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Male sexual enhancement after methoprene treatment in *Anastrepha fraterculus* (Diptera: Tephritidae): a sustained response that does not fade away after sexual maturation.

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South American fruit fly, sesquiterpene, mating success, sterile insect technique, SIT, juvenile hormone analogue

Running title: Sustained effect of methoprene on *A. fraterculus*

Abstract

The juvenile hormone (JH) of insects triggers physiological changes related to reproduction in adults of both sexes. Methoprene is a sesquiterpene with some effects that are analogous to those of JH. Treatments with methoprene accelerate sexual maturation in males of the South American fruit fly *Anastrepha fraterculus*, giving young males a mating advantage over non-treated males of the same age. Here, we evaluated the effects of methoprene treatment on *A. fraterculus* males after the sexual maturation phase and tested whether this compound provides a long-term mating advantage. Moreover, we took the first step to unravel the mechanisms that underlie male sexual enhancement. We treated males 1 day or 8 days after adult emergence and compared mate choice between recently matured (young) females and females that had been mature for ca. 10 days (aged females). We also addressed methoprene treatment effects on male sexual signalling. We found that methoprene treatment enhanced male sexual competitiveness even after the sexual maturation phase, and the effect did not decrease until males were older than 20 days. However, when methoprene treatment was carried out close to sexual maturity, the mating enhancement was no longer observed, suggesting a non-immediate effect and excluding the possibility that methoprene acts as a pheromonal compound. Young and aged females tended to mate more frequently with treated-males. This might indicate that in a context of sexual selection, the potential benefits associated with reproductive success would be similar for females of both ages. Treated males released larger amounts of pheromonal compounds than non-treated males, but their courtship behaviour was not altered to the same extent, suggesting that methoprene treatment may accelerate differently the components of male courtship. We discuss potential benefits of using methoprene to increase the efficiency of the sterile insect technique, which is an environmentally safe method to control this important South American fruit pest.

1. Introduction

Juvenile hormone (JH) is a natural, non-cyclic sesquiterpenoid that acts on the endocrine system of insects and regulates diverse aspects of physiology such as development, metamorphosis, diapause, reproduction and polyphenism (Vogel *et al.* 1979, Riddiford *et al.* 1991, Nijhout 1994, Wyatt & Davey 1996). The effect of JH depends on the individual developmental stage. It promotes development and growth at the larval stage, while preventing metamorphosis (Nijhout 1994, Klowden 2007). At the adult stage, JH triggers physiological changes related to reproduction (Nijhout 1994, Klowden 2007). In most insects, the pre-copulatory period is under hormonal control, in which JH plays a key role (Happ 1992, Wyatt & Davey 1996, Gilbert *et al.* 2000, Wilson *et al.* 2003). In females, JH titers are correlated with egg production, vitellogenesis, and lipid storage in ovarioles (Gruntenko *et al.* 2005). Furthermore, the development of ovarioles is closely coordinated with sexual receptivity (Ringo 2002). In males, JH stimulates the development and maturation of the reproductive accessory glands, promoting their growth and the production of glandular secretions (Yin *et al.* 1999, Wilson *et al.* 2003, Klowden 2007), and the production of sex pheromone (Rantala *et al.* 2003).

Because JH is photosensitive and difficult to synthesize, functionally analogous compounds have been developed with both research and applied purposes. One of these analogues is methoprene, a well-known synthetic compound with larvicidal action on insects such as mosquitoes (World Health Organization 2008) and stored-grain pests (Athanassiou *et al.* 2011). Methoprene has also been studied in relation to its potential use for the sterile insect technique (SIT) against fruit flies (Diptera: Tephritidae). In the SIT, large numbers of the pest are reared, sterilized and released in areas of interest. However, in some tephritid species from genera such as *Anastrepha* and *Bactrocera*, several days are required to attain sexual maturation after adult emergence. Several SIT programs keep

the sterile flies within the facility during the sexual maturation period to avoid field mortality, but this involves space, food, water and labor. The use of JH analogues, such as methoprene, accelerates male sexual maturation, thus reducing costs of maintenance of flies before field releases (Teal *et al.* 2011, Segura *et al*. 2013).

The impact of methoprene on sexual maturation has been explored in a number of tephritid species. Teal *et al*. (2000) showed that treatment with topical applications of methoprene during the first hours post-emergence reduced the pre-copulatory period of *Anastrepha suspensa* (Loew) males from 7 days (when non-treated) to 4 days (when treated). Acceleration of sexual maturity has also been described for *Anastrepha ludens* (Loew) (Gómez-Simuta & Teal 2010), *Anastrepha obliqua* (Macquart) (Chacón-Benavente *et al*. 2013), *Zeugodacus cucurbitae* (Coquillett) (previously described as *Bactrocera cucurbitae*) (Haq *et al*. 2013) and *Bactrocera tryoni* (Froggatt) (Collins *et al*. 2014). In *Anastrepha fraterculus* (Wiedemann), Segura *et al*. (2009, 2013) and Liendo *et al*. (2013) showed that methoprene treatment also reduced the male maturation time from 7 to an average of 4 days. This period of time during which only methoprene-treated males attain sexual maturity (i.e., 4-7 days after emergence in the case of *A. fraterculus*) is known as the 'accelerated sexual maturity phase' (ASMP) (Haq *et al*. 2010b). In other species, such as *Ceratitis capitata (*Wiedemann), this effect was not observed (Faria *et al*. 2008, Shelly *et al*. 2009).

