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Methoprene treatment reduces the pre-copulatory period in *Anastrepha fraterculus* (Diptera: Tephritidae) sterile males

D. F. Segura[1,2], M. E. Utgés[2,3], M. C. Liendo[1,2], M. F. Rodríguez[1], F. Devescovi[1], M. T. Vera[2,4], P. E. A. Teal[5] & J. L. Cladera[1]

1 Instituto de Genética ''E.A. Favret'', CNIA, INTA Castelar, Buenos Aires, Argentina
2 CONICET, Argentina
3 Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina
4 Estación Experimental Agroindustrial Obispo Colombres, Tucumán, Argentina
5 Center for Medical, Agricultural, and Veterinary Entomology, ARS-USDA, Gainesville, FL, USA

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Correspondence
Diego F. Segura (corresponding author), Instituto de Genética ''E.A. Favret'', CNIA, INTA Castelar, Los Reseros y Nicolás Repetto, Castelar, B1712DIA Argentina.
E-mail: dsegura@cnia.inta.gov.ar

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Abstract

Anastrepha fraterculus is a major fruit pest in South America. Ongoing studies support the implementation of the sterile insect technique (SIT) against this pest. Sexual readiness of sterile males is a key point for SIT application. The pre-copulatory period of *A. fraterculus* males has not been reported before, but it is expected to last several days. An acceleration of sexual maturation was achieved in other *Anastrepha* species after topical applications of juvenile hormone analogues, like methoprene. Here, we studied the effect of methoprene on male sexual maturation, mating duration and sperm transfer in *A. fraterculus* as well as the impact of acetone (methoprene solvent) on survival. We also explored a method to deliver methoprene massively. Pheromone-calling and mating ability were evaluated daily from adult emergence, and used as indicators of sexual maturity. *Anastrepha fraterculus* males showed a long pre-copulatory period (7 days approximately), as other *Anastrepha* species. This process was accelerated after methoprene treatment (2.5 μg/μl), both in non-irradiated and irradiated males which matured 2–3 days earlier. Mating duration for methoprene-treated males was longer than for mature untreated males, however, no differences in sperm transfer were detected. Survival was not affected by acetone. Dipping pupae in methoprene allowed emerging males to mature as fast as those receiving topical application as adults. Dipping of pupae is a promising method to deliver massively methoprene and should be further investigated.

Introduction

The Tephritidae family comprises many species that are pests, because their females use commercially important fruit to lay eggs. Among other methods that have been developed to control these fruit pests, the sterile insect technique (SIT) (Knipling 1959) has proven to be an effective and environmentally safe strategy (Klassen and Curtis 2005). This method relies on the mass rearing and release of sterile insects, which are expected to mate with wild conspecific insects and induce a significant level of sterility within the natural population. Developed largely to suppress, contain or eradicate Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), populations (Hendrichs et al. 1983, 2002), its use has now been extended to other genera of the Tephritidae family, with the concomitant need to modify the technology to suit new species (Cáceres et al. 2007). A good example of this is the process of sexual maturation, which can be particularly long in some species of *Anastrepha* (7–8 days) and *Bactrocera*

(2–4 weeks) when compared to *C. capitata* (3–4 days) (Teal et al. 2007; Faria et al. 2008; Shelly et al. 2009). Within *Anastrepha*, long pre-copulatory periods have been reported for *Anastrepha suspensa* (Loew) (Teal et al. 2000; Pereira 2005; Pereira et al. 2009), *Anastrepha ludens* (Loew) and *Anastrepha obliqua* (Macquart) (Teal et al. 2007). The slow maturation necessitates the release of immature flies that will be subject to predation and other losses or a long pre-release, holding period until close to maturity, increasing operational costs (food, space, and staff personnel to maintain sterile flies until maturation) (Enkerlin 2007). Moreover, holding flies for too long in confined environments before releasing them causes physical damage to the insects, forcing the release of sexually immature individuals (Teal and Gómez-Simuta 2002).

This downside for applying the SIT against *Anastrepha* species has prompted the study of factors that regulate sexual maturation in these species. Sexual maturation in insects is under hormonal control (Happ 1992) with juvenile hormone (JH) playing a key role in this process. Although JH effects have been studied mainly in females (Wyatt and Davey 1996; Gilbert et al. 2000; Wilson et al. 2003; Chen et al. 2004; Gruntenko et al. 2005), JH is also involved in male sexual maturation, with maturation of the accessory glands being identified as its principal role (Wilson et al. 2003; Klowden 2007). JH has also been reported to mediate response to sexual pheromones in insects (Anton and Gadenne 1999).

