ± 11 (8)a	–	76 ± 19 (9)a

Experiment 1. ♂A: wild males. ♂B: mass-reared males.
Experiment 2. ♂A: irradiated males. ♂B: fertile males.
Experiment 3. ♂A: wild males. ♂B: mass-reared male.

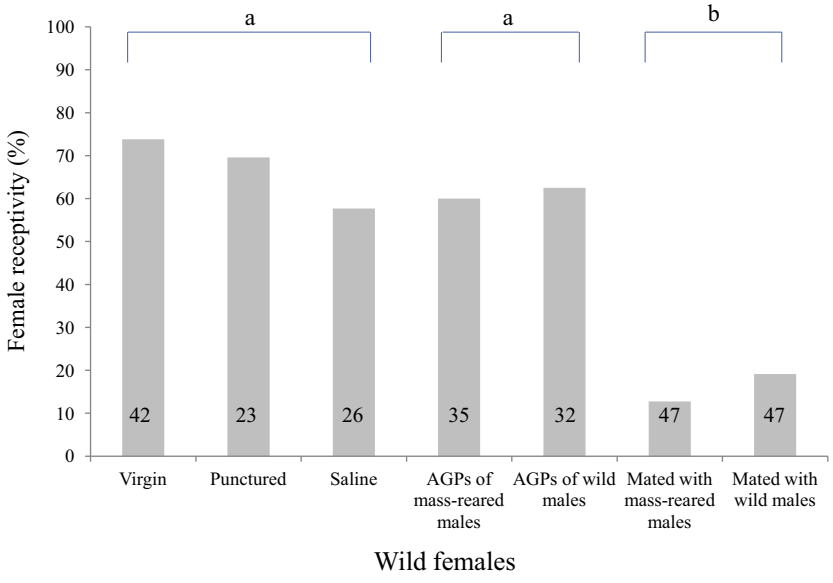

Fig. 1. Receptivity of *Anastrepha ludens* wild females of different categories: virgin, punctured and injected with saline as controls, injected with AGPs of mass-reared or wild males and previously mated with mass-reared or wild males. Different letters over bars indicate significant difference ($P < 0.001$) after the sequential Bonferroni method (Rice, 1989) following χ^2-tests.

3.3. Effect of time of dissection

3.3.1. Wild flies

Neither mating latency nor copulation duration were affected by female treatment (ANOVA, $F = 1.67$, df = 3, 31, $P = 0.193$ for mating latency, Table 1; $F = 0.92$, df = 3, 31, $P = 0.442$ for copulation duration, Table 2). There were no significant differences between the receptivity of control females (i.e., virgin females and saline females) (χ^2 test, $\chi^2 = 0.355$, df = 1, $P = 0.551$). There were no significant differences between control virgin or females injected with AGPs dissected during the evening ($\chi^2 = 0.396$, df = 1, $P = 0.529$), yet there was a significant difference between injected and mated females ($\chi^2 = 7.682$, df = 1, $P = 0.005$), injected females were more likely to mate than previously mated females (Fig. 4).

3.3.2. Mass-reared flies

Mating latency was affected by female treatment (Kruskal–Wallis test, $H = 22.35$, df = 3, 99, $P < 0.001$). Mating latency was