In the three *Anastrepha* species studied so far, methoprene-treated males going through the ASMP are equally likely to mate than non-treated, mature males (Pereira *et al*. 2009, 2013, Segura *et al*. 2009, Gómez-Simuta & Teal 2010, Liendo *et al*. 2013). Thus, the acceleration of sexual maturation, attributed to methoprene treatment, induces successful matings during the ASMP that otherwise would not occur. Teal *et al*. (2000) postulated that in the case of *A. suspensa* males, this phenomenon was related to an increase in sex pheromone release. Consistent with this hypothesis, Chacón-Benavente *et al*. (2013)

showed that *A. obliqua* males released more sex pheromone during the ASMP than non-treated males of the same age. Further, as pheromone production is regulated by JH (Rantala *et al*. 2003) and methoprene-treated males may have high JH concentrations in their haemolymph (Haq *et al*. 2010a), this provides additional support of the association between methoprene treatment and an increase in the emission of sex pheromones by treated males in the course of the ASMP.

In spite of the many examples where tephritid males treated with methoprene showed an increase in mating success, little attention has been paid to the effect of methoprene after the ASMP has ceased (hereafter, post-ASMP). In *A. suspensa*, Pereira *et al*. (2010) found that methoprene induced an increase in the mating success of 13-16 day-old males (post-ASMP). This suggests a double role of methoprene: as a sexual maturation promoter and as an enhancer of mating success in already matured males. However, the enhancing effect of methoprene demonstrated in *A. suspensa* was not observed in *B. cucurbitae* (Haq *et al*. 2010b). This phenomenon may therefore depend on the species.

At present, there is a lack of studies that aim at elucidating the mechanisms behind increased copulatory success of methoprene-treated males, either during or after the ASMP. After topical treatment, methoprene might be internalized through the integument, subsequently triggering a series of physiological changes, making males more attractive to females. Additionally, this sesquiterpene could be adsorbed by the males' cuticle and then slowly released, as a scent that attracts females. This mechanism has been observed for *C. capitata* males exposed to citrus oils and ginger root oil (Papadopoulos *et al*. 2006, Shelly *et al*. 2007) and it is known as the "perfume effect" (Shelly *et al*. 2007). In the same way, there is lack of knowledge about the potential benefits that females might obtain by mating with one of these particular types of males (exposed to, or topically treated with, compounds that enhance their mating performance).

Here we study the effect of methoprene treatment on the mating success of *A. fraterculus* males after the ASMP (> 7 days-old). We carried out further experiments in order to shed light on the physiological changes in treated males. Based on the potential benefits that females may obtain by mating with methoprene-treated males, we compared the number of females that mated with treated and non-treated males in recently sexually mature, virgin females (hereafter referred to as young females) and females that had been mature for 10 days approximately, but were still virgin (hereafter referred to as aged females). We also investigated the duration of methoprene treatment effect on males and whether the outcome of the mating competitiveness tests was affected by the females' age. Finally, we took the first step towards elucidating the mechanisms that underlie the enhancement of male sexual success by evaluating calling behaviour and sex pheromone release in treated and non-treated males.

2. Methods

2.1. Biological material

Anastrepha fraterculus flies were obtained from the laboratory colony kept at INTA Castelar and originally established at the Agricultural Zoology laboratories (Estación Experimental Agroindustrial Obispo Colombres, Tucumán, Argentina). This colony was initiated in 1997 with pupae obtained from infested guavas collected in Tafí Viejo (Tucumán) (Jaldo 2001). Rearing followed standard procedures using an artificial diet based on yeast, wheat germ, sugar, and agar for larvae (Salles *et al*. 1995) and a mixture of sugar and hydrolysed yeast for adults (Jaldo *et al*. 2007). All individuals were kept under controlled environmental conditions (Temp: 24 ± 2°C, RH: 70 ± 10%, photoperiod 12L: 12D). Flies used in the tests were all virgin and sexually mature, and to evaluate an age effect two groups were considered: 1) young flies (10-12d post-emergence) and 2) aged flies (18-22d post-emergence) (Petit-Marty *et al*. 2004).

165

166 **2.2. Effect of methoprene treatment on mating success**

167 Mating tests were carried out by offering two males (one treated and one non-treated) to an
168 individual female in a 1 L plastic cylindrical container. Each container was considered as a
169 replicate. All experiments were conducted under laboratory conditions (Temp: 25 ± 2°C and
170 70 ± 10% RH). Illumination was provided by fluorescent tubes and natural light coming from
171 a window. Mating tests began at 9 am and lasted for 3 h. Given that mating activity of the
172 Argentinean population of *A. fraterculus* starts at sunrise (Petit-Marty *et al.* 2004), flies were
173 kept in separate rooms in darkness conditions until the test in order to avoid any light before
174 starting the experiments.

175 Treated males were subjected to a topical application of 1 µl of a
176 methoprene/acetone solution (5 µg/µl) on their thorax (Teal *et al*. 2000, Segura *et al*. 2009)
177 using an automatic micropipette (Eppendorf Multipette plus, Beckman Instruments, Inc.,
178 Fullerton, California, USA). Methoprene effect was evaluated using acetone as solvent
179 (henceforth, methoprene treatment). This allowed to compare our results with previous
180 studies on *A. fraterculus* (Segura *et al*. 2009, 2013, Liendo *et al*. 2013) as well as other
181 tephritid flies (Pereira et al. 2009, 2010, 2013, Haq *et al*. 2010ab) in which acetone was
182 chosen as solvent to deliver methoprene to the flies.

183 Treated and non-treated males were of the same age and only differed in the
184 methoprene treatment. Males were marked on their thorax with a dot of non-toxic, water-
185 based paint for identification during the mating test (Petit-Marty *et al.* 2004). The type of
186 male that was marked was alternated among tests. Three experiments were performed
187 considering different ages and time of methoprene treatment (see below). Before each
188 mating test, blinds were slightly opened as to create a semidarkness condition under which
189 males were transferred to the 1L containers and were allowed to acclimatize for 15 min .
190 Following that, females were released in the container. Lights were then turned on and the

191 window blind fully lifted. Then, the occurrence of matings was continuously monitored.
192 Whenever a couple was detected, male type and the mating start time were recorded.
193 Mating pairs were observed until the flies disengaged and the mating endi time was
194 recorded.