Teal et al. (2000) have shown that a topical application of methoprene or fenoxycarb (analogues of JH) induced precocious reproductive development and accelerated sexual maturation of *A. suspensa* males. More recently, JH treatment has shown to provide similar results in other *Anastrepha* species, such as *A. ludens* and *A. obliqua* (Teal et al. 2007). Although these studies have confirmed that young JH exposed males can successfully mate with mature females, no work has addressed sperm transfer or any other trait that modulates female receptivity (and therefore remating motivation), an aspect that could affect the effectiveness of SIT (Vera et al. 2003a,b; Bonizzoni et al. 2006).

The South American fruit fly, *Anastrepha fraterculus* (Wiedemann), is a major fruit pest attacking about 80 plant species and cultivars, many of them of economic importance (Norrbom 2004). It is present in many countries of the Americas from Texas, in the USA, to central Argentina (Salles 1995; Malavasi et al. 2000). Only insecticides are currently used to control it, so the implementation of environmentally safe control techniques, such as the SIT, has been proposed (IAEA 1999) resulting in intensive research efforts (Jaldo 2001; Walder 2002; Petit-Marty et al. 2004; Vera et al. 2006, 2007; Allinghi et al. 2007a,b; Gómez Cendra et al. 2007; Sciurano et al. 2007; Segura et al. 2007). Although many aspects of the biology of *A. fraterculus* have been studied (especially those related to sexual behaviour, radiation biology, genetics and artificial rearing) and the perspectives of SIT implementation are promising, no attempts have been made to analyse the pre-copulatory period of *A. fraterculus* males and the effect of JH on this process.

The objectives of the present study were: (i) to describe the process of sexual maturation in *A. fraterculus* sterile males; (ii) to evaluate the effect of different doses of methoprene on this process; (iii) to determine if methoprene treatment affects male survival; (iv) to determine whether methoprene treatment negatively affects male mating parameters such as mating duration and sperm transfer; and (v) to evaluate, should methoprene treatment show promising results, a method for delivery on a large scale.

Materials and Methods

Insects

Anastrepha fraterculus adults were obtained from the rearing facility established at the Estación Experimental Agroindustrial Obispo Colombres. Rearing followed standard protocols: eggs were collected during a 24-h period, bubbled for 48 h, with larval development and pupae maturation being performed under controlled conditions (*T*: 25°C, RH: 70%, photoperiod: 14L–10D) (Jaldo 2001; Vera et al. 2007). Flies were separated by sex after emergence and maintained in independent cages. Adult flies were fed a diet composed of sugar, hydrolyzed yeast and hydrolyzed corn (in a 4 : 1 : 1 ratio) and vitamins (A, B_1, B_2, B_6, C and D) (Jaldo 2001). Conditions (*T*: 25°C, RH: 65%; photoperiod: 14L–10D) were kept as constant as possible to guarantee homogeneity among experiments.

Behavioural assessment of reproductive status

Sexual maturity was determined daily from the first day after adult emergence. Given that mature males show characteristic behaviours, such as joining mating aggregations (or *leks*), releasing sexual pheromone, courting females and attempting copulations

(Malavasi et al. 1983; Salles 1995; Petit-Marty et al. 2004; Segura et al. 2007), we considered that direct observation of such behaviours was an appropriate method to evaluate whether a male reached sexual maturity. One day after emergence, males were placed in an experimental arena and the day they either performed pheromone-calling or mated (depending on the type of arena) for the first time was registered.

'Calling behaviour' was assessed in groups of 10 males placed in 3-l (20 cm high, 13 cm in diameter) glass containers with water, food and an artificial green twig made of plastic. Calling was evident by the exposure of the anal gland at the tip of the male abdomen and the protrusion of the salivary glands on the sides of the abdomen. The experiment was conducted under controlled laboratory conditions (T: 24–26°C, RH: 60–70% 1000–1200 luxes). Each male was painted on the thorax with a particular colour to allow individual identification. Every day, starting at 8:30 AM, males were visually inspected, and the pheromone-calling status was noted for each one of them. The observation was repeated every 15 min until 10:30 AM (covering the main period of sexual activity in this species, Petit-Marty et al. 2004).

The ability to copulate was assessed by the acceptance of males by sexually mature virgin females. The respective experiments involved caging of five males in a 3-l glass container with water, food an artificial twig, along with 10 mature (14-day-old) virgin females. At 8:30 AM, and every 20 min after that, the activity of each male was registered. When a couple was detected, it was gently removed from the container. The observation period ended at 10:30 AM. Mature females that mated were not replaced, however the male : female ratio was never below 0.5.

