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

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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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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