Journal of Insect Physiology 88 (2016) 40–47

Contents lists available at ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

The male ejaculate as inhibitor of female remating in two tephritid flies

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ARTICLE INFO

Article history:
Received 14 December 2015
Received in revised form 28 February 2016
Accepted 2 March 2016
Available online 3 March 2016

Keywords:
Female remating
Sperm effect
Sperm depletion
Multiple mating
Accessory glands
Diptera

ABSTRACT

The inhibition of female receptivity after copulation is usually related to the quality of the first mating. Males are able to modulate female receptivity through various mechanisms. Among these is the transfer of the ejaculate composed mainly by sperm and accessory gland proteins (AGPs). Here we used the South American fruit fly *Anastrepha fraterculus* (where AGP injections inhibit female receptivity) and the Mexican fruit fly *Anastrepha ludens* (where injection of AGPs failed to inhibit receptivity) as study organisms to test which mechanisms are used by males to prevent remating. In both species, neither the act of copulation without ejaculate transfer nor sperm stored inhibited female receptivity. Moreover, using multiply mated sterile and wild males in Mex flies we showed that the number of sperm stored by females varied according to male fertility status and number of previous matings, while female remating did not. We suggest female receptivity in both flies is inhibited by the mechanical and/or physiological effect of the full ejaculate. This finding brings us closer to understanding the mechanisms through which female receptivity can be modulated.

1. Introduction

After mating, females experience a series of physiological and behavioral changes that characterize and distinguish a mated female from a virgin female (Avila et al., 2011). The most common changes across taxa are: an increase in oviposition (Yamane and Miyatake, 2010; Yu et al., 2013), food intake (Carvalho et al., 2006), production of concentrated excreta (Agper-McGlaughon and Wolfner, 2013), and a "switch off" of sexual receptivity (Jang, 1995; Radhakrishnan and Taylor, 2007; Yamane et al., 2008a,b; Shutt et al., 2010; Tripet et al., 2011; Abraham et al., 2012; Yu et al., 2013; Brent and Hull, 2014). The inhibition of female receptivity seems to be mediated by a series of mechanisms used by males such as mating plugs (Wedell, 2005; Bretman et al., 2010), mate guarding (Carroll, 1991; Alcock, 1994), the stimulus of copulation *per se* (Giebultowicz et al., 1991) and the mechanical and physiological effect of a transferred ejaculate of adequate quality and quantity (Gillott, 2003; Wedell, 2005). The two main ejaculate components studied in insects are sperm and accessory gland proteins (AGPs). However, the mechanisms used by males to inhibit female remating and delay the renewal of female receptivity vary across species.

In insects where there is no mate guarding or mating plugs as such, the inhibition of female remating has been mostly attributed to AGPs. However, this is not always the case (Klowden, 2001; Lentz et al., 2009; Abraham et al., 2014). In *Drosophila*, males that only transfer AGPs reduce receptivity for a short-term, while sperm (sperm-effect) are needed for a long-term inhibition of female receptivity (Liu and Kubli, 2003). The degree to which AGPs, sperm and the physiological or mechanical effect of the full ejaculate can affect female receptivity remains to be seen. Detangling the importance of these components will aid our understanding of how males can manipulate female post-mating behavior and deepen our understanding of sexual conflict over female remating.

In tephritid fruit flies, there are contrasting results on the importance of sperm stored on the renewal of female receptivity. For example, in the Mediterranean fruit fly (medfly), *Ceratitis capitata*, Miyatake et al. (1999), determined that almost 77% of females remated when first mated to a male that could not transfer an ejaculate (aedeagus-cut males), thus showing that the transfer of the

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http://dx.doi.org/10.1016/j.jinsphys.2016.03.001
0022-1910/

ejaculate is needed to inhibit female receptivity. In another study, females willing to remate had less sperm stored than females that were not willing to remate, thus showing that sperm by themselves play a role in female sexual inhibition (Mossinson and Yuval, 2003). Furthermore, injections of AGPs directly into the hemocoel also decreased female receptivity (Jang et al., 1999). Thus, in the medfly the full ejaculate and its components (sperm and AGPs by themselves) do have a role in decreasing female receptivity. On the contrary, in *Bactrocera* AGPs are responsible for female receptivity inhibition and not the number of sperm stored by females. In the melon fly *Bactrocera cucurbitae* and the Queensland fruit fly (Q-fly) *Bactrocera tryoni* sperm-depleted sterile males were as efficient as fertile males in inhibiting female remating (Kuba and Itô, 1993; Radhakrishnan et al., 2009). Moreover, in the Q-fly the direct injection of AGPs into the thorax reduced female sexual receptivity (Radhakrishnan and Taylor, 2007). In the sapote fruit fly *Anastrepha serpentina*, a mostly monandrous species, sperm numbers were not correlated with female likelihood to remate, suggesting a role of male AGPs as modulators of female receptivity (Landeta-Escamilla et al., 2016). In contrast, in the Caribbean fruit fly *Anastrepha suspensa* the injection of a high dose of AGPs into the females failed to inhibit female receptivity (Lentz et al., 2009). Likewise, in *Anastrepha ludens*, the Mexican fruit fly (Mex fly), the injections of aqueous homogenates of AGPs do not inhibit female receptivity two days after injection, while in the South American fruit fly (SA fly) *Anastrepha fraterculus* they do inhibit female remating (Abraham et al., 2012, 2014).

The Mexican and South American fruit fly (Diptera: Tephritidae) are well-studied organisms regarding female remating behavior (Mex fly: Aluja et al., 2009; Meza et al., 2014; Abraham et al., 2014; SA fly: De Lima et al., 1994; Abraham et al., 2011a,b, 2012, 2013, 2014). Both species exhibit important differences in mating and post-mating behavior. For example, Mex flies mate at dusk (Aluja et al., 2000), female remating varies with strain: 10–20% of wild females remate while 20–80% of mass-reared females remate (Aluja et al., 2009; Meza et al., 2014; Abraham et al., 2014). The injections of aqueous homogenates of AGPs do not inhibit female receptivity two days after injection, neither in wild nor mass-reared females (Abraham et al., 2014). By contrast, SA flies mate at dawn (De Lima et al., 1994). Female remating probability is lower than in *A. ludens*, 5 to 15% of wild and laboratory females remate (Abraham et al., 2011b). When females show willingness to remate and are prevented from doing so, fertility showed a significant drop, suggesting that remating may be a response to sperm depletion (Abraham et al., 2011a), and the injections of AGPs reduced female receptivity two days after injection, both in wild and laboratory flies (Abraham et al., 2012).