195 Three variables were used in order to analyse the mating success of the males:
196 type of chosen male, latency to mate (time elapsed since female release in the container
197 until mating), and mating duration. To assess whether female election was affected by its
198 age, each test was carried out using young (10-12 days-old) and aged (18-22 days-old)
199 females.

200

201 *Experiment 1. Effect of methoprene treatment applied at emergence, on young males*. We
202 evaluated if the methoprene treatment increases male mating success in *A. fraterculus*.
203 Methoprene was applied within the first 24 h post-emergence and the mating test was
204 performed when males reached 10 to 12 days-old (Table 1). The numbers of replicates
205 were 82 for young females and 95 for aged females.

206

207 *Experiment 2. Effect of methoprene treatment applied at emergence, on aged males.* To
208 determine whether the effect of methoprene treatment increases mating success of *A.*
209 *fraterculus* males at time intervals greater than those assessed in Experiment 1,
210 methoprene was applied within the first 24 h post-emergence and the test was performed
211 after 18-22 days (Table 1). The numbers of replicates were 103 for young females and 104
212 for aged females.

213

214 *Experiment 3. Effect of methoprene treatment on young males when applied on the eighth*
215 *day post-emergence.* In this experiment we aimed to determine whether the methoprene
216 treatment could induce an increase in male mating success when the time elapsed between

the treatment and the mating test was shorter than in the previous experiments. Here, the treatment was applied at the eighth day post-emergence and the test was performed when males were 10-12 days-old (Table 1). The numbers of replicates were 106 for young females and 87 for aged females.

Table 1. Summary of male traits involved in mating tests.

Experiment	Male age at methoprene treatment (days)	Male age at mating test (days)
1	0 (sexually immature)	10-12 (young)
2	0 (sexually immature)	18-22 (aged)
3	8 (sexually mature)	10-12 (young)

Each experiment involved mating tests with young females (10-12 days-old) and aged females (18-22 days-old).

2.3. Effect of methoprene treatment on male calling behaviour and sex pheromone

In order to shed light on the mechanisms underlying the results obtained in the mating test of experiments 1-3, calling behaviour and pheromone profiles were studied. Both the observation of calling behaviour and collection of male volatiles (including pheromone) were performed simultaneously during a 3–hours period (9.00 am -12.00 pm) by placing 10 males (treated or non-treated) inside a 250 mL glass chamber (20 cm length, 4 cm in diameter) which allowed recording their behaviour and collecting volatiles at the same time (Bachmann *et al*. 2015). Like for the mating tests, males were kept in darkness until the test began. Males were 10-12 days-old and methoprene was applied within the first 24 h post-emergence (same treatments procedures as in Experiment 1). Each group of 10 males was considered a replicate.

To describe males calling behaviour, two components of male courtship associated with pheromone emission and dispersion (Nation 1989, Gómez-Cendra *et al*. 2011) were recorded: wing fanning (hereafter "fanning") and salivary glands exposure. During the 3-hours observation period the number of males performing these behaviours was recorded following Bachmann *et al*. (2015). Eight replicates were analysed for each type of males.

To estimate the amount of pheromone emitted by the males, four characteristic compounds of *A. fraterculus* pheromone were quantified: (E,E)-α-farnesene, suspensolide, anastrephin and epianastrephin (Cáceres *et al*. 2009, Břízová *et al*. 2013, Milet-Pinheiro *et al*. 2015). The amount of each compound was estimated by the average of chromatographic areas which were relativized to an internal standard. A purified air flow (400 ml/min) was blown over the 10 males enclosed in a glass chamber and volatiles were collected using a filter containing 30 mg of Hayesept Q adsorbant (Grace, Deerfield, IL, USA) at the exit of the chamber. Trapped compounds were eluted with 200 µl of methylene chloride containing tridecane as internal standard (5 ng/µL) and kept at 20°C until analyzed. Samples were analyzed in an Agilent 7890A gas chromatograph equipped with a HP-5 column (30 m ± 0.32 mm inner diameter ± 0.25 µm film thickness), and a flame ionization detector. The initial oven temperature was 35°C, held for 1 min, and then increased to 100°C at 5°C min^{-1} and from 100°C to 230°C at 12°C min^{-1}, then held for 10 min (Bachmann *et al*. 2015). Samples were injected in the splitless mode with the injector purged at 30 sec with nitrogen as the carrier gas at 27.6 cm/sec flow velocity.

Compound identities were confirmed by comparison of retention times with those of authentic synthetic samples obtained from the Center for Medical, Agricultural and Veterinary Entomology (USDA-ARS, Gainesville, FL, USA).

2.4. Data analysis

For the mating experiments, the number of copulations achieved by treated and non-treated males were compared by a G test of goodness of fit to an equal proportion hypothesis, with Yates correction for continuity [recommended whenever the degree of freedom equals 1; Zar (1996)]. To verify whether there is a similar trend in the mating preference of young and aged females, the proportion of treated males who reached copulation was compared for each female type using a homogeneity G test (Zar 1996). Latency to mate and mating duration were analysed by means of a two-way ANOVA in which males' treatment and females' age were considered as the main factors. For experiments 2 and 3 the values of latency to mate and mating duration were transformed to "Ln (x + 0.5)" in order to meet the homoscedasticity assumption.

The number of males performing sexual calling behaviours and the amount of each pheromone compound were compared between treated and non-treated males by means of Student's *t*-tests for paired samples (assumptions of normality and homogeneity of variance were checked in all cases).