For both behavioural parameters (calling and mating), the observations continued daily until every male in the arena called or mated.

Experiments

(1) Effect of juvenile hormone treatment on male sexual maturation

The effect of JH treatment on male sexual maturation was assessed by applying 1 μl of methoprene dissolved in acetone (5 μg/μl) on newly emerged males (3 h after emergence). Methoprene was applied on the thorax of the fly using a micropipette (Multipipette Plus Eppendorf, Hamburg, Germany). Dose and application method followed Teal et al. (2000). The following day a set of males was evaluated for pheromone-calling activity and another set for mating ability. Males were randomly assigned to each experiment. We carried out parallel observations of males from two control groups: untreated males and males treated with 1 μl of pure acetone. Fifteen replicates (glass containers) were performed for the treatments in which the males were treated with methoprene or pure acetone, and 20 for the control treatment.

(2) Effect of dose of methoprene on males sexual maturity

To determine the minimum amount of methoprene required to achieve a significant reduction in the age of sexual maturation (minimum effective dose), four doses of methoprene were applied to newly emerged males: 0 μg/μl (pure acetone), 1, 2.5 and 5 μg/μl. Each male received 1 μl of one of the four doses. After topical application, calling and mating assays were performed as described above. Ten replicates (glass containers) were performed for each treatment.

(3) Effect of juvenile hormone treatment on sexual maturation of irradiated males

To determine if methoprene treatment induced sexual maturation in sterile males, behavioural assessment of maturity was carried out in gamma-irradiated males. Pupae were irradiated at the Comisión Nacional de Energía Atómica 48 h before emergence with a dose of 70 Gy, which ensures 100% of sterility without side effects on male copulatory success (Allinghi et al. 2007b). After emergence, males were topically treated with methoprene at a dose of 5 μg/μl. Sexual maturation was evaluated on four types of males: (i) irradiated and treated with methoprene; (ii) irradiated and untreated; (iii) not irradiated and treated with methoprene; and (iv) not irradiated and untreated (control test). Ten replicates (glass containers) were performed for each treatment.

(4) Mating duration and stored sperm

To determine if methoprene treatment affected copulation duration or amount of sperm transferred, two mating arenas were arranged with ten 14-day-old untreated females and either five 5- to 7-day-old methoprene-treated males or five 10-day-old untreated males. Treated males were topically treated with 1 μl of methoprene at a dose of 5 μg/μl. Males and females were marked on the thorax to allow individual tagging of both sexes, and hence each male and female forming a mating couple could be identified. Mating start and ending time

was registered for each couple. After the mating couple had separated, flies were removed from the arena, and females were dissected. Spermathecae were removed, placed on a slide, and a drop of orcein was added. After 30 min, the drop of orcein was covered with a cover slide and a soft squash was performed to break the walls of each spermatheca. The preparations were visualized using a phase contrast microscope Olympus BX40 (Tokyo, Japan). Each spermatheca was assigned into one of the following three categories: empty (no sperm found), sperm traces (few spermatozoids, easily individualized) or large amounts of sperm (large bundles of sperm in which no spermatozoid could be individualized) (fig. 1). For each female, the condition of each spermatheca was registered.

(5) Effect of acetone on survival

Because acetone was used as solvent of methoprene, we studied its effect on male survival. Males were either treated after emergence with 1 μl of acetone or left untreated and placed in 3-l glass containers with water and food in groups of 10. After 4 days, food was removed from half of the containers. In the rest of the containers, food was supplied throughout the experiment. Water was supplied throughout the experiment for both dietary groups. A total of 40 containers (replicates) were analysed per treatment. The number of living flies inside each flask was checked daily. Replicates in which the food was removed at day 4 were discarded when all males died. Replicates in which flies were fed throughout the experiment were discarded whenever half of the males had died.

(6) Methoprene delivery at large scale

To evaluate an alternative method to deliver the methoprene to large numbers of flies, we immersed pupae in solutions containing methoprene. We analysed two solvents: water and acetone. In both cases, a 1 : 100 dilution of a 5 μg/μl solution of methoprene in acetone was used. Forty-eight hours before emergence, pupae were immersed into one of these solutions for 5 min, and then dried by placing them onto a tissue paper for 10 min. As controls, we included three additional treatments: dipping in pure acetone, individual topical application (1 μl of a 5 μg/μl solution of methoprene) and no treatment. After emergence, males were placed in glass containers with adult diet and water. After 3 days, males were marked and then transferred to poly-methylmethacrylate cylindric cages (20 cm in diameter, 16 cm long, ca. 5 l) in groups of five. The cages contained food, water, an artificial twig and 10 mature females. On day 4 after emergence, and for three consecutive days, we recorded the number of mating pairs.