Here we carried out three sets of experiments to separate the effects of sperm or ejaculate transfer on female remating inhibition.

(1) To determine if the full ejaculate inhibits female remating, we mated Mex and SA females to males that could not transfer an ejaculate (aedeagus-cut males). If the transferred ejaculate plays a role in inhibiting female receptivity, we expected more females to remate when mated with aedeagus-cut males, compared to females mated with control intact males.
(2) To elucidate the role of the number of sperm transferred and stored during copulation in the renewal of female receptivity, we counted sperm stored in remating and non-remating females for both species. If the number of sperm transferred plays a role in inhibiting female receptivity, we expected that remating females would have less sperm stored in their storage organs, compared with females that did not show a willingness to remate.
(3) To further elucidate the role of sperm in female remating in Mex flies, we registered the number of sperm stored and the remating behavior of females mated with virgin or previously mated males using sterile and fertile males. If sterile and fertile males transfer different numbers of sperm over successive copulations, and if sperm play a role in inhibiting female receptivity, we expected higher female remating when sperm storage was lower.

Overall, as there is apparently no effect of AGPs in Mex flies, we expected to find a strong effect of sperm storage in the probability of Mex fly female remating, and no effect of sperm stored in the SA fly, where AGPs do inhibit female receptivity.

2. Methods

2.1. Insects

Mass-reared Mex fly 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. Mex wild flies were recovered from infested oranges collected at Tuzamapam, Veracruz, Mexico. 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 sand was sieved and recovered pupae were placed in 27 L cages at 26 ± 2 °C and 80 ± 10 RH until adult emergence. On the day of emergence, flies were sorted by sex and were transferred to 27 L cages in groups of approximately 100 adults, with water and food provided *ad libitum*. Flies were fed with adult diet consisting of sugar and hydrolyzed yeast (Yeast Hydrolyzed Enzymatic, MP Biomedicals®) in a 3:1 ratio. Wild Mex flies were tested 31–35 days after adult emergence and mass-reared flies (fertile and sterile) at 15–25 days after adult emergence. Mex fly experiments were carried out at the Instituto de Biotecnología y Ecología Aplicada (INBIOTECA), Universidad Veracruzana, Xalapa, Veracruz, Mexico.

SA adults were obtained from a laboratory colony established at the LIEMEN-PROIMI, Tucumán, Argentina. This colony was initiated in 2006 with pupae obtained from the semi-massive colony in Estación Experimental Agroindustrial Obispo Colombres, founded with infested guavas collected in the vicinity of Tafí Viejo, Tucumán province, north-western Argentina. Rearing followed methods described by Jaldo et al. (2001) and Vera et al. (2007). On the day of emergence, flies were sorted by sex and were transferred to 1 L plastic containers in groups of 25 adults, with water and food provided *ad libitum*. Flies were fed with adult diet consisting of sugar (57.9%) (Ledesma S.A., Jujuy, Argentina), hydrolyzed yeast (14.5%) (Yeast Hydrolyzed Enzymatic, MP Biomedicals®), hydrolyzed corn (27.3%) (Gluten Meal, ARCOR®, Tucumán, Argentina), and vitamin E (0.3%) (Parafarm®, Buenos Aires, Argentina) (w/w) (Jaldo et al., 2001). Laboratory SA flies were tested at 20–27 days of age. These experiments were carried out at the laboratories of LIEMEN-PROIMI, Tucumán, Argentina.

2.2. Experimental procedures

2.2.1. Remating of females mated with aedeagus-cut or control males

Following Miyatake et al. (1999) we mated females to males whose tip of the aedeagus was cut ($N = 50$). These adeagus-cut males could copulate with females but could not transfer an ejaculate at mating. Aedeagus-cut males could court, mount females and intromit their aedeagus. As a control we mated females to intact males ($N = 50$). In a pilot study we determined that for SA flies 19 out of 20 females mated with aedeagus-cut males did

not store any sperm in their four storage organs (three spermathecae and a ventral receptacle), only one female had one sperm; while 9 out of 20 females mated with control males stored sperm. In the Mex fly, none of the 15 females mated with aedeagus-cut males stored sperm in their four storage organs, while 19 out of 21 females mated with control males stored sperm.

For the aedeagus-cut group, males were cooled on ice and placed under a dissecting stereomicroscope (Olympus SZX7). Their long aedeagus was uncoiled and the tip was cut with sharp forceps. Control males were similarly anaesthetized, their aedeago was uncoiled but the tip was not cut. This procedure was done in the morning of the day the males were mated to the experimental females (Mex fly) or in the morning of the day before copulation (SA fly). For Mex fly we used mass-reared and wild flies and for the SA fly, laboratory flies.

Remating frequencies of Mex fly females first mated to aedeagus-cut or intact control males were compared. Each virgin female was placed with a virgin aedeagus-cut or control male in a 250 ml plastic containers at 16:00 h. Matings were observed continuously for four hours before lights-off. The time at which the copulation was detected was recorded. Pairs were checked every five min until copulation finished and this time was recorded. After mating, males were removed and females were kept singly in 250 ml plastic containers, with diet and water *ad libitum*. To verify willingness of the females to remate, 48 h later, one standard, intact male of the same female origin [that is, mass-reared males × mass-reared females (Mex fly); wild males × wild females (Mex fly); laboratory males × laboratory females (SA fly)] was placed with the female at 16:00 h. Remating was checked continuously for four hours before lights-off.

The same methodology was used for the SA fly except that flies were observed at 7:30 h in the morning continuously for two hours.