3. RESULTS

3.1. Effect of methoprene treatment on mating success

Experiment 1. Effect of methoprene treatment applied at emergence, on young males. Methoprene-treated males achieved greater percentage of matings than non-treated males for both type of females (Fig. 1), yet these differences were statistically significant for young females ($G_y = 8.398$, $p < 0.01$, $N = 82$), but not for aged females ($G_y = 1.784$, $p = 0.182$, $N = 95$). Even when significant differences were detected only for young females, the proportion of females that mate with treated males was similar between young and aged females, since the percentage of matings obtained by treated males did not statistically differ between both types of females ($G = 1.710$, $p = 0.191$, $N = 177$). Latency to mate did not

differ between treated and non-treated males and was independent of the age of the females (Table 2). No interaction was detected between factors (i.e., males' treatment and females' age) (Table 2). Mating duration was significantly longer for aged females than for young females and there was no effect of male type (Table 3). No interaction between factors was detected (Table 3).

Figure 1

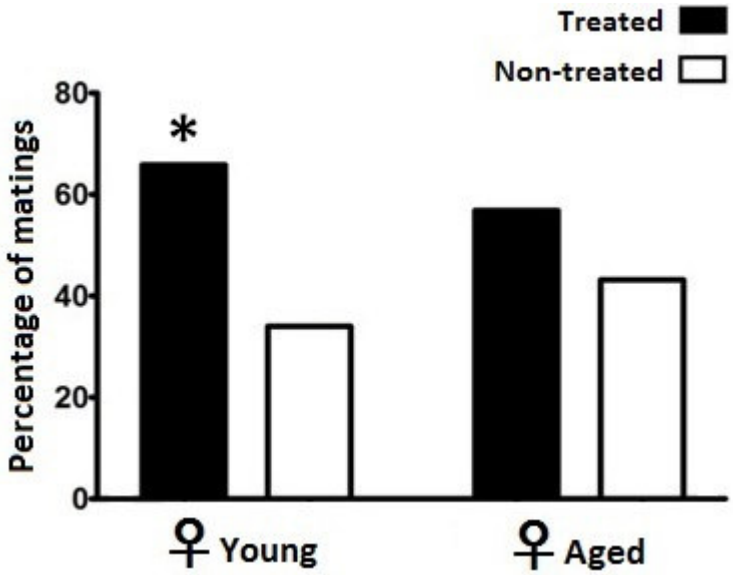


Figure 1. ***Effect of methoprene treatment applied to males at emergence, on mature (10-12 days-old) males mating performance with mature young and aged females (Experiment 1).*** Males were treated within the first 24 h post-emergence and their mating success was evaluated at day 10-12 post emergence. Asterisk indicates significant differences ($p < 0.05$) between treated and non-treated males (G test of goodness of fit).

Experiment 2. Effect of methoprene treatment applied at emergence, on aged males. Aged females mated more frequently with treated males than with non-treated males ($G = 4.689$, $p = 0.030$, $N = 104$) but this was not the case for young females ($G = 1.645$, $p = 0.200$, $N = 103$) (Fig. 2). As in Experiment 1, the percentage of matings obtained by treated males did not differ between types of females ($G = 0.398$, $p = 0.533$, $N = 207$). Latency to mate and mating duration were not affected by the type of male (Tables 2 and 3). However, young females started to mate sooner and for shorter duration than aged females (Tables 2 and 3). No interaction was detected for either time variable, except for latency in experiment 2 (Tables 2 and 3).

Figure 2

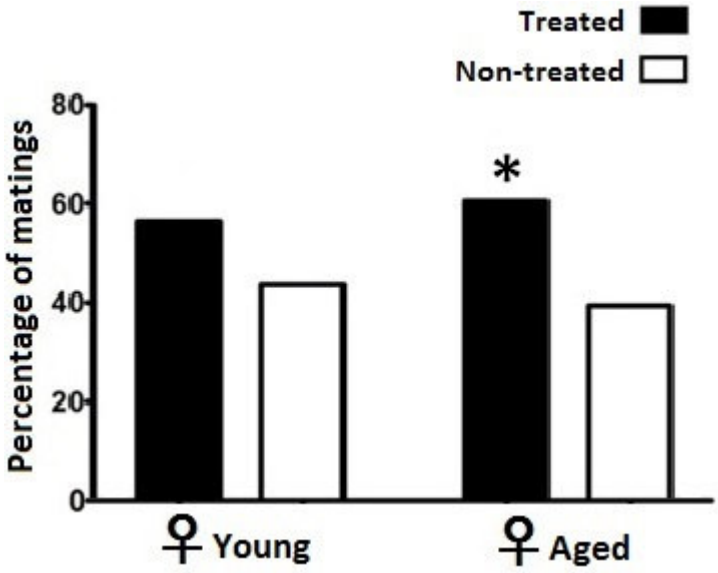


Figure 2. ***Effect of methoprene treatment applied to males at emergence, on aged (18-22 days-old) males mating performance with mature young and aged females (Experiment 2).*** Males were treated within the first 24 h post-emergence and their mating success was evaluated at day 18-22 post emergence. Asterisk indicates significant differences ($p < 0.05$) between treated and non-treated males (G test of goodness of fit).

Experiment 3. Effect of methoprene treatment on young males when applied on the eighth day post-emergence. The percentage of matings was not affected by methoprene treatment and was also independent of females´ age (young females: $G = 2.994$, $p = 0.084$, $N = 86$, aged females: $G = 0.103$, $p = 0.748$, $N = 87$) (Fig. 3). The analysis showed that the percentage of treated males that mated with both types of females was not statistically different ($G = 1.007$, $p = 0.316$, $N = 173$). No differences were found between treated and non-treated males in terms of latency to mate (Table 2) and mating duration (Table 3). As in experiment 2, young females showed lower latency to mate and lower duration than aged females (Tables 2 and 3, respectively). No interaction (male x female) was found between the main factors for these two variables (Tables 2 and 3).