Data analysis

In experiments 1–3, the mean age at which males started to release sexual pheromone and to mate were analysed by ANOVA. One-way ANOVA was used in experiments 1 and 2, and a two-way ANOVA in experiment 3 (with irradiation and methoprene treatments as factors). Whenever significant differences were detected, means were separated using the Tukey's HSD test.

In experiment 4, mating duration was compared by a *t*-test for independent samples, with the type of male as the main factor. The amount of sperm stored was ranked for each female by considering all three spermathecae. A value was assigned to each spermatheca according to the amount of sperm found: 0 when it was empty; 1 when sperm traces were detected and 2 when sperm bundles were found. The condition for each female was obtained by adding the values assigned to each spermatheca. A Mann–Whitney was performed using this value as dependent variable and the type of male as factor.

In experiment 5, estimated survival curves were computed using the Kaplan–Meier method and a

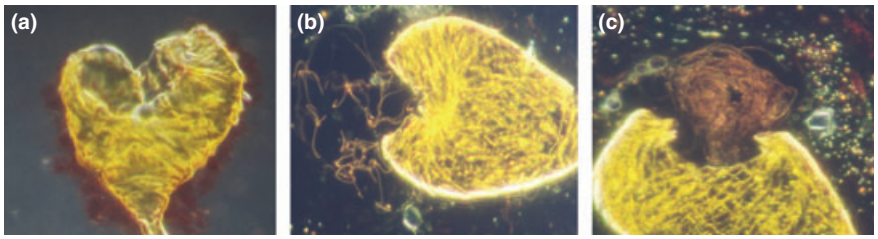

Fig. 1 Spermathecae of *Anastrepha fraterculus* corresponding to the different categories in terms of sperm storage: (a) empty; (b) traces; (c) large amounts.

parametric survival regression model was fitted assuming an exponential and a Weibull distribution for survival (response variable). Afterwards, and to select the best model, we performed an ANOVA. To compare both groups, we also performed a log-rank test. While data from the 4-day fed group was considered uncensored, data from flies that were fed throughout the experiment had censored and uncensored data, so the mean and its variance are based on a truncated estimator. The restricted mean was calculated using the maximum time for all the curves as a common upper limit for the AUC (area under the curve) calculation. The analyses were performed using the survival package for R (v. 2.9.0) (R Development Core Team 2009; Therneau and Lumley 2009).

In experiment 6, the percentage of mated males was submitted to a one-way ANOVA. ANOVA was performed separately for data obtained at days 4, 5 and 6. Whenever significant differences were detected, means were separated using the Tukey's HSD test.

All analyses (except survival analysis) were performed with STATISTICA for Windows (StatSoft Inc 2000).

Results

(1) Effect of juvenile hormone treatment on male sexual maturation

The cumulative per cent of calling males found each day of observation is presented in fig. 2a. Methoprene-treated males initiate sexual calling in younger ages compared to non-treated and acetone-treated males. Interestingly, acetone-treated males perform calling in younger ages than control males. Significant differences were found among types of males in the mean age for calling (ANOVA for mean age: $F_{2,47} = 34.748$, $P < 0.001$). Multiple comparisons show that methoprene treatment significantly reduced the age at which males started to call, with intermediate values for the males treated with acetone (table 1). The cumulative per cent of mated males is shown in fig. 2b. Similarly to calling behaviour, methoprene-treated males initiate mating earlier than non-treated and acetone-treated males. Significant differences were detected among types of males in the mean age of mating (ANOVA for mean age, $F_{2,35} = 49.000$, $P < 0.001$). Multiple comparisons show that methoprene-treated males matured faster than acetone-treated males, which in turn matured faster than untreated males (table 1).