2.3. Effect of sperm on renewal of female receptivity

2.3.1. Mex fly

On the day of testing, approximately 100 virgin wild males and 100 virgin wild females were released into a 27 L cage at 17:00 h. The cage was checked for copulating pairs at 5-min intervals for 3 h after releasing the flies. Copulating pairs were carefully coaxed into test tubes (20 mL), which were then plugged and numbered. Pairs were checked every five min until copulation finished. Copulation start and end time were recorded. After the end of copulation, females were kept singly in 250 mL plastic containers with water and food (adult diet described above).

On the day following copulation, females were marked and placed in 27 L cage with water and food. The mark consisted of a small piece of paper glued with a dot of white glue on their notothorax (Resistol®). A letter (Microsoft Word Arial, size 4) printed on the paper was used to individually recognize all the females. This technique does not affect the sexual behavior of tephritid flies (McInnis et al., 2002; Vera et al., 2003; Meza et al., 2005; Segura et al., 2007).

Two days after copulation, females were offered virgin wild males in a 1:1 ratio at 17:00 h. Cages were checked for copulating pairs continuously for 3 h after releasing flies. Copulating pairs were carefully coaxed into test tubes (20 mL) and sprayed with ethyl chloride (Traumazol®) immediately before the second mate could transfer any sperm. In a pilot study we determined that females did not store any sperm in their four storage organs when the copulating pairs were sprayed with ethyl chloride and separated within the first minute of copulation ($N = 21$ for Mex fly; $N = 13$ for SA fly).

Females were separated from the males and dissected. Females that had not accepted a partner for copulation after 3 h were also removed and sprayed with ethyl chloride. Females from both groups were dissected and the number of sperm in their spermathecae counted. This experiment was repeated two times.

2.3.2. SA fly

A similar methodology to Mex fly was used except that 50 pairs of virgin males and females were released in 250 mL plastic containers at 7:30 h (one pair per container). After the end of copulation, females were maintained singly in 250 mL plastic containers with water and food (adult diet described above) and two days after copulation, two virgin males per females were offered.

2.3.3. Sperm counts

Females were dissected under a dissecting microscope (Leica S8AP0 for the Mex fly and Arcano ZTX for the SA fly) using a 60× magnification, following Taylor et al. (2000) and Twig and Yuval (2005). Reproductive tracts were removed and placed over a slide with a 50 µl drop of saline solution (NaCl 0.9%, PISA®). Spermathecae were dissected and placed together on slides with 10 µl of saline solution containing 0.1% of soap (Triton®). Spermathecae were broken with fine forceps and the drop was stirred quickly with entomological pins for one minute. A 18 × 18 mm coverslip was then placed on top of the storage organs and secured with transparent nail polish. Sperm for the ventral receptacle was not quantified as this is not an important sperm storage organ in the SA fly (Abraham et al., 2011b); while for the Mex fly, by the end of a copulation there is very little sperm left in the ventral receptacle as it has migrated to the three spermathecae (Pérez-Staples et al., 2014). Spermatozoids were counted under a phase contrast microscope (Leica CME) at 200× magnification. The whole coverslip was covered by counting all spermatozoids in 50 randomly selected fields, which corresponds to 12.11% of the total area. To obtain the total number of sperm stored, a conversion factor of 8.25 was applied to the sperm counted (Pérez-Staples and Aluja, 2006). When no sperm was counted in 50 fields, a coverslip screening was carried out to ensure that there was no sperm in the storage organs of the female.

2.4. Sperm and sexual inhibition of females mated with multiply mated males in Mex flies

General procedures followed Kuba and Itô (1993) and Radhakrishnan et al. (2009). We used sequentially mated sterile and fertile males and assessed sperm stored and remating probability of mated females according to male mating history. The basis of this experiment was to obtain females storing different numbers of sperm, according to male fertility status (fertile or sterile) and/or number of previous matings (virgin, once or twice mated), and correlate it with female remating probability. If sperm quantity determines remating behavior, then there should be higher remating when sperm storage is lower and *vice versa*.

Since mass-reared fertile females are not consistently inhibited from remating, we used wild females as ~50% of females do not remate (Meza et al., 2014). Wild males were also used as the fertile standard. Thus, virgin females mated with either a virgin (Fw_0,) once mated (Fw_1), or twice mated wild male (Fw_2); or with either a virgin (Fs_0), once mated (Fs_1), or twice mated sterile male (Fs_2).

Day 1. Wild virgin males were paired with wild virgin females ($N = 70$; Fw_0) and sterile virgin males were paired with wild virgin females ($N = 70$; or Fs_0) in 250 ml plastic containers. At 16:00 h flies were placed together and copulation duration and the number of copulations were registered. At the end of copulations, all mated males were kept singly with water and food. A subset of mated females was dissected for sperm count with the methodology described above. The other subset of females was maintained in

individual plastic cages with water and food for the remating test two days later (Day 3).

Day 2. Wild males mated in Day 1 were paired with wild virgin females (Fw_1) and sterile males mated in Day 1 were paired with wild virgin females (Fs_1) in 250 ml plastic containers. At 16:00 h flies were placed together and copulation duration and the number of copulations were registered. At the end of copulations, all mated males were kept singly with water and food. A subset of mated females was dissected for sperm count with the methodology described above. The other subset of females was maintained in individual plastic cages with water and food for the remating test two days later (Day 4).

Day 3. Wild males mated on Day 1 and Day 2 were paired with wild virgin females (Fw_2) and sterile males mated in Day 1 and Day 2 were paired with wild virgin females (Fs_2) in 250 ml plastic containers. At 16:00 h flies were placed together and copulation duration and the number of copulations were registered. At the end of copulations, all mated males were discarded. A subset of mated females was dissected for sperm count with the methodology described above. The other subset of females was maintained in individual plastic cages with water and food for the remating test two days later (Day 5).

Females mated on Day 1 (Fw_0, Fs_0) with virgin wild or sterile males were paired with wild virgin males in 250 ml plastic containers. At 16:00 h flies were placed together and the number of rematings was registered.

Day 4. Females mated on Day 2 with previously (one time) mated wild or sterile males (Fw_1, Fs_1) were paired with wild virgin males in 250 ml plastic containers. At 16:00 h flies were placed together and the number of rematings was registered.

