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Female remating behaviour in pest tephritid fruit flies and its implication for the Sterile Insect Technique

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Abstract

Background: The efficiency of the Sterile Insect Technique (SIT) targeting tephritid fruit flies depends not only on sterile males mating with wild females, but also on their ability to transmit an ejaculate and inhibit female remating.

Methods: Here we review female remating in tephritid flies of economic importance, inhibition of female remating by males and focus on the factors that can modulate post-copulatory mating behaviour.

Results: Remating by females can vary greatly between fruit fly species, both in mating frequency and time elapsed between matings (sexual refractory period). While some species seem to be monandrous, others vary in their degree of polyandry - ranging from only two matings in their lifetime to 8 matings per day. Remating inhibition can occur through sperm, accessory gland products (AGPs) or copulatory courtship. However, the mechanisms by which males inhibit female mating are still poorly understood.

Conclusions: Despite many studies on the sexual behaviour of tephritids, we still know little about the processes occurring during the copula and how the different components of the ejaculate can affect female post-copulatory behaviour. AGPs have been shown to affect mating inhibition in *Ceratitis capitata*, *Bactrocera tryoni* and *Anastrepha fraterculus* but not in *Anastrepha ludens* or *Anastrepha suspensa*. Thus, the effect of male AGPs should not be generalized throughout tephritids. Understanding how AGPs modify female post-copulatory behaviour can be useful in developing alternative control tactics such as the use of antiafrodisiac substances.

Keywords: accessory gland products, copulation, polyandry, sperm.

Female remating

Polyandry in insects is common across a wide range of taxa (Ridley, 1988; Arnqvist & Nilsson, 2000; Torres-Vila et al., 2004). The family Tephritidae is no exception; there are many studies in several species documenting female remating. Most studies have focused on the Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) (reviewed by Mossinson & Yuval,

2003). Within the genus *Bactrocera*, female remating has been studied for at least four species, *Bactrocera cucurbitae* (Coquillett) (Kuba & Ito, 1993; Haq et al., 2013), *Bactrocera tryoni* (Froggatt) (Harmer et al., 2006), *Bactrocera cucumis* (French) and *Bactrocera cacuminata* (Hering) (Song et al., 2007; Chinajariyawong et al., 2009). Within the genus *Rhagoletis*, female remating has been documented in *Rhagoletis zoqui* (Bush) (Aluja et al., 2001; Rull et al., 2012; Tadeo et al., 2013), *Rhagoletis solanophaga* (Hernández-Ortiz & Frías) (J. Rull personal communication), *Rhagoletis cingulata* (Loew) (Tadeo-Hernández, 2011), *Rhagoletis ramosae* (Hernández-Ortiz) (Tadeo-Hernández, 2014), *Rhagoletis turpinia* (Hernández-Ortiz) (Aluja et al., 2001) and *Rhagoletis completa* (Cresson) (Rull et al., 2012; Tadeo et al., 2013). In *Rhagoletis pomonella* (Walsh), Opp & Prokopy (2000) observed that females mated up to eight times in a day.

Within *Anastrepha*, the presence of multiple mating in females has been studied in *Anastrepha suspensa* (Loew) (Sivinski et al., 1988), *Anastrepha obliqua* (Macquart) and *Anastrepha ludens* (Loew) (Pérez-Staples et al., 2008a; Aluja et al., 2009; Abraham et al., 2014; Meza et al., 2014), *Anastrepha fraterculus* (Wiedemann) (De Lima et al., 1994; Abraham et al., 2011a;b; 2013; 2014), *Anastrepha bistrigata* (Bezzi) (Silva et al., 1985) and *Anastrepha serpentina* (Wiedemann) (Landeta-Escamilla et al., 2016); while for *Anastrepha sororcula* (Zucchi) (Silva et al., 1985) and *Anastrepha zuelanie* (Stone) (unpublished data S.A.), rematings are more rare.