Figure 3

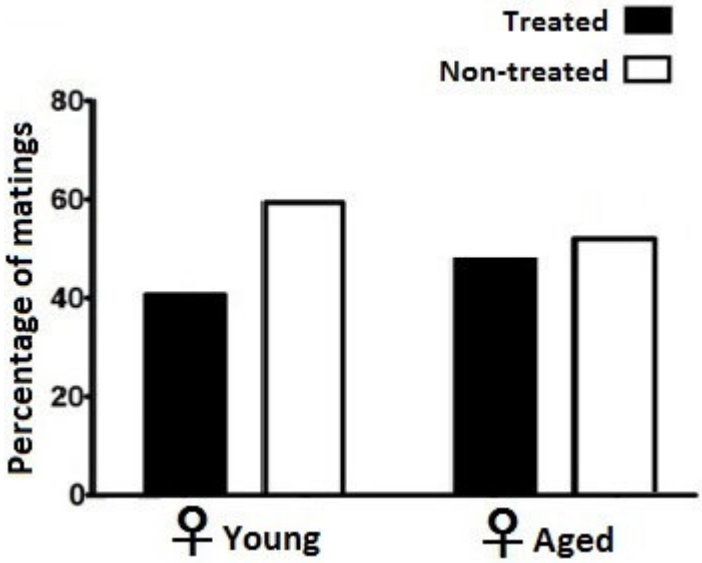


Figure 3. ***Effect of methoprene treatment applied to males on the eighth day after emergence, on young (10-12 days) males mating performance with mature young and aged females (Experiment 3).*** Males were treated at day 8 after emergence and their mating success was evaluated at day 10-12 post emergence.

Table 2. Mean (±S.E.) latency to mate for each experiment. Latency is presented for each type of mating pair. Results of the two-way ANOVA are also presented.

Experiment	Females	Males	Latency (min) ± S.E.	n	Male effect	Female effect	Interaction
1	Young	T	13.9 ± 2.5	54	d.f. = 1, 175 F = 0.283 p = 0.595	d.f. = 1, 175 F = 0.597 p = 0.441	d.f. = 1, 175 F = 0.146 p = 0.703
		NT	14.3 ± 3.3	28			
	Aged	T	14.9 ± 2.0	54			
		NT	17.2 ± 2.2	41			
2	Young	T	11.2 ± 2.4	58	d.f. = 1, 205 F = 0.040 p = 0.852	d.f. = 1, 205 F = 11.220 p = 0.001	d.f. = 1, 205 F = 5.780 p < 0.001
		NT	16.2 ± 3.0	45			
	Aged	T	21.7 ± 2.6	63			
		NT	14.5 ± 1.9	41			
3	Young	T	13.8 ± 1.7	35	d.f. = 1, 172 F = 1.281 p = 0.259	d.f. = 1, 172 F = 4.243 p = 0.041	d.f. = 1, 172 F = 0.369 p = 0.544
		NT	13.4 ± 2.1	51			
	Aged	T	24.9 ± 3.6	42			
		NT	22.1 ± 3.2	45			

Experiment 1: 10-12d-old males which were treated within the first 24 h post-emergence vs. 10-12d-old males which were not treated. **Experiment 2**: 18-22d-old males which were treated within the first 24 h post-emergence vs. 18-22d-old males which were not treated. **Experiment 3**: 10-12d-old males which were treated at day 8 post-emergence vs. 10-12d-old males which were not treated. Young females: 10-12d-old. Aged females: 18-22d-old. T = treated; NT = non-treated. n = number of replicates; d.f. = degrees of freedom; F = ANOVA statistic; p = statistical significance (α = 0.05).

Table 3. Mean (±S.E.) of mating duration for each experiment. Mating duration is presented for each type of mating pair. Results of the two-way ANOVA are also presented.

Experiment	Females	Males	Duration (min) ± S.E.	n	Male effect	Female effect	Interaction
1	Young	T	68.6 ± 4.0	54	d.f. = 1, 175 F = 0.410 p = 0.524	d.f. = 1, 175 F = 14.240 p < 0.001	d.f. = 1, 175 F = 0.440 p = 0.507
		NT	68.7 ± 6.4	28			
	Aged	T	91.5 ± 5.2	54			
		NT	84.8 ± 4.6	41			
2	Young	T	71.6 ± 3.2	58	d.f. = 1, 205 F = 0.120 p = 0.725	d.f. = 1, 205 F = 13.440 p < 0.001	d.f. = 1, 205 F = 1.140 p = 0.286
		NT	67.0 ± 3.2	45			
	Aged	T	83.5 ± 5.2	63			
		NT	82.8 ± 3.8	41			
3	Young	T	56.3 ± 7.8	35	d.f. = 1, 172 F = 0.309 p = 0.579	d.f. = 1, 172 F = 9.402 p = 0.003	d.f. = 1, 172 F = 0.518 p = 0.473
		NT	53.4 ± 2.6	51			
	Aged	T	72.0 ± 4.9	42			
		NT	64.7 ± 3.5	45			

Experiment 1: 10-12d-old males which were treated within the first 24 h post-emergence vs. 10-12d-old males which were not treated. **Experiment 2**: 18-22d-old males which were treated within the first 24 h post-emergence vs. 18-22d-old males which were not treated. **Experiment 3**: 10-12d-old males which were treated at day 8 post-emergence vs. 10-12d-old males which were not treated. Young females: 10-12d-old. Aged females: 18-22d-old. T = treated; NT = non-treated. n = number of replicates; d.f. = degrees of freedom; F = ANOVA statistic; p = statistical significance (α = 0.05).