Day 5. Females mated on Day 3 with previously (two times) mated wild or sterile males (Fw_2, Fs_2) were paired with wild virgin males in 250 ml plastic containers. At 16:00 h flies were placed together and the number of rematings was registered.

2.5. Statistical analysis

2.5.1. Remating of females mated with aedeagus-cut or control males

For each species, a *t*-test of independent samples was used to compare copulation duration between females mated with aedeagus-cut males versus intact control males. A Wilcoxon test for independent samples was used when assumptions were not achieved. A χ^2-test was used to compare the differences in remating frequency two days after mating between females mated with aedeagus-cut males versus intact control males.

2.5.2. Effect of sperm on renewal of female receptivity

For each species, the copulation duration and the number of sperm stored in the three spermathecae of the females, was compared between females that remated and those that did not with a *t*-test of independent samples. The relationship between number of sperm stored and copulation duration was determined with a Pearson's correlation analysis. The proportion of females with empty spermathecae was tested with a χ^2 of Homogeneity.

2.5.3. Sperm and sexual inhibition in females mated with multiply mated males in Mex flies

Differences in mating latency (time from the male and female encounter until mating) and copulation duration across the sequential matings of individual males were tested using a mixed model analysis of variance using male fertility (i.e., wild fertile or sterile), number of previous matings (0, 1 or 2) and their interaction as fixed factors and individual identity as random factor. For copulation duration two outliers were deleted from the analysis. For mating latency all data were used.

A χ^2-test was used to compare male mating probability across the three days of mating (mated = 1; unmated = 0) between wild fertile or sterile males. We used a Kruskal–Wallis analysis to compare the number of sperm stored by mates of wild fertile and sterile males in sequential matings. Only those pairing that resulted in sperm storage were considering.

A logistic regression was used to analyze the ability of males to inhibit female remating across sequential matings (female remated = 1; females did not remate = 0). Fertility, number of previous matings and their interaction were used as predicted variables. Analyses were carried out in JMP ver. 9 (SAS Institute Inc., Cary, NC, U.S.A.).

3. Results

3.1. Remating of females mated with aedeagus-cut or control males

3.1.1. Mex fly

In mass-reared flies, no difference was found in copulation duration between females mated with aedeagus-cut (78 ± 8 min, $N = 31$) or control intact males (96 ± 12 min, $N = 31$) (*t*-test, $t = 1.24$, $df = 60$, $P = 0.221$). In wild flies, copulation duration in females mated with aedeagus-cut (45 ± 3 min, $N = 33$) were slightly shorter than copulations with control intact males (56 ± 3 min, $N = 33$) (*t*-test, $t = 2.27$, $df = 64$, $P = 0.026$).

Both wild and mass-reared females were more likely to remate when first mated with an aedeagus-cut male compared to a normal male (Fig. 1; Table 1). This was in line with the prediction that the transferred ejaculate does play a role in inhibiting female receptivity.

3.1.2. SA fly

No difference was found in copulation duration between females mated with aedeagus-cut [77–115–145 min (Q1-Median-Q3), $N = 28$] or control intact males [76–97–123 min (Q1-Median-Q3), $N = 37$] (Wilcoxon test, $W = 1056.5$, $df = 63$, $P = 0.078$). Laboratory females were more likely to remate when first mated with an aedeagus-cut male compared to a normal male (Fig. 1; Table 1), similar to that found in Mex fly.

3.2. Effect of sperm on renewal of female receptivity

3.2.1. Mex fly

Females choosing to remate had on average 327.9 (±105) ($N = 18$) sperm in their spermathecae compared to females who did not remate (412.7 ± 67, $N = 55$). There was no significant difference in the number of sperm in remating or non-remating females (*t*-test, $t = 0.64$, $df = 71$, $P = 0.521$) (Fig. 2). A sample size and power test revealed a 98% probability of detecting a significant difference at an alpha of 0.05. Also, 16.7% of the rematers and 16.4% of the females that did not remate had no sperm at all in their spermathecae ($\chi^2 = 0.01$, $df = 1$, $P = 0.976$). This shows that number of sperm stored by themselves do not determine female remating. There was no correlation between copulation duration and number of sperm stored (Pearson's, $r = 0.19$, $P = 0.150$). No difference was found in copulation duration between the first mating by females that remated (79.7 ± 15.2 min, $N = 18$) or did not remate (87.8 ± 7 min, $N = 43$) (*t*-test, $t = 0.55$, $df = 56$, $P = 0.585$).

3.2.2. SA fly

Females choosing to remate had 46.4 (±23) ($N = 17$) sperm in their spermathecae. Similarly, females who did not remate had 54.2 (±11) ($N = 67$) sperm in their storage organs (*t*-test, $t = 0.32$, $df = 82$, $P = 0.751$) (Fig. 2), thus sperm numbers *per se* did not determine female remating in this fly either. A sample size and power

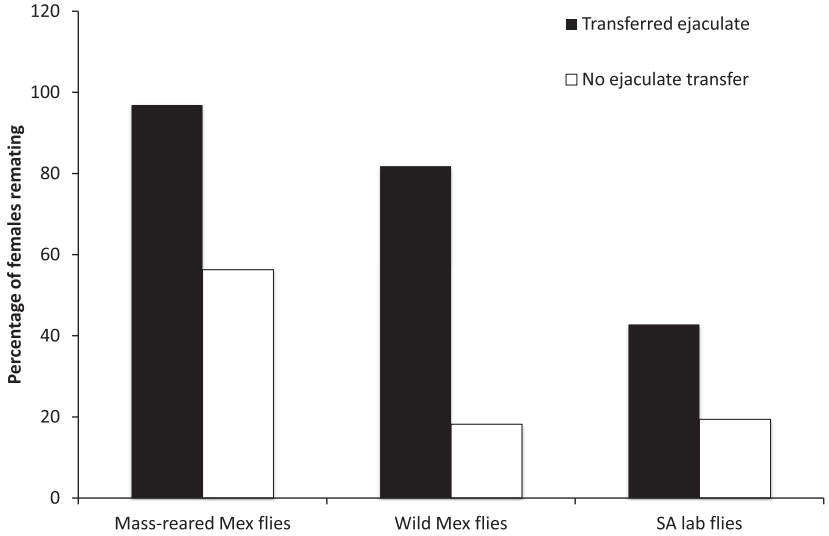

Fig. 1. Percentage of females remating after first mating with a control intact male (transferred an ejaculate) or an aedeagus-cut male (could not transfer an ejaculate) in mass-reared or wild *Anastrepha ludens* (Mex) flies or *Anastrepha fraterculus* (SA) flies.