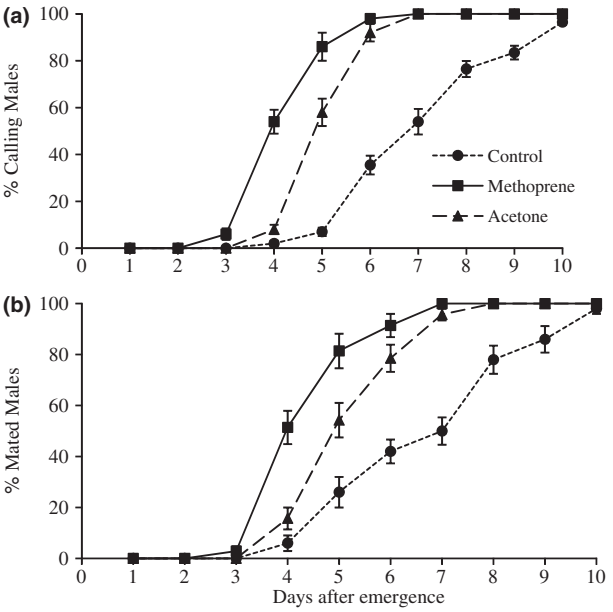

Fig. 2 Cumulative percentage (+SE) of *Anastrepha fraterculus* males engaged in sexual calling (a) or mating (b) for the three types of males studied in experiment 1: males treated with methoprene dissolved in acetone-, acetone-treated males and untreated males.

Table 1 Mean age (SE) in days at which males of *Anastrepha fraterculus* started to perform sexual calling or engaged in mating in experiments 1, 2 and 3. Within each experiment and behavioural parameter, means followed by the same letter did not differ statistically. Irr, irradiated males; JH, males treated with methoprene; Control, untreated and non-irradiated

Experiment	Treatments	Pheromone calling	Mating
1. Effect of Methoprene	Untreated	7.33 (0.11)a	7.05 (0.19)a
	Acetone	5.73 (0.11)b	5.56 (0.14)b
	JH	4.56 (0.12)c	4.73 (0.15)c
2. Effect of methoprene dose ($\mu g/\mu l$)	0	5.71 (0.11)a	5.57 (0.32)a
	1	5.27 (0.12)b	5.43 (0.25)a
	2.5	4.59 (0.10)c	4.32 (0.12)b
	5	4.43 (0.12)c	4.53 (0.19)b
3. Effect of irradiation and methoprene treatment	Irr+JH	4.80 (0.09)a	4.84 (0.19)a
	JH	4.91 (0.14)a	4.80 (0.20)a
	Irr	6.68 (0.08)b	6.24 (0.16)b
	Control	6.57 (0.06)b	6.28 (0.24)b

(2) Effect of dose of methoprene on males sexual maturity

Figure 3 shows the sexual maturation curve for males treated with different doses of methoprene. For both behavioural variables, males that received a dose of 5 $\mu g/\mu l$ and those that received a dose of 2.5 $\mu g/\mu l$ generally showed a similar pattern of

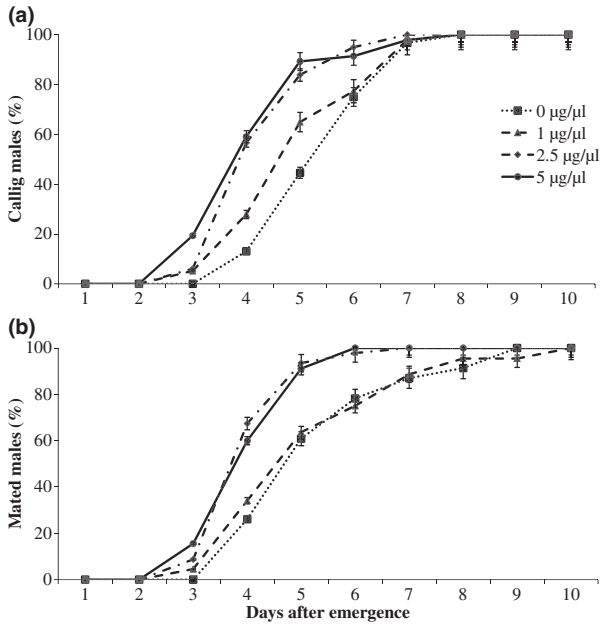

Fig. 3 Cumulative percentage (+SE) of *Anastrepha fraterculus* males engaged in sexual calling (a) or mating (b), for the four doses of methoprene evaluated in experiment 2.