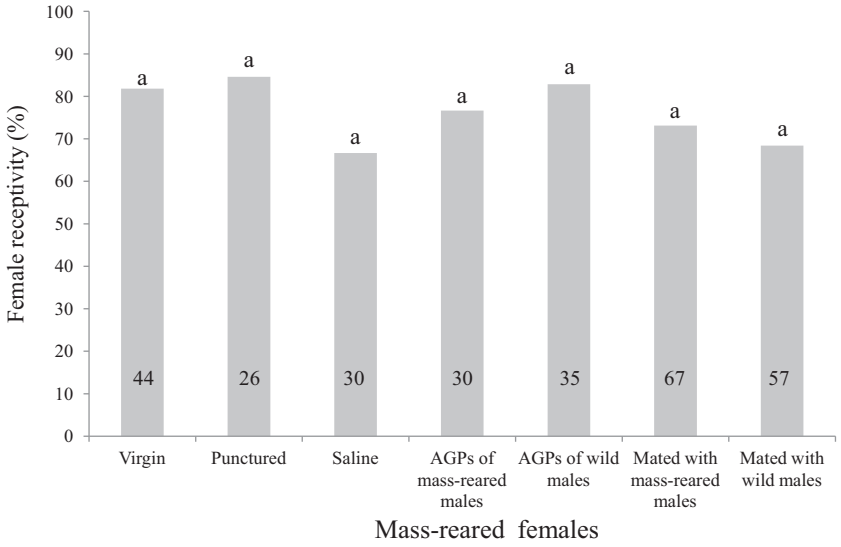

Fig. 2. Receptivity of *Anastrepha ludens* mass-reared females of different categories: virgin, punctured and injected with saline as controls, injected with AGPs of mass-reared or wild males and previously mated with mass-reared or wild males. Different letters over bars indicate significant difference ($P < 0.001$) after the sequential Bonferroni method (Rice, 1989) following χ^2-tests.

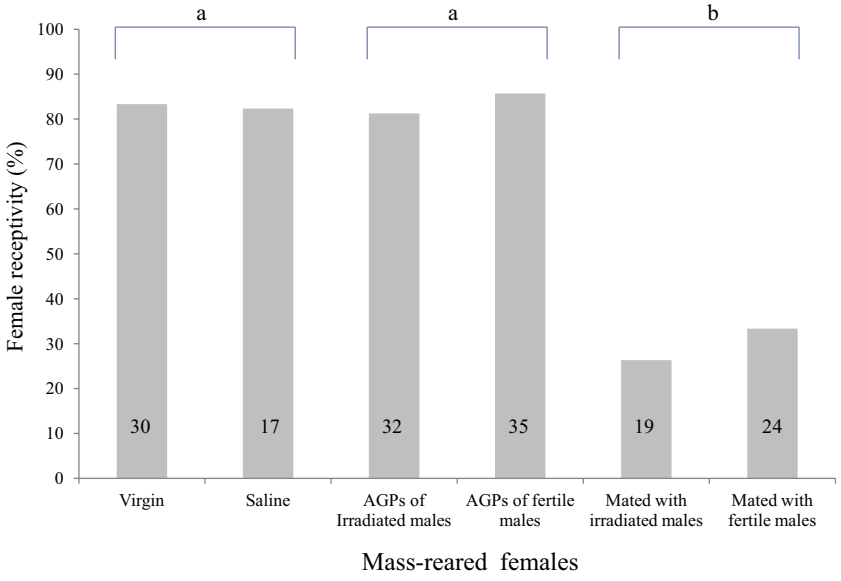

Fig. 3. Receptivity of *Anastrepha ludens* mass-reared females of different categories: virgin and injected with saline as controls, injected with AGPs of irradiated or fertile males and previously mated with irradiated or fertile males. Different letters over bars indicate significant difference ($P < 0.001$) after the sequential Bonferroni method (Rice, 1989) following χ^2-tests.

shorter in virgin females compared with saline, injected and mated females (Table 1). Copulation duration was not affected by female treatment (ANOVA, $F = 1.52$, df = 3, 44, $P = 0.221$) (Table 2). There were no significant differences in female receptivity among the four female treatments (χ^2 test, $\chi^2 = 4.142$, df = 3, $P > 0.246$) (Fig. 5).

4. Discussion

Injections of male accessory gland products (AGPs) did not reduce female sexual receptivity in *A. ludens*. Injection of AGPs homogenates mediates the receptivity switch off of virgin females in *Callosobruchus maculatus*, *Callosobruchus chinensis* (Yamane et al., 2008a,b), *Spodoptera litura* (Yu et al., 2013), *Lygus hesperus* (Brent and Hull, 2014), anophelines and *Aedes* mosquitoes (Shutt et al., 2010; Tripet et al., 2011). For the few pest tephritids were this has been studied *Ceratitis capitata* (Jang et al., 1999), *B. tryoni* (Radhakrishnan and Taylor, 2007) and *A. fraterculus* (Abraham et al., 2012) these products do inhibit female remating at the same dose used in the present study. Fly strain, male irradiation, male AGP dose or time of dissection had no effect on female receptivity. Moreover, while wild female receptivity decreased after mating, in most experiments mass-reared females did not switch off their receptivity after mating, irrespective of male strain. Thus, there was a strong effect of female strain on their post-mating behavior. While this coincides with previous studies where mass-reared female remating behavior is not switched off from mating with a mass-reared male (Meza et al., 2014), here during one trial (Fig. 3), mated mass-reared females were inhibited from remating.