Below we outline the mechanisms by which males can inhibit females from remating and some factors that can modulate female post-copulatory behaviour.

Male mechanisms to inhibit female receptivity

Males will not necessarily gain full paternity from mating with a female (Simmons, 2001). Female insects have sperm storage organs such as the spermathecae and the ventral receptacle where sperm mixing or stratification can take place from rival males (Pérez-Staples et al., 2007; Bertin et al., 2010; Collins et al., 2012; Scolari et al., 2014; Thomas et al., 2014). Thus, in terms of fitness it is in a male's best interest to inhibit females from remating with other rival males after they have mated. Male mechanisms to inhibit female remating and to delay the renewal of sexual receptivity have been studied in detail mostly in *Drosophila melanogaster* (Meigen) (Wolfner, 2002) and certain mosquitoes species. In general, males can inhibit female remating through sperm, copulatory courtship or through particular peptides in the products of the male accessory glands (AGPs). During mating, these secretions are transferred to the female together with sperm. In particular a sex peptide has been found in *Drosophila melanogaster* and *C. capitata* that can inhibit female remating (Chapman & Davies, 2004; Davies & Chapman, 2006). However, for tephritids, sperm, AGPs or both can act in succession to inhibit female remating, but there seems to be great differences between species in the importance of either component of the ejaculate in rendering a female sexually unreceptive.

Female sexual receptivity is not necessarily a single event, it can be “turned off” in the short term but then females can regain receptivity. Thus, despite male investment in manipulating female receptivity, such receptivity usually returns after a certain period of time. On the other hand female receptivity can be turned off in the long term if, with a single mating, females do not remate. The sexual refractory period refers to the time between successive copulations and is generally related to "mating quality", i.e., how much sperm and AGPs (or of what quality) are transferred to the female during copulation.

Sperm effect

The effect of sperm on female receptivity can be studied directly counting the amount of sperm in females that show willingness to remate, compared with mated females that do not show willingness to remate. In this experimental design (Mossinson & Yuval, 2003; Harmer et al., 2006), for both *C. capitata* and *B. tryoni*, remating females had significantly lower numbers of sperm stored, compared to non-remating females. In *A. fraterculus*, *A. ludens* and *A. serpentina*, sperm numbers by themselves seem to play no role in female sexual inhibition (Landeta-Escamilla et al., 2016; Abraham et al., 2016).

The role of sperm numbers on female remating can also be studied using multiply mated sterile males, which transfer decreasing numbers of sperm to their mates, after each successive copulation. In contrast to *B. cucurbitae* and *B. tryoni*, fertile *A. obliqua* males do not suffer this decrease in sperm numbers with increasing matings, thus no sperm depletion is apparent (Kuba & Ito, 1993; Radhakrishnan et al., 2009; Pérez-Staples & Aluja, 2006). For the two *Bactrocera* species studied, remating inhibition did not depend on the number of sperm transferred during copulation, since sterile males without sperm were equally successful in inhibiting female remating compared to fertile males (Kuba & Ito, 1993; Radhakrishnan et al., 2009). In *B. tryoni* this lack of an effect of sperm numbers was seen up to 30 days after the initial mating (Radhakrishnan et al., 2009).

While the mechanisms responsible for changes that occur in females after copulation is not fully understood, the "sperm effect" seems to act mechanically on spermathecae receptors (Fritz & Turner, 2002), which could trigger a physiological response in females to elicit the production of hormones. The sperm effect is usually related to quantity (sperm numbers). However, sperm quality (sperm viability or motility) could also be related to sexual inhibition.

AGP effects

Accessory gland products of insect are produced in the male accessory glands and are composed of carbohydrates, lipids, other materials (uric acid, prostaglandin, juvenile hormone), and in a great amount of proteins (from simple peptides to large structural molecules) (Gillot, 2003; Perry et al., 2013). This is why they are called "secretions", "products" or directly "proteins" of male accessory glands.

