JOURNAL OF APPLIED ENTOMOLOGY

J. Appl. Entomol.

Exposure of sterile Mediterranean fruit fly (Diptera: Tephritidae) males to ginger root oil reduces female remating

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Keywords

Ceratitis capitata, polyandrous behaviour, α-copaene

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Received: April 16, 2010; accepted: September 16, 2010.

doi: 10.1111/j.1439-0418.2010.01584.x

Abstract

Females of *Ceratitis capitata* are facultative polyandrous, with remating more common in laboratory strains rather than wild ones. In the application of the Sterile Insect Technique (SIT) against this pest, large overflooding ratios of sterile : wild males can increase the remating frequency. Females that mate for the first time with a sterile male tend to remate more frequently. The exposure of sterile males to ginger root oil (GRO) is used in *C. capitata* SIT programmes to increase the sterile male mating success. Exposing males to an 'aromatherapy' with GRO may also increase the remating frequency among wild females. The frequency of wild females remating, number of matings per female, the refractory period between the first and second mating, and the duration of the first and second matings of wild females were determined under laboratory conditions for three mating scenarios that included wild males only or wild males competing with sterile males (either GRO-treated or non-treated). Wild females first mated with sterile males exposed to GRO had their remating rate over the following 6 days and the mean number of matings per female reduced in comparison to those first mated with non-exposed sterile males, from 62.5% to 32.2% and from 3.1 to 1.6 respectively. The remating parameters of females mated with sterile GRO-exposed males resembled those of females mated with wild males.

Introduction

The Sterile Insect Technique (SIT) is often used as a component of area-wide integrated pest management programmes for controlling insect pests (Dyck et al. 2005). This technique is widely used to reduce populations of the Mediterranean fruit fly (*Ceratitis capitata* Wied.) (Diptera: Tephritidae) in prevention, suppression, containment or eradication programmes (Hendrichs et al. 2005). In the field, sterile Mediterranean fruit fly males must compete with wild males in attracting and mating The sterile male production process subjects the insects to artificial and adverse conditions that modify their biology and behaviour and result in the decreased biological quality of sterile males (Cayol 2000; Lux et al. 2002b). Sterile males aggregate in mating arenas or *leks* with wild males, where they court attract females (Field et al. 2002; Lux et al. 2002a), but they 'call' (i.e. release the sexual pheromone) less frequently than wild individuals (Papadopoulos et al. 1998). Irradiated males perform the entire courtship pattern, but differ in the time spent on each activity, with a reduction of 40–50% matings in relation to non-irradiated males, according to Lux *et al.* (2002b).

Low sterile male competitiveness is a challenge that must be overcome to improve the SIT. Some efforts have been made to decrease the negative

effects of the production and sterilization process (Fisher 1997; Calkins and Parker 2005; Parker 2005) and to enhance sterile male sexual performance (McInnis et al. 2002; Shelly and Kennelly 2002; Yuval et al. 2007). The exposure of *C. capitata* sterile males to ginger root oil (GRO) volatiles before they are released is a simple procedure that can be implemented on a large scale (Shelly et al. 2004a,b), allowing lower overflooding ratios in the field (Barry et al. 2003) as sterile male mating competitiveness may be increased by up to three times (Shelly and McInnis 2001; McInnis et al. 2002; Shelly *et al*. 2002, 2003; Paranhos et al. 2008). However, the sterile males either exposed or not to GRO exhibit similar dispersal behaviour and post-release survival in the field (Paranhos et al. 2010).

After a successful courtship, mating occurs, and sperm transfer begins. Sterile males have viable sperm that carries dominant lethal mutations and they transfer it in association with accessory gland fluid into the female's sperm storage organs (Twig and Yuval 2005). Accessory gland fluid is a secretion rich in peptides that changes female physiology and behaviour in many aspects, including the olfactory 'switch' from attraction to the male pheromone to attraction to host fruit odours (Jang 2002). Irradiation does not affect the capacity of accessory gland fluid to mediate female post-mating behaviour (Jang 2002), but sperm transfer is quantitatively reduced by irradiation (Seo et al. 1990; Taylor et al. 2001).

In *C. capitata*, the propensity of a female to remate depends on the male's courtship behaviour, sexual performance, and ejaculate transferred, and is greater when the first mating occurred with a sterile individual (Hendrichs et al. 2002; Vera et al. 2002; Mossinson and Yuval 2003; Kraaijeveld and Chapman 2004). It has been shown that some Mediterranean fruit fly females remate in few hours after the initial mating and may not wait for the sperm supply to decrease to some critical threshold prior to remating (Yuval et al. 1996; Blay and Yuval 1997; Vera et al. 2003).