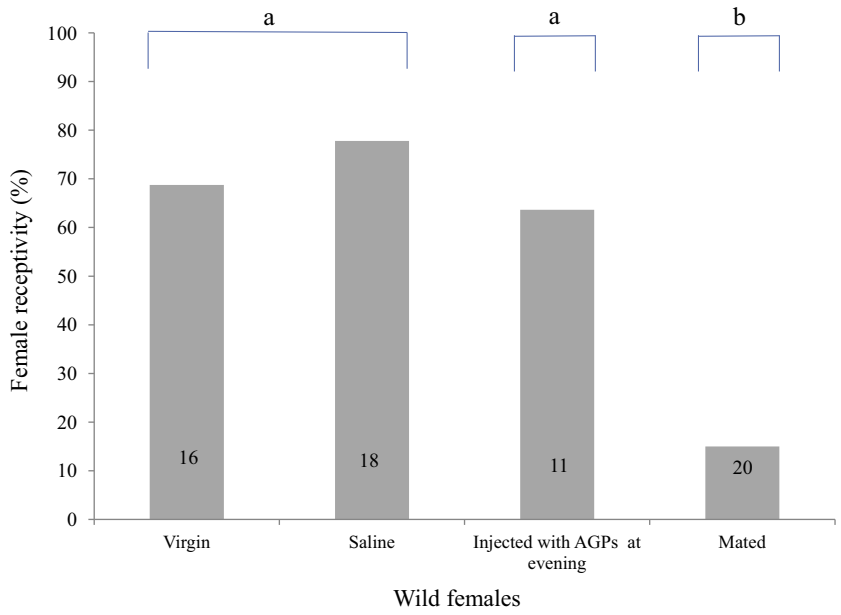

Fig. 4. Receptivity of *Anastrepha ludens* wild females of different categories: virgin and injected with saline as controls, injected with AGPs of wild males at evening (with accessory glands dissected at evening) and previously mated with wild males. Different letters over bars indicate significant difference ($P = 0.005$) after the sequential Bonferroni method (Rice, 1989) following χ^2-tests.

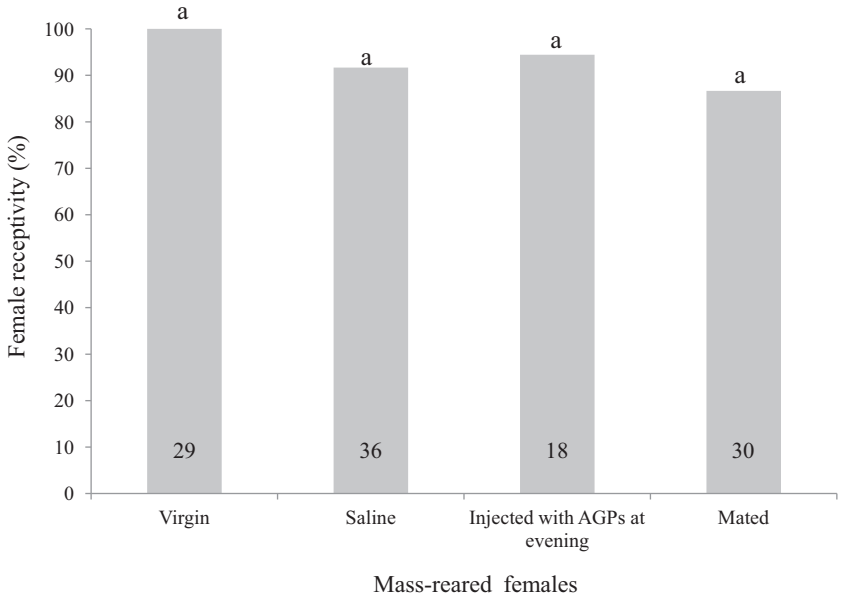

Fig. 5. Receptivity of *Anastrepha ludens* mass-reared females of different categories: virgin and injected with saline as controls, injected with AGPs of mass-reared males at evening (with accessory glands dissected at evening) and previously mated with mass-reared males. Different letters over bars indicate significant difference ($P < 0.001$) after the sequential Bonferroni method (Rice, 1989) following χ^2-tests.

Although there were no differences in mass-rearing procedures between these batches, there are other factors during handling or transportation that we cannot control.

4.1. Mating latency and copulation duration

Mating latency and copulation duration were not affected by injections of AGPs, and when there was an effect, injected females (including saline injected) behaved more like control virgin females than like mated ones. Overall, our results showed that the injection of AGPs into virgin females do not affect mating latency, copulation duration and sexual refractoriness in *A. ludens* females.