Table 1
Receptivity of females (number of females remating with intact males) 2 days after mating with aedeagus-cut or intact control males, in *Anastrepha ludens* (Mex) and *Anastrepha fraterculus* (SA) flies.

	Mass-reared Mex fly flies		Wild Mex fly flies		Laboratory SA fly flies	
	Aedeagus-cut males	Intact control males	Aedeagus-cut males	Intact control males	Aedeagus-cut males	Intact control males
N	50	50	50	50	47	50
N° of copulations	32	32	33	33	28	36
Rematers	31	18	27	6	12	7
Non-rematers	1	14	4	27	16	29
χ^2 (*P* value)	14.72 (<0.0001)		32.18 (<0.0001)		4.13 (0.042)	

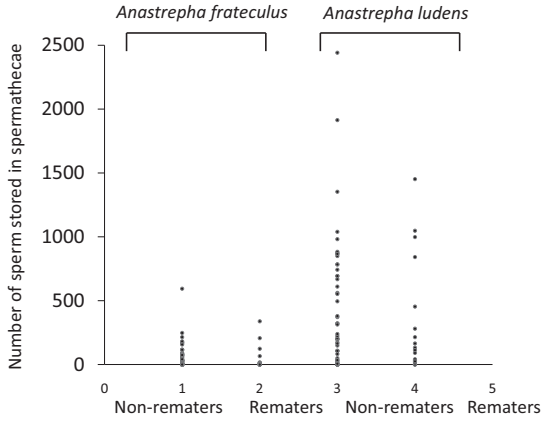

Fig. 2. Number of sperm stored in the three spermatecae of the females willing to remate (rematers) or not (non-rematers) 48 h after their first copulation, in *Anastrepha fraterculus* (SA fly) and *Anastrepha ludens* (Mex) flies.

test revealed a 98% probability of detecting a significant difference at an alpha of 0.05. However, here 43% of females choosing to remate had no sperm in their storage organs while only 23.8% of females that did not remate had no sperm ($\chi^2 = 3.57$, $df = 1$, $P = 0.058$). There was no correlation between copulation duration and number of sperm stored (Pearson's, $r = 0.02$, $P = 0.820$). The initial copulation duration of remating females was shorter (56.8 ± 8 min, $N = 17$) compared to females that did not remate (83 ± 5 min, $N = 67$) (*t*-test, $t = 2.4$, $df = 82$, $P = 0.018$).

3.3. Sperm and sexual inhibition of Mex flies females mated with multiply mated males

3.3.1. Mating probability, latency and copulation duration

There was no significant difference in mating probability between sterile and wild Mex fly fertile males ($\chi^2 = 1.31$; $df = 329, 1$; $P = 0.287$). Sterile flies had significantly shorter mating latencies than wild flies ($F_{1,13} = 13.37$; $P = 0.0028$). Number of previous matings had no effect on mating latency ($F_{1,245} = 1.43$;

Table 2
Number of *Anastrepha ludens* (Mex fly) mating pairs, mating latency (min) and copulation duration (min) of sterile and wild male first, second and third matings. Different letters indicate significant differences.

N° of day	Type of males	N° of pairs	Matings (%)	Mating latency	Copulation duration
Day 1 (virgin males)	Sterile	70	56 (80%)	73 ± 9 a	56 ± 5 a
	Wild	70	43 (61.4%)	164 ± 6 b	64 ± 5 a
Day 2 (once mated males)	Sterile	56	51 (91%)	95 ± 6 a	55 ± 4 ab
	Wild	43	38 (88.4%)	135 ± 3 b	62 ± 4 ab
Day 3 (twice mated males)	Sterile	50	33 (66%)	94 ± 10 a	48 ± 5 b
	Wild	38	29 (76.3%)	163 ± 6 b	53 ± 5 b

$P = 0.233$), nor was there a significant interaction between male fertility and number of previous matings ($F_{1,245} = 3.54$; $P = 0.0610$). Male fertility had no effect on copulation duration ($F_{1,2} = 1.64$; $P = 0.296$). However, the number of previous matings had a significant effect on copula duration ($F_{1,243} = 4.03$; $P = 0.046$), the last copulation (third copulation) was shorter than the first copulation (Table 2). There was no significant interaction between male fertility and number of matings on copulation duration ($F_{1,243} = 0.06$; $P = 0.805$).

3.3.2. Sperm storage and remating by females

Females mated to either sterile or wild males showed different and opposite patterns of sperm storage (Fig. 3). There was no significant difference between the sperm stored by females mating first (Fw_0) or second (Fw_1) with wild males. Fewer sperm numbers were observed in the third female (Fw_2) ($H = 6.60$; $df = 30, 2$; $P = 0.036$). On the contrary, the first females mating with virgin sterile males (Fs_0) showed lower sperm storage compared to the second (Fs_1) and third female (Fs_2) ($H = 9.48$; $df = 34, 2$; $P = 0.008$) (Fig. 3). The number of females without sperm was very similar irrespective of male fertility or number of previous matings (Fw_0 3/16, Fw_1 2/14, Fw_2 2/15, Fs_0 2/15, Fs_1 3/15, Fs_2 3/14).

Based on the sperm storage patterns between sterile and wild males (Fig. 3), and if female remating is influenced by sperm storage, then we expected higher remating probability for the first females mated to sterile males compared to the second or third female, and no difference between the second and third female. For females mated sequentially to wild males we expected them to have increasing remating tendencies. However, there was no effect of male fertility ($F_{153,1} = 1.50$; $P = 0.222$), number of previous matings ($F_{153,2} = 0.60$; $P = 0.548$), nor their interaction ($F_{153,2} = 1.34$; $P = 0.264$) in the probability of female remating (Fig. 4). Once again, we found that the number of sperm stored is not an important predictor of female remating.