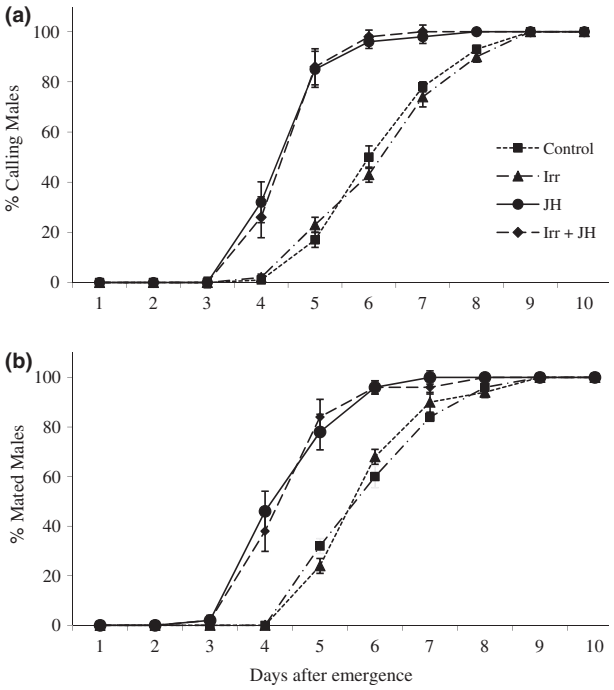

Fig. 4 Cumulative percentage (+SE) of males of *Anastrepha fraterculus* engaged in sexual calling (a) or mating (b), for the four types of males studied in experiment 3: irradiated and treated with methoprene (Irr+JH); irradiated and untreated (Irradiated); treated with methoprene and non-irradiated (JH); untreated and non-irradiated (Control).

sexual maturation. The same is true for the doses of 1 and 0 $\mu g/\mu l$. The mean age at which males started to call differed significantly among types of male (ANOVA: $F_{3,36} = 27.514$, $P < 0.001$, table 1, fig. 3a). Multiple contrasts show that the treatments with a dose of 2.5 and 5 $\mu g/\mu l$ were not statistically different and the remaining comparisons were all statistically significant ($P < 0.05$). The mean age at which males mated differed significantly among treatments (ANOVA: $F_{3,36} = 8.308$, $P < 0.001$, table 1, fig. 3b). Multiple contrasts show no significant differences between acetone-treated males and those that received a dose of 1 $\mu g/\mu l$, and between the doses of 2.5 and 5 $\mu g/\mu l$, but the remaining comparisons were all statistically significant ($P < 0.05$) (table 1).

(3) Effect of juvenile hormone treatment on sexual maturation of irradiated males

The temporal pattern of sexual maturation is presented in fig. 4. Our results demonstrate that irradiation status did not affect the onset of calling or mating behaviour, and only an effect of methoprene is evident. The mean age for calling and mating is shown in table 1 for each type of male. Significant differences in this variable were found in calling assays between methoprene-treated and untreated males, regardless of their irradiation status (two-way ANOVA: $F_{1,36}$ methoprene = 340.392, $P < 0.001$; $F_{1,36}$ irradiation = 0.012, $P = 0.936$; $F_{1,36}$ interaction = 1.223, $P = 0.277$). The ANOVA showed similar significant results for the mating experiments: differences in mean age of mating between treated and untreated males, and no effect of irradiation (two-way ANOVA: $F_{1,36}$ methoprene = 42.483, $P < 0.001$; $F_{1,36}$ irradiation = 0.002, $P = 0.991$; $F_{1,36}$ interaction = 0.039, $P = 0.844$).

(4) Mating duration and stored sperm

Copulations of methoprene-treated males lasted significantly longer than those of mature untreated males (45.17 ± 2.61 and 18.16 ± 1.64 min, respectively, *T*-test: $t_{104} = 8.476$, $P < 0.001$). On the other hand, no significant differences were detected in the amount of sperm stored (Mann–Whitney: $U_{104} = 1080.5$; $P = 0.078$).

(5) Effect of acetone on survival

The Weibull age-specific hazard model fit better than the exponential model ($P < 0.001$) for both diet regimes. We found no significant differences in sur-

vival between acetone-treated males or untreated males when males were fed only for the first 4 days after emergence [log-rank test $\chi^2(1) = 0.2$, P = 0.65; Weibull hazard scale = 0.18; mean survival (±SE) treated: 9.40 ± 0.10 days; $n_i = 389$; untreated: 9.33 ± 0.10 days; $n_i = 382$; fig. 5]. As in the previous case, when flies were fed throughout the experiment, no significant differences in survival were found between treated and untreated males [log-rank test $\chi^2(1) = 0.8$, P = 0.38; Weibull hazard scale = 0.45; mean survival (±SE) treated: 123.00 ± 2.50 days; $n_i = 400$; 45% censored data; untreated: 126.00 ± 2.35 days; $n_i = 399$; 43.4% censored data; fig. 5].