4.2. Sexual receptivity

An important and positive point for the SIT was that irradiation or mass-rearing did not seem to affect a male's ability to inhibit female remating, as there were no differences in the remating probability of wild females mating either with a mass-reared or wild male (Fig. 1). Similarly, in this same species, male origin (wild or mass-reared) had no effect on wild female remating (Meza et al., 2014). Three important questions arise from our findings. First, if AGPs are not involved in the inhibition of female sexual receptivity, what are the mechanisms used by males to induce refractoriness in this species? Second, why are there such stark differences in sexual inhibition between *A. ludens* and other species of the same family

(*C. capitata*, *B. tryoni*) and even the same genus (*A. fraterculus*)? And third, what is the function of AGPs in *A. ludens*?

Males can use a variety of mechanisms to inhibit female post-mating receptivity and delay the renewal of receptivity. Both the act of copulation *per se* as well as different components of the ejaculate can be used by males for this purpose. The amount of sperm stored by females play an important role inhibiting female receptivity in the moth *Plodia interpunctella* (Lewis et al., 2013), while in the melon fruit fly *Bactrocera cucurbitae* and *B. tryoni* sperm numbers play no role and AGPs are responsible for female inhibition (Kuba and Itô, 1993; Harmer et al., 2006; Radhakrishnan and Taylor, 2007). In many cases more than one mechanism may be involved, combined together through a synergetic effect. For example, both in the tsetse, *Glossina morsitans*, and the Hessian fly, *Mayetiola destructor*, physical stimuli from the act of copulation along with AGPs reduce female receptivity (Gillott and Langley, 1981; Bergh et al., 1992). In the moth *Lymantria dispar* the introduction of male genitalia into the bursa copulatrix produce a transient suppression of female sexual pheromone production. However, permanent suppression of pheromone production resulted from an adequate supply of sperm in the spermatheca (Giebultowicz et al., 1991). Mating inhibition may occur not only on a combination of factors but also on the succession of events, with copulation duration, sperm transfer and AGPs acting in succession to reduce female sexual receptivity (Mossinson and Yuval, 2003). In *A. fraterculus*, AGP injections inhibit female receptivity but this effect does not have the same magnitude as that produced by copulation (Abraham et al., 2011). This shows that other mechanisms, such as physical stimulation during copulation and/or the effect of the ejaculate in the spermathecae are also involved in mating inhibition (Abraham et al., 2012). As the ejaculate is a complex substance (e.g., see Perry et al., 2013), more research is needed to clarify the mechanisms behind female remating inhibition in *A. ludens*.

As in our study of *A. ludens*, AGP injections in *Anastrepha suspensa* had no effect on female remating inhibition or oviposition (Lentz et al., 2009). However, a high dose of 1 male equivalent used in *A. suspensa* may have obscured effects. Likewise, in *B. tryoni* a high dose of 1 or 2 male equivalents do not reduce female receptivity, while a smaller dose of 0.2 male equivalents, as that used here, does inhibit female remating (Radhakrishnan and Taylor, 2007). *A. suspensa*, *A. ludens* and *A. fraterculus* have a lek mating system where females are very choosy, they have similar sperm storage patterns between the spermathecae and ventral receptacle (Fritz, 2004; Pérez-Staples et al., 2014), and have infrequent remating. Currently, it is unclear why there is an effect of AGPs in *C. capitata*, *A. fraterculus*, *B. tryoni* and not in *A. ludens*. Females could be more monandrous due to effective AGPs compared to polyandrous females (Pitnick et al., 2001; Perry et al., 2013), or males of species where females are more monandrous may not need to produce complex substances or peptides and instead rely on other physical stimuli to manipulate female receptivity (Lentz et al., 2009). Clearly, the function of AGPs in modulating female receptivity needs further investigation within Tephritidae. Beyond inhibiting female receptivity, AGPs may have other functions in *A. ludens* such as changing female olfactory-driven behaviors (Jang, 2002), increasing oviposition, female excretion or feeding (Avila et al., 2011; Agper-McGlaughon and Wolfner, 2013).