Some of the effects of AGPs on female postmating behaviour have been studied using direct injections of these secretions into virgin females. Among these effects, the most relevant for

pest tephritids are a decrease in sexual receptivity observed in *B. tryoni* (Radhakrishnan & Taylor, 2007), *A. fraterculus* (Abraham et al., 2012) and *C. capitata* (Jang et al., 1999). On the other hand, there are cases where the injection of AGPs homogenates do not induce refractoriness in females, such in *A. suspensa* (Lentz et al., 2009) and *A. ludens* (Abraham et al., 2014), it is still unclear why there are such marked differences between species. In *B. dorsalis* there is an increase in male accessory gland size after mating, and males can inhibit females on successive copulations (Wei et al., 2015a). However, it remains to be seen if AGPs in this species cause female mating inhibition.

Copulation effect

Additionally, the *stimulus* of the introduction of the male aedeago could suppress female remating, at least in the short-term. In order to test this hypothesis, Miyatake et al. (1999) used “penis-cut” *C. capitata* males (males with part of the aedeago cut, such that they can copulate but there is no ejaculate transfer), and then evaluated remating of females first mated with penis-cut males, compared with control intact males. There was no female remating inhibition when males were prevented from transferring the ejaculate (sperm and AGPs), thus this demonstrates that in *C. capitata* the stimulus of copulation *per se* did not inhibit female remating. Recently, the same was observed in *A. fraterculus* and *A. ludens* (Abraham et al., 2016). However, we cannot ignore the fact that the tip of the aedeagus, with its many facets, spines and crenellations, may be very important in copulatory courtship, with effects on sperm transfer and storage, as well as subsequent effects on receptivity (Eberhard & Pereira, 1993; 1995; Marchini et al., 2001). Thus, the microsurgery would have totally eliminated any such effect. Nevertheless, this approach demonstrates that the full ejaculate in those species is needed for mating inhibition.

In *C. capitata*, and possibly *A. fraterculus* and *A. ludens* more than one mechanism may be involved, combined together through a synergetic effect. Currently it is postulated that different elements may act at different time scales. Thus, the ejaculate can inhibit receptivity in the short-term, by acting on receptors in sperm storage organs and finally, the secretions of the accessory glands of the male can act in the long-term, to change the response of the female to male courtship signals (Delrio & Cavaloro, 1979; Jang, 1995; Miyatake et al., 1999; Mossinson & Yuval, 2003; Gavriel et al., 2009). This change can include modulation of female olfactory behaviour. For example, in *C. capitata*, injections of the AGPs cause chemoreceptive changes in females, producing a switch from attraction to male pheromone to attraction for host volatiles (Jang, 2002).

Factors affecting female remating

Several fruit flies of economic importance are controlled all over the world with the Sterile Insect Technique (SIT). Mass-rearing and irradiation are necessary for SIT, and both factors can affect male post-copulatory success. For insects controlled through SIT, it is desirable that females remain monandrous so that there will be no additional matings after wild females mate with sterile males. Since SIT programs require that males survive to reach sexual

maturity in the field and are able to mate and induce a refractory period in wild females, numerous attempts to enhance male sexual competitiveness and accelerate the process of sexual maturation have been made (called post-teneral pre-release treatments, reviewed in Caceres et al., 2007; Pereira et al., 2013). Examples are the incorporation of protein into the adult male diet, the acceleration of sexual maturation with a Juvenile hormone mimic (methoprene) or aromatherapy (e.g., with ginger root oil) (Fig.1).

Mass-rearing

The process of mass-rearing *per se* can influence female remating. *Anastrepha ludens* represents an iconic case where as many as 80% of mass-reared females remate (Abraham et al., 2014; Meza et al., 2014). This effect was not observed in females of other species such as *A. fraterculus* and *B. cucurbitae* (Abraham et al., 2011a; Haq et al., 2013), although in those studies females stemmed from a laboratory mass-rearing colony and not from a mass-rearing facility, as was the case for *A. ludens*.