(6) Methoprene delivery at large scale

Males that were obtained from pupae dipped in methoprene diluted in acetone and those treated by topical application as adult had similar pre-copulatory periods, which were shorter than those with of the remaining treatment groups (table 2). The ANOVA showed significant differences among treatments for the three ages analysed (ANOVA: day 4: $F_{4,70} = 3.861$, P = 0.017; day 5: $F_{4,70} = 10.714$, P < 0.001; day 6: $F_{4,70} = 19.738$, P < 0.001). Multiple comparison analyses show that topically treated males and those that emerged from pupae dipped in methoprene diluted in acetone mature at the same time and significantly faster than the rest of the males, among which no differences were found (table 2).

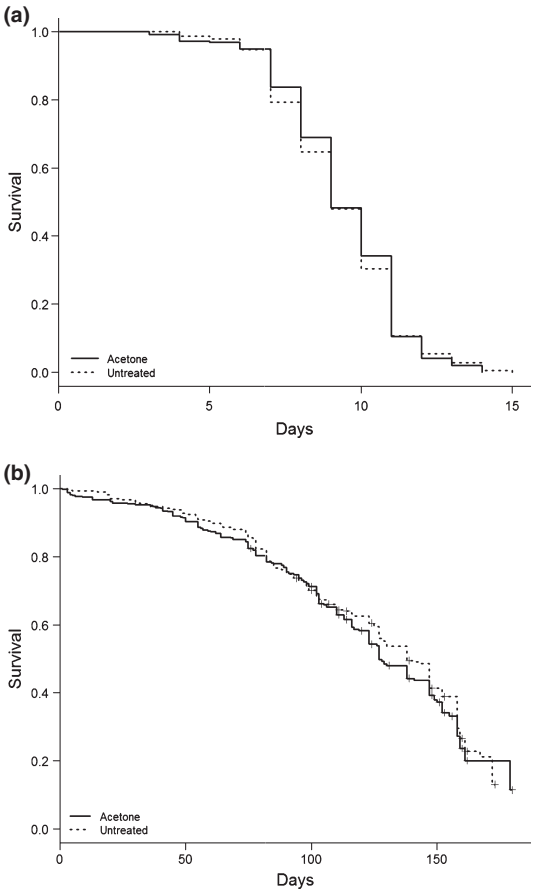

Fig. 5 Cumulative survival curves for *Anastrepha fraterculus* males treated with acetone and untreated males. (a) Cumulative proportion of 4-day-fed surviving males by group (Kaplan–Meier), uncensored data. (b) Cumulative proportion of surviving males fed throughout the experiment by group (Kaplan–Meier), censored (+) and uncensored data.

Discussion

In the present work, we have determined the time needed for *A. fraterculus* males to become sexually mature. Our results indicate that *A. fraterculus* males have a temporal pattern of sexual maturation similar to *A. suspensa*, particularly on mating behaviour (Teal et al. 2000). Males started to mate on the fourth day after emergence, with almost all males mated by day 10. Calling behaviour followed a similar trend, with the number of calling males increasing gradually to 50% by day 7. The fact that both parameters (calling and mating) followed the same dynamic supports the use of these behaviours as indicators of sexual maturation. *Anastrepha fraterculus* has a long maturation process compared to other tephritids, such as *C. capitata* (Faria et al. 2008), which could be viewed as a disadvantage to the use of the SIT against this species. However, our results showed that this can be overcome by the application of the JH analogue methoprene, as in other *Anastrepha* species (Teal et al. 2007). Males that were treated with methoprene matured significantly faster than untreated males, reaching sexual maturity at around day 4 after emergence. Although we do not know the mechanism by which methoprene affects male's behaviour, the response found is in agreement with JH's pivotal role in reproduction reported in adult insects (Klowden 2007). In addition, we found a faster maturation as the methoprene dose increased, suggesting a dose-dependent response to methoprene treatment.

The SIT relies on the principle that released males are sterile and any additional treatment to improve their effectiveness should take into account potential adverse effects on aspects related to their sexual behaviour and competitiveness (Cáceres et al. 2007). The method that is currently used to sterilize flies is irradiation, in particular gamma irradiation. Here,

Table 2 Mean percentage of matings obtained, at days 4, 5 and 6, of *Anastrepha fraterculus* males dipped (at pupae stage) in a methoprene solution using acetone (Dip acet+metho) or water as solvent (Dip H_2O+metho). Males from three control groups were examined: Control (untreated males); Dip acet (males dipped in pure acetone at pupal stage) and Top (males treated topically with methoprene). Mean percentages followed by the same letter did not differ between types of males