3.2. Effect of methoprene treatment on male calling behaviour and sex pheromone

There were no significant differences between methoprene-treated and non-treated males in the rate at which they performed wing fanning ($t_7 = 0.769$, $p = 0.233$) (Fig. 4a). Salivary glands exposure showed a similar result ($t_7 = 1.672$, $p = 0.070$) (Fig. 4b), however in this case there is a tendency favouring treated males which might reflect a lack of replication as the statistical power for this specific test was 59.6%. Methoprene-treated males released larger amounts of the four pheromonal compounds than non-treated males (suspensolide:

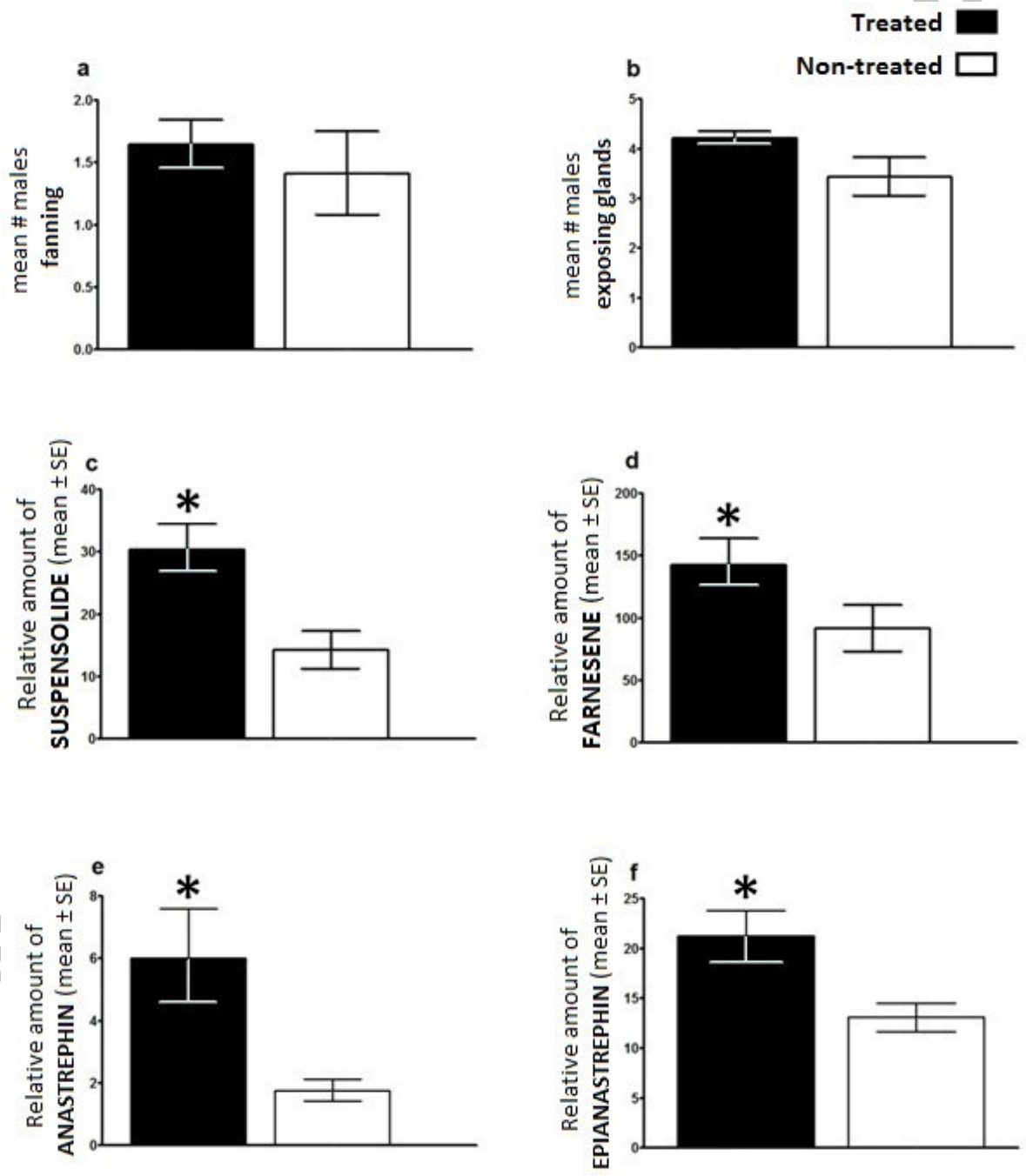
376 t_7 = 2.606, p = 0.018; farnesene: t_7 = 2.148, p = 0.034; anastrephin: t_7 = 2.817, p = 0.013;

377 epianastrephin: t_7 = 2.440, p = 0.022) (Fig. 4c-f).

378

379

380 **Figure 4**



381

382 **Figure 4.** ***Males' calling behaviour and pheromone release.*** a) Mean (±S.E.) number of
383 males performing wing fanning or b) salivary glands exposure. c-f) Mean (±S.E.) amount of
384 each of the four pheromonal compounds under study released per male. Asterisks indicate
385 significant differences ($p < 0.05$) between treated and non-treated males (Student's *t*-test for
386 paired samples).
387

388 **4. Discussion**

389 Most studies on the role of JH analogues on the physiology and behaviour of tephritid fruit
390 fly adults have focused on its effect during the accelerated sexual maturation phase
391 (ASMP) (Gómez-Simuta & Teal 2010, Chacón-Benavente *et al*. 2013, Haq *et al*. 2013,
392 Segura et al. 2013, Collins *et al*. 2014). Here, we found that topical treatment with
393 methoprene on recently emerged *A. fraterculus* males resulted in a generalized
394 enhancement of their mating success even when these males were tested after this phase,
395 showing a similar trend when evaluating young or aged females. In contrast, when
396 methoprene treatment was applied close to sexual maturation (8 days-old), there was no
397 improvement of their mating success for either of the two types of females. Methoprene-
398 treated males were found to release larger amounts of pheromonal compounds when
399 compared with non-treated males.