4. Discussion

The mechanisms by which female sexual receptivity can be induced or inhibited are key for an in depth understanding of how male and female behavior can determine their mating success. In a variety of species male accessory glands (AGPs) or sperm have been found to influence female mating inhibition (Avila et al., 2011). As AGPs have been found to have an effect in decreasing

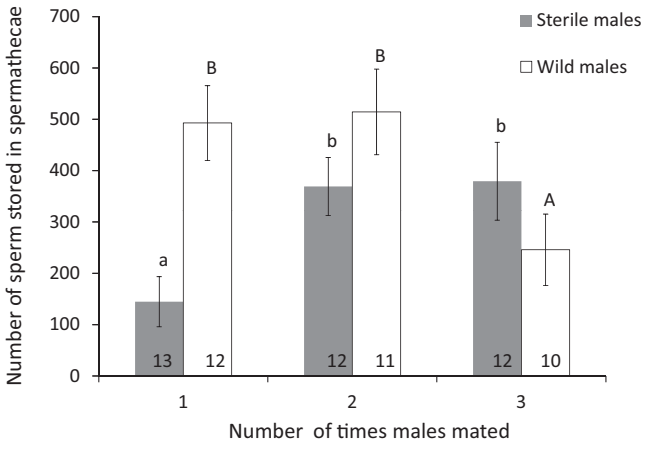

Fig. 3. Number of sperm stored (mean ± SE) in the three spermathecae by the first three sequential mates of sterile or wild *Anastrepha ludens* males. Different letters indicate significant differences within sterile and wild males in post hoc multiple comparisons by Dunns test. Numbers inside bars are sample sizes.

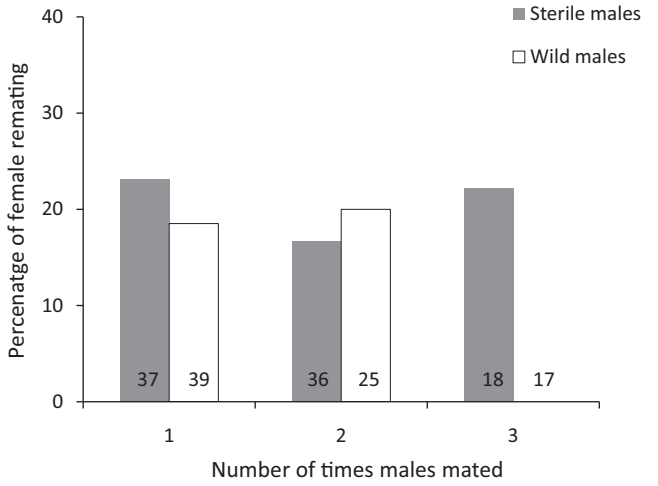

Fig. 4. Percentage of wild *Anastrepha ludens* female remating after mating with previously mated sterile or wild virgin males. Numbers inside bars are sample sizes. Females mating with thrice mated wild males (Fw_2) did not remate.

receptivity in the SA fly but not the Mex fly, we expected sperm numbers to have a marked effect on female receptivity in the Mex fly. While in the SA fly we expected a weak or no effect of sperm. Our results suggest that for both species the full ejaculate diminishes female receptivity and sperm numbers by themselves do not play a role in inhibiting female sexual receptivity.

4.1. Remating of females mated with aedeagus-cut or control males and sperm numbers

When males of both species were unable to deliver an ejaculate (aedeagus-cut males) they were less likely to inhibit female remating compared to intact control males. This means that there is an effect (mechanical and/or physiological) of the ejaculate in the renewal of female receptivity. However, we cannot ignore that aedeagus-cut males are also lacking the tip of the aedeagus, which has many facets, spines and crenellations with effects on ejaculate transfer and storage and may be important in copulatory courtship and female receptivity (Eberhard and Pereira, 1993; Marchini et al., 2001; Briceño et al., 2011). Thus, both copulatory courtship and the full ejaculate may be involved in mating inhibition. For example, in the gypsy moth *Lymantria dispar*, a transient suppression of pheromone is caused by the introduction of male genitalia into the bursa copulatrix, which results in mechanical pressure being transmitted to the bursa. However, a permanent suppression of pheromone production and therefore receptivity inhibition resulted from an adequate supply of sperm in the spermatheca (Giebultowicz et al., 1991). Similar to our results, in medflies, females mated with aedeagus-cut males were also more likely to remate than females mated to control males (Miyatake et al., 1999).

In both species studied here, the number of sperm stored had no effect on the renewal of female receptivity. This is in concordance to a previous study in *A. serpentina* (Landeta-Escamilla et al., 2016) but not in medflies (Mossinson and Yuval, 2003). There seems to be a certain consistency within *Anastrepha* but not within the Tephritidae family.

4.2. Mating probability, latency and copulation duration of multiply mated males

Sterile Mex fly males had shorter latencies to mate compared to wild males. Likewise, in the Q-fly, sterile males had shorter mating latencies than fertile males (Radhakrishnan et al., 2009).

Mass-reared fertile Mex fly males call more frequently, earlier and for a longer period than wild males (Meza Hernández and Díaz-Fleischer, 2006), thus males may be able to attract females more quickly and exhibit shorter latencies to mate. Alternatively, a possibility is that the courtship sequence of sterile flies is shorter, and missed some of the courtship behaviors.

In the Mex fly, copulation duration was shorter for the last copulation (third copulation), irrespective of male fertility. In contrast, in the Q-fly, copulation duration was longer for the last copulation (fifth copulation) (Radhakrishnan et al., 2009) and in the West Indies fruit fly *Anastrepha obliqua*, copulation duration did not vary with number of previous matings (Pérez-Staples et al., 2008).

4.3. Sperm storage and remating by females mated to multiply mated males

In the Mex fly, two findings deserve attention: Firstly, the number of sperm stored in females mated with sterile males increased from the first to the second mate, and was maintained up to the third mate. In the melon fly and Q-fly the number of sperm stored by females mating with sterile males decreased with consecutive matings (Kuba and Itô, 1993; Radhakrishnan et al., 2009). Differences between these species could be due to testicle size and capacity, as the Mex fly is bigger than the other two species.