4.3. Final considerations

We cannot completely rule out other factors that might have influenced the lack of effect of AGPs as inhibitors of sexual receptivity of females in *A. ludens*, such as: (i) the location of the injection, and (ii) the time when the AGPs can take effect: short-term (24 h) or long-term (beyond 48 h). For example, in *Anopheles gambiae*, AGPs initially were thought not to control female receptivity (Klowden, 2001). However, recently Shutt et al. (2010) demonstrated that AGPs injections induce refractoriness in *An. gambiae* and *Anopheles stephensi* and the authors hypothesized that this discrepancy could have resulted from the location of injection and subsequent access to AGPs receptors. However, this possibility is highly unlikely in our case because the injections were located in the same place and under the same methodology as in the study with *A. fraterculus* (Abraham et al., 2012). Although, it cannot be ruled out completely, as the locations of specific receptors of AGPs in Tephritidae fruit flies, as in *Aedes*, have not been identified yet (Helinski et al., 2012). In *Drosophila melanogaster*, sex peptide enters the hemolymph, and receptors for this protein have been found in the reproductive tract and central nervous system (Yapici et al., 2008; Häsemeyer et al., 2009; Yang et al., 2009). Finally, it is also possible that AGPs may have short-term effects (a few hours after injection), as in the moths *Helicoverpa zea* and *S. litura* (Kingan et al., 1993; Yu et al., 2013), or long-term effects on sexual inhibition, beyond 48 h, as in *Aedes aegypti*, *D. melanogaster* and *C. capitata* (Craig, 1967; Mossinson and Yuval, 2003; Chapman et al., 2003). This remains to be tested in *A. ludens*.

4.4. Conclusions

As in a previous study (Meza et al., 2014), wild females were overall less likely to remate compared to mass-reared females. Although we do not know the mechanism by which females are inhibited from mating, mass-reared females may have reduced expression or sequence changes in particular receptors that regulate their sexual receptivity. Indeed, it does not seem that mass-reared males have lost their ability to inhibit female remating, but rather that mass-reared females may be under strong selection to avoid male harassment and quickly remate, given the high densities that they are maintained in the mass rearing facilities. This opens interesting questions as to how fast remating inhibition can evolve or disappear from a population, as well as how sexual conflict has evolved between *A. ludens* males and females.

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References

Abraham, S., Goane, L., Cladera, J., Vera, M.T., 2011. Effects of male nutrition on sperm storage and remating behavior in wild and laboratory *Anastrepha fraterculus* (Diptera: Tephritidae) females. J. Insect Physiol. 57, 1501–1507.

Abraham, S., Cladera, J., Goane, L., Vera, M.T., 2012. Factors affecting *Anastrepha fraterculus* female receptivity modulation by accessory gland products. J. Insect Physiol. 58, 1–6.

Abraham, S., Liendo, M.C., Devescovi, F., Peralta, P.A., Yusef, V., Ruiz, J., Cladera, J., Vera, M.T., Segura, D.F., 2013. Remating behaviour in *Anastrepha fraterculus* (Diptera: Tephritidae) females is affected by male juvenile hormone analogue treatment but not by male sterilization. Bull. Entomol. Res. 103, 310–317.

Agper-McGlaughon, J., Wolfner, M.F., 2013. Post-mating change in excretion by mated *Drosophila melanogaster* females is a long-term response that depends on sex peptide and sperm. J. Insect Physiol. 59, 1024–1030.

Aluja, M., Celedonio-Hurtado, H., Liedo, P., Cabrera, M., Castillo, F., Guillen, L., Rios, E., 1996. Seasonal population fluctuations and ecological implications for management of *Anastrepha* fruit flies (Diptera: Tephritidae) in commercial mango orchards in southern Mexico. J. Econ. Entomol. 89, 654–667.

Aluja, M., Piñeiro, J., Jácome, I., Díaz-Fleischer, F., Sivinski, J., 2000. Behavior of flies in the genus Anastrepha (Trypetinae: Toxotripanini). In: Aluja, M., Norrbom, A.L.