On the other hand, mass-rearing can influence male ability to modulate female remating. For example, *A. fraterculus* females injected with AGPs of laboratory males were less likely to mate, compared with females injected with AGPs of wild males (Abraham et al., 2012), showing that mass-rearing is not always detrimental. In the same way, laboratory (sterile) males are equally capable in suppressing female receptivity compared to wild males, through a natural copulation (Abraham et al., 2013). Similarly, laboratory-reared males were as efficient as wild males in inhibiting female remating in *B. cucurbitae* (Haq et al., 2013). Likewise in *A. ludens*, mass-reared and wild males were just as efficient in inhibiting either wild or mass-reared females (Abraham et al., 2014).

Irradiation

Sterile *C. capitata* males were less able to inhibit female remating, and females mated with sterile males had the shorter refractory periods compared to females mating with wild males (Vera et al., 2003; Gavriel et al., 2009). In *A. serpentina*, females mated with sterile males had higher remating propensity, compared to females mated with fertile males (Landeta-Escamilla et al., 2016). In *A. fraterculus* females injected with AGPs of sterile males had higher remating compared with females injected with AGPs of fertile male AGPs (Abraham et al., 2012). However, sterile males were as efficient as wild males in inhibiting female remating throughout a natural copulation, thus AGPs are not the only component of the ejaculate responsible of female inhibition in this species (Abraham et al., 2013). Similarly, irradiation had no effect on female remating propensity in *B. cucurbitae* (Haq et al., 2013).

Male diet

Male diet can affect sperm numbers and indirectly female remating (Yuval et al., 2002; 2007). Male diet affects sperm production and female remating in at least *C. capitata*, *B. tryoni*, *B. cucurbitae*, *A. fraterculus* and *A. obliqua* (Blay & Yuval, 1997; Taylor & Yuval, 1999; Yuval et al., 2002; Pérez-Staples et al., 2008a;b; Aluja et al., 2009; Gavriel et al., 2009, Abraham et al., 2011b; Costa et al., 2012; Haq et al., 2014) but not in *R. pomonella* (Hendrichs et al.,

1992). In *A. fraterculus*, for example, wild females mated with sugar-fed males remate more often, remate sooner and stored less sperm than females mated with protein-fed males (Abraham et al., 2011b). On the other hand, in this same fly, male diet affected AGPs capacity to inhibit female receptivity (Abraham et al., 2012). Similarly, in *C. capitata* the ability of sterile males to inhibit female receptivity is greatly improved when they are fed a diet rich with protein (Gavriel et al., 2009; but see Shelly & Kennelly, 2002). In *B. tryoni* feeding males with only 24 or 48 h of yeast hydrolysate after emergence increases sperm numbers and decreases female remating (Pérez-Staples et al., 2008b). Overall evidence suggests that in general ingestion of protein at the adult stage in tephritid fruit flies increases their post-copulatory success and should be incorporated into pre-release diets when possible.

Juvenile Hormone treatment

Juvenile hormone (JH) is a sesquiterpene, which along with other hormones regulates growth and passage through the early development stages of the life cycle. JH in some species also regulates the beginning of the process of sexual maturation in females, males or both sexes (Ringo, 2002). It has been postulated that this hormone is primarily responsible for coordinating reproductive maturity in *A. suspensa* males and induces early development (Teal et al., 2000). The use of methoprene (an analog of JH) in males of *A. fraterculus* allows reaching sexual maturity at an early stage (Segura et al., 2011). Since the genera *Anastrepha* and *Bactrocera* require several days to reach sexual maturity, the use of JH allows SIT programs to reduce the storage time of the flies in the facility and reduce time in the field to achieve male sexual maturity (Teal et al., 2000). The use of methoprene affects the process of sexual maturation, but in *A. fraterculus* females injected with AGPs of 6 d-old methoprene-treated males had higher receptivity, compared to females injected with AGPs of sexual mature untreated-males (Abraham et al., 2012). This suggests, that methoprene matures males up to a certain point. Methoprene treated young males can mate, but perhaps methoprene did not mature their accessory glands. In this same species, wild females mated with methoprene-treated males had higher remating rate and shorter refractory periods than wild females mated with sterile untreated-males or wild males (Abraham et al., 2013). On the contrary, methoprene treatment alone or accompanied with protein had no effect on female remating propensity in *B. cucurbitae* (Haq et al., 2014). Clearly, this is a topic that deserves further research in a variety of species.