400 Previous works with *A. fraterculus* and two other *Anastrepha* species evaluated the
401 mating competitiveness of males treated with methoprene during the ASMP, finding that the
402 proportion of sexually mature males is much higher in treated males compared to non-
403 treated ones and suggesting that the increase in the proportion of sexually mature
404 individuals is due to an accelerated development (Segura *et al*. 2009, Pereira *et al*. 2010,
405 Gómez-Simuta & Teal 2010, Liendo *et al*. 2013). Here, we focused on the effects of
406 methoprene treatment on these males at a later stage (post-ASMP), and found that these
407 effects do not decrease over time, at least up to approximately 20 days post-emergence.
408 Our results are in agreement with those recorded for *A. suspensa* (Pereira *et al*. 2010),
409 which suggest that methoprene would act as a mating enhancer. This enhancing effect has

410 also been found after exposure to guava fruit volatiles in *A. fraterculus* (Vera *et al*. 2013,
411 Bachmann *et al*. 2015), and ginger root and orange essential oils in *C. capitata* (Shelly &
412 McInnis 2001, Papadopoulos *et al*. 2006). However, in these works males were exposed to
413 odour sources without physical contact (only volatiles). Given that methoprene belongs to
414 the sesquiterpenoid family, like many of the compounds released by guava (Bachmann *et*
415 *al*., 2015), it would be interesting to see the outcome of exposing males to methoprene
416 volatiles rather than to topical treatment. It might be hypothesized that the mechanisms
417 behind the mating enhancement are similar for both types of applications; yet this idea
418 remains to be properly addressed.

419 Latency to mate and mating duration were not affected by methoprene treatment,
420 even in those cases where the treatment positively affected males mating success.
421 Interestingly, we found that aged females took longer than young females to engage in
422 mating. This may indicate that aged females require a prolonged stimulation before
423 accepting a partner to mate: *A. fraterculus* females used here were sexually mature 10
424 days after emergence; and keeping them for another 10 days (18-22 days-old) without
425 access to males may have induced a state of reproductive arrestment (Tatar & Yin, 2001),
426 which would be slowly reverted after perceiving a calling male. Moreover, the copulations of
427 these females lasted longer than those of young ones. Fritz (2004) and Abraham *et al*.
428 (2011) found a positive correlation between the mating duration and the number of sperm
429 stored by females of *A. suspensa* and *A. fraterculus*, respectively. Therefore, we might
430 expect that aged females were supplied with more sperm than young ones. One possible
431 interpretation is that aged females encountering a male for the first time and having a
432 reduced possibility to copulate again in the future, extend the current copulation to obtaining
433 larger volumes of sperm. In any case, further studies on the behaviour and physiology of
434 the female are needed.

435 Many works have found that methoprene treatment affects sexual maturation and
436 competitiveness of tephritid fruit fly males, but practically none of them have elucidated the
437 mechanisms underlying these phenomena. Pereira *et al*. (2010), based on previous work
438 by Teal *et al*. (2000), and Teal & Gomez-Simuta (2002), hypothesized that mating
439 enhancement in *A. suspensa* was related to an increase in sexual signalling and
440 pheromone release, but they did not evaluate this hypothesis. Here, 10 days-old, treated *A.*
441 *fraterculus* males released more pheromone than non-treated males. This corresponds to
442 the higher mating success recorded for treated males, suggesting that females are
443 attracted to the partner that emits a higher amount of pheromone. In contrast, methoprene
444 treatment did not affect so strongly males' calling behaviour. Wing fanning did not differ at
445 all between treated and non-treated males, whereas salivary gland exposure showed a
446 marginally non-significant tendency favouring treated males. Abraham *et al*. (2013) found
447 that during the ASMP, treated males were unable to induce, on females, a mating refractory
448 period (time between the first and the second mating) comparable to that induced by
449 naturally matured males, even when both types of males mated in similar proportions.
450 These authors suggested that methoprene treatment may accelerate some components of
451 the sexual system, but a time lag of other maturation components may occur. Our results
452 also agree with the idea that this juvenile hormone analogue might not act evenly in all the
453 components of the sexual system, given the observed decoupling between pheromone
454 emission and calling behaviour (which was affect only marginally). In a related study,
455 Chacón-Benavente *et al*. (2013) found that treated males of *A. obliqua* performed sexual
456 behaviours and released farnesene at a higher rate than non-treated ones, during and after
457 the ASMP, suggesting that in some species methoprene may act on different reproductive
458 parameters more synchronically than on others.

459 When methoprene treatment was applied close to sexual maturity, the improvement
460 of mating success was no longer observed (as when applied at emergence), suggesting its

effect is not immediate. Shelly *et al*. (2007) proposed that exposure of males of *C. capitata* to ginger root oil resulted in the retention of some compounds in the cuticle which would elicit attraction in the females and, therefore, confer a mating advantage ("perfume effect"). Although Experiment 3 was not specifically designed to test the perfume effect, our results do not provide evidence to support this kind of mechanism when methoprene treatment is carried out after the ASMP in *A. fraterculus* males. In fact, as found in previous experiments, it seems that the mode of action of methoprene is triggering a physiological change that makes males to release sexual pheromone at higher rates. This supports the idea that methoprene is internalized through the integument.