(Eds.), Fruit Flies (Tephritidae): Phylogeny and Evolution of Behavior. CRC Press, Washington, DC, USA, pp. 375–408.
Aluja, M., Jácome, I., Macías-Ordóñez, R., 2001. Effects of adult nutrition on male sexual performance in four neotropical fruit fly species of the genus *Anastrepha* (Diptera: Tephritidae). J. Insect Behav. 14, 759–775.
Aluja, M., Pérez-Staples, D., Sivinski, J., Sánchez, A., Piñero, J., 2008. Effects of male condition on fitness in two tropical tephritid flies with contrasting life histories. Anim. Behav. 76, 1997–2009.
Aluja, M., Rull, J., Sivinsky, J., Trujillo, G., Pérez-Staples, D., 2009. Male and female condition influence mating performance and sexual receptivity in two tropical fruit flies (Diptera: Tephritidae) with contrasting life histories. J. Insect Physiol. 55, 1091–1098.
Avila, F.W., Sirot, L.K., LaFlamme, B.A., Rubinstein, C.D., Wolfner, M.F., 2011. Insect seminal fluid proteins: identification and function. Annu. Rev. Entomol. 56, 21–40.
Bergh, J.C., Harris, M.O., Rose, S., 1992. Factors inducing mated behavior in female Hessian flies (Diptera: Cecidomyiidae). Ann. Entomol. Soc. Am. 85, 224–233.
Birke, A., Aluja, M., Grany, P., Bigurra, E., Pérez-Staples, D., McDonald, R., 2006. Long aculeus and behavior of *Anastrepha ludens* render gibberellic acid ineffective as an agent to reduce 'Ruby Red' grapefruit susceptibility to the attack of this pestiferous fruit fly in commercial groves. J. Econ. Entomol. 99, 1184–1193.
Brent, C.S., Hull, J.J., 2014. Characterization of male-derived factors inhibiting female sexual receptivity in *Lygus hesperus*. J. Insect Physiol. 60, 104–110.
Calcagno, G.E., Manso, F., Vilardi, J.C., 2002. Comparison of mating performance of medfly (Diptera: Tephritidae) genetic sexing and wild type strains: field cage and video recording experiments. Fla. Entomol. 85, 41–50.
Cayol, J.P., 2000. Changes in sexual behavior and life history traits of tephritid species caused by mass-rearing processes. In: Aluja, M., Norrbom, A.L. (Eds.), Fruit Flies: Phylogeny and Evolution of Behavior. CRC, Boca Raton, FL, pp. 843–860.
Chapman, T., Bangham, J., Vinti, G., Seifried, B., Lung, O., Wolfner, M.F., Smith, H.K., Partridge, L., 2003. The sex peptide of *Drosophila melanogaster*: female post-mating responses analyzed by using RNA interference. Proc. Natl. Acad. Sci. USA 100, 9923–9928.
Chen, P.S., Stumm-Zollinger, E., Aigaki, T., Balmer, T., Bienz, M., Böhlen, P., 1988. A male accessory gland peptide that regulates reproductive behavior of female *D. melanogaster*. Cell 54, 291–298.
Craig Jr., G.B., 1967. Mosquitoes: female monogamy induced by a male accessory gland substance. Science 156, 1499–1501.
Fernández, N.M., Klowden, M.J., 1995. Male accessory gland substances modify the host-seeking behavior of gravid *Aedes aegypti* mosquitoes. J. Insect Physiol. 41, 965–970.
Fritz, A.H., 2004. Sperm storage patterns in singly mated female of the Caribbean fruit fly, *Anastrepha suspensa* (Diptera: Tephritidae). Ann. Entomol. Soc. Am. 97, 1328–1335.
Gavriel, S., Gazit, Y., Yuval, B., 2009. Remating by female Mediterranean fruit flies (*Ceratitis capitata*, Diptera: Tephritidae): temporal patterns and modulation by male condition. J. Insect Physiol. 55, 637–642.
Giebultowicz, J.M., Raina, A.K., Uebel, E.C., Ridgway, R.L., 1991. Two-step regulation of sex-pheromone decline in mated gypsy moth females. Arch. Insect Biochem. Physiol. 16, 95–105.
Gillott, C., 2003. Male accessory gland secretions: modulators of female reproductive physiology and behavior. Annu. Rev. Entomol. 48, 163–184.
Gillott, C., Langley, P.A., 1981. The control of receptivity and ovulation in the tsetse-fly, *Glossina morsitans*. Physiol. Entomol. 6, 269–281.
Harmer, A.M.T., Radhakrishnan, P., Taylor, P.W., 2006. Remating inhibition in female Queensland fruit flies: effects and correlates of sperm storage. J. Insect Physiol. 52, 179–186.