Secondly, in wild Mex fly males there was a decrease in sperm transfer after the male's second copulation. This is opposite to what occurs in the West Indies fly, where wild males allocate similar numbers of sperm in their first three copulations (Pérez-Staples and Aluja, 2006). In this species, selection may favor males to partition sperm, as females use sperm reserves quickly to oviposit in their native host which is ephemeral and abundant. In contrast, the native host for Mex flies is available for months at a time, thus males may have less pressure to quickly inseminate females and partition sperm equally (Díaz-Fleischer and Aluja, 2003; Pérez-Staples et al., 2014).

For the Q-fly and the melon fly, the number of sperm stored by females mated to sterile males decreased dramatically after the second males' copulation. These sperm-depleted males were equally successful in inhibiting female remating compared to fertile males, who were capable of partitioning sperm numbers in successive matings (Kuba and Itô, 1993; Radhakrishnan et al., 2009). Thus, remating inhibition did not depend on the number of sperm transferred during copulation in these species. Here, female remating was not correlated with the number of sperm stored, and neither male fertility nor number of previous matings affected the males' capacity to inhibit female remating. Thus, we can conclude that the number of sperm by itself plays no role in female sexual inhibition in the Mex fly.

If AGPs and sperm numbers by themselves do not inhibit female receptivity, then what causes remating inhibition in the Mex fly? Most probably, there is a synergic combination of factors, such as sperm plus other components of the seminal fluid. The ejaculate is complex, and contains parasperm, seminal proteins, water, and macromolecules (Perry et al., 2013). We are just beginning to understand the multiple effects that the ejaculate can have on female physiology and behavior. Furthermore, in many cases more than one mechanism may be involved in mating inhibition. In *Drosophila melanogaster*, for example, the combined effect of the sex peptide (SP) and sperm is well-documented. The SP is responsible for the short and long-term post mating effect in females, and the presence of sperm is necessary as a "carrier" of SP (Chapman et al., 2003; Liu and Kubli, 2003; Peng et al., 2005). The persistence of post-mating effects requires that the SP binds to and is released from sperm (Agper-McGlaughon and Wolfner, 2013). In *L. dispar*, as mentioned above, two male mechanisms are involved for females to cease pheromone production and become non-receptive, introduction of male genitalia during copulation and an adequate sperm supply being transferred (Giebultowicz et al., 1991). Another noteworthy example is the ground beetle *Leptocarabus procerulus*, in which the seminal substances transferred during copulation produce a double effect in females. Seminal fluids are used by the male as a physical instrument (through the formation of a mating plug) and as a physiological mechanism (by delaying the renewal of receptivity). Although females seek to counteract these mechanisms (by removing the mating plug), both mechanisms are needed together to reduce female remating (Takami et al., 2008).

4.4. Final conclusions

A. ludens and *A. fraterculus* are two closely related species, within the fraterculus species group (Frey et al., 2013). However, in the context of male mechanisms used to inhibit female sexual receptivity, there are interesting differences. While SA fly females are inhibited by male AGPs (Abraham et al., 2012) but not by the number of sperm stored by females (present study), in the Mex fly we failed to find an effect of injected AGPs (Abraham et al., 2014) or the number of sperm stored, although the full ejaculate inhibited receptivity in both species. On the other hand, copulation duration differs between females that wanted to remate and those that refused to remate in the SA fly but not in the Mex fly, suggesting that the role of copulation duration may also differ between species. Clearly, the mechanisms used by males and females to influence mating decisions and ultimately paternity allocation are still not well understood beyond a few studied species. Experiments using male mutant AGP-deficient and the study of intrinsic factors influencing female behavior could shed light into this matter.

Acknowledgments

We thank José Gutierrez Ruelas, Dina Orozco, and technicians at the MoscaFrut facility (SAGARPA-IICA) for mass-reared pupae. We thank Evaristo Calihua Chipahua, Carlos Carmona, Cruz Perea-Castellanos, and Diego Lendechi for colony maintenance and technical assistance with the experiments. We thank Dinesh Rao and two anonymous reviewers for comments on the manuscript. Funding was provided by CONACYT Mexico grant number Ciencia Básica 179741 awarded to DPS. We thank CONICET Argentina for a postdoctoral scholarship for SA.

References

Abraham, S., Goane, L., Rull, J.A., Cladera, J., Willink, E., Vera, M.T., 2011a. Multiple mating in *Anastrepha fraterculus* females and its relationship with fecundity and fertility. Entomol. Exp. Appl. 141, 15–24.

Abraham, S., Goane, L., Cladera, J., Vera, M.T., 2011b. 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.

Abraham, S., Nuñez-Beverido, N., Contreras-Navarro, Y., Pérez-Staples, D., 2014. Female receptivity in *Anastrepha ludens* (Diptera: Tephritidae) is not modulated by male accessory gland products. J. Insect Physiol. 70, 41–48.

Alcock, J., 1994. Postinsemination associations between males and females in insects: the mate-guarding hypothesis. Annu. Rev. Entomol. 39, 1–21.