The accessory reproductive glands of males of many insect species produce bioactive secretions called accessory gland products (AGPs) (Guillott 2003), which are transferred to females with the ejaculate (Clifton *et al*. 2014). In *Heliothis virescens* (Ochsenheimer) (Lepidoptera: Noctuidae), Park *et al*. (1998) and Shu *et al*. (1998) showed that male AGPs contain JH as well as components that induce the endogenous synthesis of JH in females, which, in turn, stimulates egg production. Later, Pszczolkowski *et al*. (2006) demonstrated that the fertility of females increases due to the JH transferred in the AGPs. In *Aedes aegypti* (L.) (Diptera: Culicidae), Fernández & Klowden (1995) found that males treated with methoprene contained more AGPs than non-treated males. By mating with males with high JH levels (either acquired naturally or induced by methoprene treatment), females may have an advantage in terms of fecundity over females mated with males with low JH levels. This may be especially beneficial for females at the beginning of the reproductive process, when females are ready to mate and JH level may be still low, and egg maturation has not yet been completed. If this holds true in *A. fraterculus*, then females that mate with males with high levels of JH may increase their reproductive potential and this might explain the higher mating success of methoprene treated males. The main function of JH in adult females of many insects is related to egg production (Guillott 2003,

Gruntenko *et al*. 2005). Thus, the increase in mating success may be related to direct benefits for the females in terms of fecundity and fertility (Gwynne 1984, Reinhold 1999, Arnqvist & Nilsson 2000, Kumaran *et al*. 2013), particularly to oogenesis, as was found in other insect species (Fernández & Klowden 1995, Pszczolkowski *et al*. 2006). In addition, indirect benefits by increasing the fitness of their offspring (Fedorka & Mousseau 2002) cannot be discarded. These hypotheses concerning sexual selection remain to be explored and further studies are needed to fully understand the possible advantages (through direct or indirect benefits) that females gain from mating with sexually enhanced males.

Unlike most studies on other species of Tephritidae, in which the effect of JH analogues was studied during the ASMP, here we focused on the sexual behaviour and performance of males after they had passed the ASMP. This treatment induced artificially matured males to release larger amounts of pheromone and to engage in mating more frequently than males that matured naturally. Like the exposure to guava fruit volatiles (Bachmann *et al*. 2015), methoprene treatment may be used to increase the efficiency of the SIT by improving the quality of sterile males. The enhancement of mating success observed in this study would add an important benefit to the use of methoprene to accelerate the sexual maturation of sterile males. Furthermore, the persistence of the effect observed in 20 days-old males represents an additional advantage for the SIT. Note, nonetheless, that males were treated with methoprene dissolved in acetone. Alternative methods that do not require this solvent should be considered. First, because using acetone at large scales (as those required in the framework of SIT) is not feasible. Second, because methoprene effect cannot be separated from a potential effect of acetone under our experimental design, and even when previous studies showed that methoprene accelerates sexual maturation in absence of acetone (Gómez-Simuta *et al*. 2013; Adnan 2015), none of these studies were conducted in *A. fraterculus*. Likewise, it should be considered that this work was done under laboratory conditions with fertile (i.e., not

irradiated) males. Further experiments under semi-field conditions in which sterile males treated with methoprene compete with fertile males from the wild are also needed.

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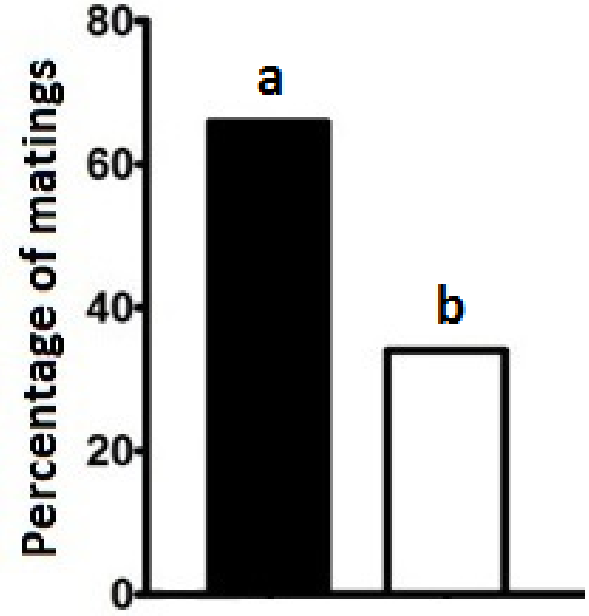
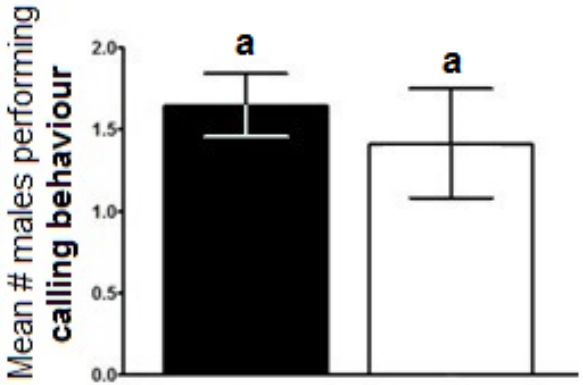
METHOPRENE TREATMENT
0 d-old males

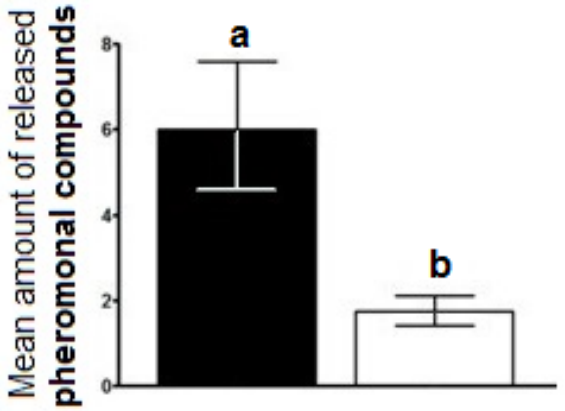
Treated

Non-treated

Mature males







735 Highlights

736

737 - Methoprene treatment at emergence enhanced male mating success after sexual maturity

738 - Both young and old females mated more frequently with methoprene-treated males

739 - Methoprene applied close to sexual maturation did not increase male mating success

740 - Methoprene-treated males released larger amounts of sex pheromone

741 - Males' calling behaviour was affected by methoprene treatment to a much lesser extent

742