Aromatherapy

In order to counteract the detrimental effect of mass-rearing process and irradiation, some effort has been made using different substances to increase male mating success. In *C. capitata*, the use of ginger root oil (GRO) increase male mating success (Shelly et al., 2002) and females mated with such males had lower remating rate (Shelly et al., 2004; Morelli et al., 2010). Similarly, females of *B. tryoni* mated with lure-fed males (males fed with plant derived chemicals to enhance their mating competitiveness) had lower remating propensity (Kumaran et al., 2013). Our understanding of how female remating behaviour is modulated by chemicals beyond a few substances tested for aromatherapy is still incipient. Certainly the changes in

female olfactory behaviour observed as a result of the transfer of AGPs during mating, suggests that the relationship between female remating, male pheromones and lures is a complex one. Further research is needed on the interaction between chemical ecology and the sexual behaviour of tephritid flies of economic importance.

Male age

Male age is an important factor that can influence their pre and post-copulatory success. Sperm storage by females and sperm number decline with male age in *C. capitata* and *B. tryoni*, respectively (Taylor et al., 2001; Pérez-Staples et al., 2008b; but see Papanastasiou et al., 2011 and Costa et al., 2012 for *C. capitata*). In *C. capitata* middle-aged males (11 d-old) are more effective in reducing female sexual receptivity than younger (4 d-old) or older (18 d-old) males (Gavriel et al., 2009). Similarly, Shelly et al. (2007) found that middle aged males (5 or 10 d-old) are more effective, compared to younger males (3-4 d-old). A recent study found that in *C. capitata* female remating was not influenced by male age (from 4 to 20 d-old) when males were well-nourished (Costa et al., 2012). In contrast, in *B. tryoni*, male age does not affect their ability to inhibit female remating (Pérez-Staples et al., 2008b).