Häsemeyer, M., Yapici, N., Heberlein, U., Dickson, B.J., 2009. Sensory neurons in the *Drosophila* genital tract regulate female reproductive behavior. Neuron 61, 511–518.
Helinski, M.E.H., Deewatthanawong, P., Sirot, L.K., Wolfner, M.F., Harrington, L.C., 2012. Duration and dose-dependency of female sexual receptivity responses to seminal fluid proteins in *Aedes albopictus* and *Ae. aegypti* mosquitoes. J. Insect Physiol. 58, 1307–1313.
Hendrichs, J., Robinson, A.S., Cayol, J.P., Enkerlin, W., 2002. Medfly areawide sterile insect technique programs for prevention, suppression or eradication: the importance of mating behavior studies. Fla. Entomol. 85, 1–13.
Himuro, C., Fujisaki, K., 2008. Males of the seed bug *Togo hemipterus* (Heteroptera: Lygaeidae) use accessory gland substances to inhibit remating by females. J. Insect Physiol. 54, 1538–1542.
Jang, E.B., 1995. Effects of mating and accessory gland injections on olfactory-mediated behavior in the female Mediterranean fruit fly, *Ceratitis capitata*. J. Insect Physiol. 41, 705–710.
Jang, E.B., 2002. Physiology of mating behavior in Mediterranean fruit fly (Diptera: Tephritidae): chemoreception and male accessory gland fluids in female post-mating behavior. Fla. Entomol. 85, 89–93.
Jang, E.B., McInnis, D.O., Kurashima, R., Carvalho, L.A., 1999. Behavioural switch of female Mediterranean fruit fly, *Ceratitis capitata*: mating and oviposition activity in outdoor field cages in Hawaii. Agric. For. Entomol. 1, 179–184.
Kingan, T.G., Thomas-Laemont, P.A., Raina, A.K., 1993. Male accessory glands factors elicit change from "virgin" to "mated" behaviour in the female corn earworm moth *Helicoverpa zea*. J. Exp. Biol. 183, 61–76.
Klowden, M.J., 2001. Sexual receptivity in *Anopheles gambiae* mosquitoes: absence of control by male accessory gland substances. J. Insect Physiol. 47, 661–666.
Knipling, G.F., 1955. Possibilities of insect control of eradication through the use of sexually sterile males. J. Econ. Entomol. 48, 459–462.
Knipling, E.F., 1959. Sterile-male method of population control. Science 130, 902–904.
Kuba, H., Itô, I., 1993. Remating inhibition in the melon fly, *Bactrocera* (=*Dacus*) *cucurbitae* (Diptera: Tephritidae): copulation with spermless males inhibits female remating. J. Ethol. 11, 23–28.
Lentz, A.J., Miller, J.R., Spencer, J.L., Keller, J.E., 2009. Effect of male accessory gland extract on female oviposition and sexual receptivity of Caribbean fruit fly (Diptera: Tephritidae). Fla. Entomol. 42, 415–420.
Lewis, Z., Lizé, A., Wedell, N., 2013. The interplay between different stages of reproduction in males of the moth *Plodia interpunctella*. Anim. Behav. http://dx.doi.org/10.1016/j.anbehav.2013.08.006.
Liimatainen, J., Hoikkala, A., Shelly, T., 1997. Courtship behavior in *Ceratitis capitata* (Diptera: Tephritidae) comparison of wild and mass-reared males. Ann. Entomol. Soc. Am. 90, 836–843.
Lux, S.A., Munyiri, F.N., Vilardi, J.C., Liedo, P., Economopoulos, A.P., Hasson, O., Quilici, S., Gaggl, K., Cayol, J.P., Rendon, P., 2002a. Consistency in courtship pattern among populations of medfly *Ceratitis capitata*: comparison among wild strains and strains mass-reared for SIT operations. Fla. Entomol. 85, 113–125.
Lux, S.A., Vilardi, J.C., Liedo, P., Gaggl, K., Calagno, G.E., Munyiri, F.N., Vera, M.T., Manso, F., 2002b. Effects of irradiation on the courtship behavior of medfly mass reared for the sterile insect technique. Fla. Entomol. 85, 102–112.
Meza, J.S., Arredondo, J., Orozco, D., Pérez-Staples, D., 2014. Disparity in sexual behaviour between wild and mass-reared Mexican fruit flies. Physiol. Entomol. 39, 263–270.
Moreno, D.S., Sanchez, M., Robacker, D.C., Worley, J., 1991. Mating competitiveness of irradiated Mexican fruit fly (Diptera: Tephritidae). J. Econ. Entomol. 8, 1227–1234.