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., 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.
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.
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.
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.
Bretman, A., Lawniczak, M.K.N., Boone, J., Chapman, T., 2010. A mating plug protein reduces early female remating in *Drosophila melanogaster*. J. Insect Physiol. 56, 107–113.
Briceño, R.D., Orozco, D., Quintero, J.L., Hanson, P., Hernández, M.R., 2011. Copulatory behaviour and the process of intromission in *Anastrepha ludens* (Diptera: Tephtiridae). Int. J. Trop. Biol. 59, 291–297.
Carroll, S., 1991. The adaptive significance of mate guarding in the soapberry bug, *Jadera Haematoloma* (Hemiptera: Rhopalidae). J. Insect Behav. 4, 509–530.
Carvalho, G.B., Kapahi, P., Anderson, D.J., Benzer, S., 2006. Allocrine modulation of feeding behavior by the sex peptide of *Drosophila*. Curr. Biol. 16, 692–696.
Chapman, T., Bangham, J., Vinti, G., Seifried, B., Lung, O., Wolfner, M., Smith, H.K., C Patridge, L., 2003. The sex peptide of *Drosophila melanogaster*: female post-mating responses analyzed by using RNA interference. Proc. Natl. Acad. Sci. 100, 9923–9928.
De Lima, I.S., Howse, P.E., Salles, L.A.B., 1994. Reproductive behavior of the South American fruit fly *Anastrepha fraterculus* (Diptera: Tephritidae): laboratory and field studies. Physiol. Entomol. 19, 271–277.
Díaz-Fleischer, F., Aluja, M., 2003. Behavioural plasticity in relation to egg and time limitation: the case of two fly species in the genus Anastrepha (Diptera: Tephritidae). Oikos 100, 125–133.
Eberhard, W.G., Pereira, F., 1993. Functions of the male genitalic surstyli in the Mediterranean fruit fly, *Ceratitis capitata* (Diptera: Tephritidae). J. Kansas Entomol. Soc. 66, 427–433.
Frey, J.E., Guillén, L., Frey, B., Samietz, J., Rull, J., Aluja, M., 2013. Developing diagnostic SNP panels for the identification of true fruit flies (Diptera: Tephritidae) within the limits of COI-based species delimitation. BMC Evol. Biol. 13, 1–19.
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. Ann. Rev. Entomol. 48, 163–184.
Jaldo, H.E., Gramajo, M.C., Willink, E., 2001. Mass rearing of *Anastrepha fraterculus* (Diptera: Tephritidae): a preliminary strategy. Florida Entomol. 84, 716–718.
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., 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.
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.
Klowden, M.J., 2001. Sexual receptivity in *Anopheles gambiae* mosquitoes: absence of control by male accessory gland substances. J. Insect Physiol. 47, 661–666.
Landeta-Escamilla, A., Hernández, E., Arredondo, J., Díaz- Fleischer, F., Pérez-Staples, D., 2016. Male irradiation affects female remating behavior in *Anastrepha serpentina* (Diptera: Tephritidae). J. Insect Physiol. 85, 17–22.
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). Florida Entomol. 42, 415–420.
Liu, H., Kubli, E., 2003. Sex-peptide is the molecular basis of the sperm effect in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. U.S.A. 100, 9929–9933.
McInnis, D.O., Rendon, P., Komatsu, J., 2002. Mating and remating of medflies (Diptera: Tephritide) in Guatemala: indvidual fly marking in field cages. Florida Entomol. 85, 126–137.
Marchini, D., Bene, G.D., Falso, L.F., Dallai, R., 2001. Structural organization of the copulation site in the medfly *Ceratitis capitata* (Diptera: Tephritidae) and observations on sperm transfer and storage. Arthropod Struct. Dev. 30, 39–54.
Meza, J.S., Díaz-Fleischer, F., Orozco, D., 2005. Pupariation time as a source of variability in mating performance in mass-reared *Anastrepha ludens* (Diptera: Tephritidae). J. Econ. Entomol. 98, 1930–1936.
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.
Meza Hernández, J.S., Díaz-Fleischer, F., 2006. Comparison of sexual compatibility between laboratory and wild Mexican fruit flies under laboratory and field conditions. J. Econ. Entomol. 99, 1979–1986.
Miyatake, T., Chapman, T., Partridge, L., 1999. Mating-induced inhibition of remating in females Mediterranean fruit flies *Ceratitis capitata*. J. Insect Physiol. 45, 1021–1028.
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.
Peng, J., Chen, S., Busser, S., Liu, H., Honegger, T., Kubli, E., 2005. Gradual release of sperm bound sex-peptide controls female postmating behavior in Drosophila. Curr. Biol. 15, 2017–2213.
Pérez-Staples, D., Aluja, M., 2006. Sperm allocation and cost of mating in a tropical tephritid fruit fly. J. Insect Physiol. 52, 839–845.
Pérez-Staples, D., Aluja, M., Macías-Ordóñez, R., Sivinski, J., 2008. Reproductive trade-offs from mating with a successful male: the case of the tephritid fly *Anastrepha obliqua*. Behav. Ecol. Sociobiol. 62, 1333–1340.
Pérez-Staples, D., Córdova-García, G., Aluja, M., 2014. Sperm dynamics and cryptic male choice in tephritid flies. Anim. Behav. 89, 131–139.
Perry, J.C., Sirot, L., Wigby, S., 2013. The seminal symphony: how to compose an ejaculate. Trends. Ecol. Evol. 28, 414–422.
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.
Segura, D.F., Petit-Marty, N., Sciurano, R.B., Vera, M.T., Calcagno, G., Allinghi, A., Gomez-Cendra, P., Cladera, J.L., Vilardi, J.C., 2007. Lekking behavior of *Anastrepha fraterculus* (Diptera: Tephritidae). Florida Entomol. 90, 154–162.
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., Yuval, B., 2000. Copula duration and sperm storage in Mediterranean fruit flies from a wild population. Physiol. Entomol. 25, 94–99.
Takami, Y., Sasabe, M., Nagata, N., Sota, T., 2008. Dual function of seminal substances for mate guarding in a ground beetle. Behav. Ecol. 19, 1173–1178.
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.
Twig, E., Yuval, B., 2005. Function of multiple sperm organs in females Mediterranean fruit flies (*Ceratitis capitata,* Diptera: Tephritidae). J. Insect Physiol. 51, 67–74.
Vera, M.T., Cladera, J.L., Calcagno, G., Vilardi, J.C., McInnis, D.O., 2003. Remating of wild *Ceratitis capitata* (Diptera: Tephritidae) females in field cages. Ann. Entomol. Soc. Am. 96, 563–570.
Vera, M.T., Abraham, S., Oviedo, A., Willink, E., 2007. Demographic and quality control parameters of *Anastrepha fraterculus* (Diptera: Tephritidae) artificial rearing. Florida Entomol. 90, 53–57.
Wedell, N., 2005. Female receptivity in butterflies and moths. J. Exp. Biol. 208, 3433–3440.
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.
Yamane, T., Miyatake, T., 2010. Induction of oviposition by injection of male-derived extracts in two *Callosobruchus* species. J. Insect Physiol. 56, 1783–1788.
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. 27, 105–116. http://dx.doi.org/10.1007/s10905-013-9414-4.