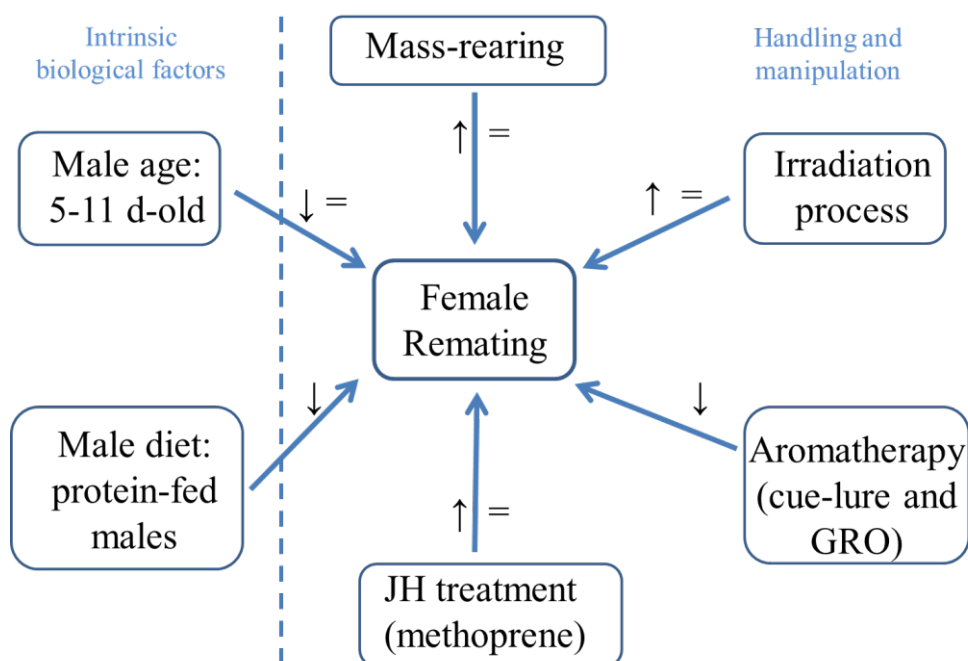


Fig. 1. Principal factors affecting female remating behavior in tephritid flies of economic importance. Symbols indicate that the factor increases (↑), decreases (↓) or has no effect (=) on female remating, depending on the studied species. JH: juvenile hormone. GRO: ginger root oil.

Impact of remating in SIT

If sterile males transfer lower quality and/or quantity of sperm and/or AGPs, wild females could remate more often, or earlier, when mating with a sterile male. In *C. capitata* sterile males are less able to inhibit female receptivity (Kraaijeveld & Chapman, 2004; Gavriel et al., 2009; Morelli et al., 2013). Also, the sexual refractory period for medfly females is shorter

when mated to sterile males than to wild males (Vera et al., 2003; Gavriel et al., 2009). A high female remating rate and a rapid renewal of receptivity may compromise the efficiency of SIT, as a female could remate with a wild fertile male, thereby leaving viable offspring (Bloem et al., 1993; Kraaijeveld & Chapman, 2004). Additionally, we know little about how female choice changes after mating with a sterile male, perhaps females could change their choice after mating with a sterile male and prefer a wild male as second partner.

Future perspectives

Some points could be highlighted as “black holes” in the study of the role of sperm and male accessory glands in the Tephritidae family, due to the difficulty of separating one factor from the other. The use of molecular techniques such as producing AGP-deficient males, RNA interference (e.g. Gabrieli et al., 2016), or the use of genetically modified insects could greatly aid in this endeavour. Also, attempting artificial insemination with only sperm could help disentangling these two factors. A deeper understanding on the physiological and chemical mechanisms that govern female remating as well as how post-teneral treatments affect these physiological processes is needed. For example, a further knowledge gap is if and how both internal and external copulatory courtship influences female remating behaviour.

Furthermore, there are relatively few species where the genes expressed in the male reproductive system and the proteins of the seminal fluid have been characterized in detail. Information acquired through the genome, proteome or transcriptome will also aid our understanding on the function of AGPs (e.g., Scolari et al., 2012; Wei et al., 2015b; 2016). The genomic and transcriptomic data for tephritids remains limited. Despite the evolutionary constraints at the functional level, genes with reproductive functions are evolving faster than other genes not associated with reproduction (Wagstaff & Begun, 2005). Thus, efforts must continue in the search for new genes and proteins in the male and female reproductive tract. In particular, control methods may benefit from finding genes that are differentially regulated in the female reproductive tract in response to mating, and corroborating the transfer of AGPs (transcripts and proteins) from males to females during copulation (e.g., Scolari et al., 2012; 2014; Kumaran et al., 2014). Identification of molecular and genetic mechanisms that are involved in mating inhibition, as well as displacement or incapacitation of ejaculates are also interesting areas to pursue. It will also be useful to compare sequences in other species to find putative orthologs, and to study the post-copulatory molecular interactions between sexes and the molecular mechanisms underlying tephritid reproductive biology.

Our knowledge on female remating is constrained to a small number of species, generally of economic importance. A large number of species have been relatively ignored inside the Tephritidae family and in related families. Many of these species will represent valuable model organisms for the study of cryptic post-copulatory process, such as cryptic female choice and sperm competition. One curious example is that of the Agave fly *Euxesta bilimequi* (Diptera: Ulidiidae), where females remate repeatedly, expel the sperm after copulation and

consume the sperm (Rodríguez et al., 2013). This and others insects provide us with the opportunity to study polyandry, sexual conflict, and cost and benefits of these behaviours to females.

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