Mossinson, S., Yuval, B., 2003. Regulation of sexual receptivity of female Mediterranean fruit flies: old hypotheses revisited and new synthesis proposed. J. Insect Physiol. 49, 561–567.
Pérez-Staples, D., Córdova-García, G., Aluja, M., 2014. Sperm dynamics and cryptic male choice in tephritid flies. Anim. Behav. 89, 131–139.
Pérez-Staples, D., Martinez-Hernandez, M.G., Aluja, M., 2010. Male age and experience increases mating success but not female fitness in the Mexican fruit fly. Ethology 116, 778–786.
Perry, J.C., Sirot, L., Wigby, S., 2013. The seminal symphony: how to compose an ejaculate. Trends Ecol. Evol. 28, 1–9.
Pitnick, S., Brown, W.D., Miller, G.T., 2001. Evolution of female remating behavior following experimental removal of sexual selection. Proc. R. Soc. Lond. B 268, 557–563.
Poiani, A., 2006. Complexity of seminal fluids: a review. Behav. Ecol. Sociobiol. http://dx.doi.org/10.1007/s00265-006-0178-0.
Radhakrishnan, P., Taylor, P.W., 2007. Seminal fluids mediate sexual receptivity and copula duration in Queensland fruit flies. J. Insect Physiol. 53, 741–745.
Radhakrishnan, P., Pérez-Staples, D., Weldon, C.W., Taylor, P.W., 2009. Multiple mating and sperm depletion in male Queensland fruit flies: effects on female remating behaviour. Anim. Behav. 78, 839–846.
Reyes, J., Santiago, G., Hernández, P., 2000. The Mexican fruit fly eradication programme. In: Tan, K.H. (Ed.), Area-Wide Control of Fruit Flies and Other Insect Pests. Penerbit Universiti Sains Malaysia, Penang, Malaysia, pp. 377–380.
Rice, W.R., 1989. Analyzing tables of statistical tests. Evolution 43, 223–225.
Rull, J., Brunel, O., Mendez, M.E., 2005. Mass rearing history negatively affects mating success of male *Anastrepha ludens* (Diptera: Tephritidae) reared for the sterile insect technique. J. Econ. Entomol. 98, 1510–1516.
Shutt, B., Stables, L., Aboagye-Antwi, F., Moran, J., Tripet, F., 2010. Male accessory gland proteins induce female monogamy in anopheline mosquitoes. Med. Vet. Entomol. 24, 91–94.
Taylor, P.W., Kaspi, R., Mossinson, S., Yuval, B., 2001. Age-dependent insemination success of sterile Mediterranean fruit flies. Entomol. Exp. Appl. 98, 27–33.
Thomas, D.B., 2003. Reproductive phenology of the Mexican Fruit Fly, *Anastrepha ludens* (Loew) (Diptera: Tephritidae) in the Sierra Madre Oriental, Northern Mexico. Neotropical Entomol. 32, 385–397.
Tripet, F., Lounibos, L.P., Robbins, D., Moran, J., Nishimura, N., Blosser, E.M., 2011. Competitive reduction by satyrization? Evidence for interspecific mating in nature and asymmetric reproductive competition between invasive mosquito vectors. Am. J. Trop. Med. Hyg. 85, 265–270.
Xu, J., Wang, Q., 2011. Seminal fluid reduces female longevity and stimulates egg production and sperm trigger oviposition in a moth. J. Insect Physiol. 57, 385–390.
Yamane, T., Miyatake, T., 2010. Induction of oviposition by injection of male-derived extracts in two *Callosobruchus* species. J. Insect Physiol. 56, 1783–1788.
Yamane, T., Miyatake, T., Kimura, Y., 2008a. Female mating receptivity after injection of male-derived extracts in *Callosobruchus maculatus*. J. Insect Physiol. 54, 1522–1527.
Yamane, T., Kimura, Y., Katsuhara, M., Miyatake, T., 2008b. Female mating receptivity inhibited by injection of male-derived extracts in *Callosobruchus chinensis*. J. Insect Physiol. 54, 501–507.
Yang, C.H., Rumpf, S., Xiang, Y., Gordon, M.D., Song, W., Jan, L.Y., Jan, Y.N., 2009. Control of the postmating behavioral switch in *Drosophila* females by internal sensory neurons. Neuron 61, 519–526.
Yapici, N., Kim, Y.J., Ribeiro, C., Dickson, B.J., 2008. A receptor that mediates the post-mating switch in *Drosophila* reproductive behaviour. Nature 451, 33–37.
Yu, J.F., Li, C., Xu, J., Liu, J.H., Ye, H., 2013. Male accessory gland secretions modulate female post-mating behavior in the moth *Spodoptera litura*. J. Insect Behav. http://dx.doi.org/10.1007/s10905-013-9414-4.