Treatment	% Mated day 4 (SE)	% Mated day 5 (SE)	% Mated day 6 (SE)
Control	1.33 (0.61)a	12.00 (3.26)a	44.00 (4.67)a
Dip H_2O+metho	2.67 (0.80)a	22.67 (6.28)a	45.33 (5.20)a
Dip acet	1.33 (0.60)a	20.00 (4.27)a	34.67 (3.06)a
Top	12.00 (1.34)b	42.67 (6.67)b	73.33 (6.00)b
Dip acet+metho	17.33 (1.41)b	48.00 (5.33)b	81.33 (6.96)b

we evaluated the effect of methoprene application to irradiated males using the optimal radiation dose established for *A. fraterculus* (Allinghi et al. 2007a,b) and found no interaction between radiation and methoprene treatment, supporting the joint implementation of these two pre-release treatments.

The SIT not only requires that released males are able to mate with wild females, but also that they effectively inseminate females with sterile sperm. Thus, a thorough evaluation of a male type to be release for pest control is not complete until some reproductive parameters (other than calling or mating) are evaluated (Vera et al. 2003a,b; Briceño et al. 2007). Here, we found no significant differences in the amounts of sperm stored in females mated with methoprene-treated males compared to those mated with mature untreated males. Thus, females not only accept younger treated males, but they also store similar amounts of sperm after mating treated or mature males. However, our method to estimate transferred sperm was qualitative, and sperm counting assays should be carried out to validate this result. Likewise, remating tendency should be addressed in females mated with treated and untreated males, to reveal whether females use young males sperm or will seek a different source of sperm to fertilize the eggs (Vera et al. 2003a,b; Bonizzoni et al. 2006). It is also interesting to note, that copulation time was longer for methoprene-treated males. This shows that longer copulations do not necessarily imply higher amounts of transferred sperm (Taylor et al. 2000, 2001). In fact, in some cases, very long copulations result in no sperm transfer at all (Mossinson and Yuval 2003).

To recommend the use of methoprene, it is not sufficient to test its effect on reproductive success and other aspects, such as survival, should also be assessed. An application of 1 μl of acetone did not affect the survival of the males when they were fed for only 4 days. When males were fed throughout the experiment, a difference was detected and around day 100 the survival curve for treated males decreased faster than that of untreated males. As methoprene-treated males would be sexually mature in the open field by day 7 after emergence, it can be concluded that there is no hazard in treating them with 1 μl of acetone. A rather unexpected result, concerning acetone application, was the apparent effect that this solvent had on sexual maturation, as acetone-treated males showed intermediate values between methoprene-treated males and untreated males. Contamination can be ruled out, because a different tip was used for pure acetone and methoprene solutions. We have no records of such effect in other *Anastrepha*; however, in another tephritid fly, *Bactrocera oleae* (Gmelin), acetone exposure prolonged immature developmental time (egg to adult) (Cosmidis et al. 2002). The fact that acetone exposure was attained in Cosmidis et al. (2002) by adding it to larval artificial medium makes any direct comparison difficult, but shows that acetone can affect development in a close-related species.

In the context of the SIT, the topical application of methoprene is not feasible, as millions of insects need to be periodically released. Based on promising results in *A. suspensa* and *A. ludens* (Pereira et al., this volume), we evaluated a mass method to deliver the methoprene to the flies based on dipping pupae in a methoprene solution. This method was effective, and males emerging from dipped pupae matured as fast as topically treated males, but only when acetone was the solvent. Although this is a fast and clean alternative to topical treatment, using large amounts of acetone may be unfeasible due to its high flammability and noxious fumes. Perhaps methoprene diluted in water did not show good results because of its poor solubility in this solvent. Conceivably another JH analogue with higher solubility in water could render similar results to methoprene in acetone. This should be further evaluated before dipping in water is ruled out as a plausible method to massively deliver methoprene to emerging males (especially since we found that with an appropriatesolvent, dipping can effectively accelerate maturation).

In conclusion, the present work provides a description of the dynamics of sexual maturation evaluated through pheromone-calling and mating

behaviour of *A. fraterculus* males. We found that this process can be accelerated by treating the males with methoprene without an impact on mortality. Methoprene treatment proved to be efficient and allowed males to engage in sexual signalling, mating and transferring of sperm on equal grounds as mature males, for fertile and for sterile males. Therefore, there is no hazard if the two treatments (gamma irradiation and methoprene exposure) are applied to the same fly. We also found that mass delivery of methoprene can be attained by dipping pupae in a methoprene solution, but more work in this regard is still needed to develop a fast and safe method that does not affect sterile male quality.

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