As aromatherapy with GRO improves the mating success of sterile males and female propensity to remate increases when the first mate is sterile, we might expect the use of GRO-treated males to increase the occurrence of female remating (above that observed using non-treated sterile males). Shelly *et al*. (2004a) found that laboratory females initially mated to non-irradiated laboratory males were more likely to remate with GRO-exposed sterile males than with non-exposed ones. However, when initially mated with sterile males, the GRO status of the initial mate had no effect on female tendency to remate with wild males.

In this study, we continue investigation of the possible effect of GRO exposure to sterile males on the remating propensity of wild females. We examined for the three mating scenarios (wild males only or wild males competing with sterile males, either GRO-treated or non-treated) and, the following parameters were recorded: female mating frequency, duration of the refractory period between first and second matings, and the duration of the first and second matings.

Materials and Methods

Study site

The experiments were carried out at the Entomology Laboratory, Embrapa Semi Arid, Petrolina-PE, Brazil. Temperature and relative humidity were not controlled (27–31°C and 10–80% RH), except inside the rooms where the sterile males were kept until the beginning of the tests (25–27°C, 50–60% RH and a 12 : 12 L : D cycle).

Insects

Wild flies were obtained from infested guava fruits collected in the São Francisco River Valley. Fruits were held over vermiculite, which provided a pupation substrate. One day after adult emergence, males and females were separated and maintained in plastic screened cages (0.003 m^3) with a maximum of 30 flies per cage, which were kept in the laboratory under natural conditions (27–31°C and 10–80% RH). Flies had free access to water and food (1 part yeast hydrolyzate enzymatic, 3 parts sugar and 0.4 parts wheat germ) until they became sexually mature (8–9 days old; Mossinson and Yuval 2003).

Sterile flies were provided as pupae by Moscamed Brazil Facility, Juazeiro-BA, Brazil. Sterile males belonged to the *tsl*-Vienna 8 genetic sexing strain and were irradiated as pupae 2 days before emergence at 95 Gy of gamma irradiation from a ^{60}Co source. Irradiated pupae were placed in two acrylic screened cages of 30 × 30 × 30 cm (30 ml of pupae per cage) for adult emergence. The adults had free access to water and food (the same of wild flies). The cages were maintained in the laboratory (25–27°C, 50–60% RH and a 12 : 12 L : D cycle) until the adults become sexually mature (5 days old; Papadopoulos *et al*. 1998).

Ginger root oil exposure

One day prior to the test, when they were 4 days old, half of sterile males were aromatically treated by exposing them to 2.7 ml of GRO (Citrus and Allied Essences Ltd, Lake Success, NY) in a separate small room (27 m^3; 0.1 ml of GRO/m^3) where the scent was dispersed by fans for 20 h at 25–27°C, 50–60% RH and a 12 : 12 L : D cycle. The other half (non-exposed sterile males) continued in the same condition as described above.

Marking

In order to be able to differentiate sterile and wild males, the sterile (either treated or non-treated with GRO) or wild males were alternately marked with a white dot of water soluble non-toxic ink (Faber-Castell®, São Paulo, SP, Brazil) on the thorax 1 day before the experiments, according the methodology described in the quality control manual (FAO/IAEA/USDA, 2003). The sterile males were marked 2 h before exposure to GRO.

Experiments

To determine the effect of GRO on remating, wild females were first allowed to mate under each one of the three mating scenarios (wild males only or wild males competing with sterile males, either GRO-treated or non-treated) and then they were offered the opportunity to remate over 6 days. For the first mating, 100 virgin wild males and 500 virgin sterile males (either exposed or non-exposed to GRO) were placed in a 30 × 30 × 30 cm acrylic cage at 6:30 AM with free access to water and food. Ten minutes later, 100 virgin wild females were added. The same procedure was followed with only wild males (600 wild males and 100 wild females). The two situations involving sterile males (either exposed or not to GRO + wild males) and the situation with only wild males represent the scenarios of either a Mediterranean fruit fly suppression area under SIT releases, where the sterile males compete with wild ones for females, or a wild population respectively.

As matings formed, the couples were collected and kept in acrylic tubes until they decoupled. Females were then separated from the males, and half of them were marked at random with a dot of paint on the thorax. Pairs of females, one painted and one unpainted were placed in 20 × 20 × 20 cm acrylic cages with free access to water, food and grape berries for oviposition. The grape berries, which were changed every 24 h, provided the females the opportunity to lay eggs and discharge their spermathecae.

From the next day, and during the next 5 days, females were allowed to remate in the same mating scenario. Every day at 6:30 AM, the females were removed from their cage and placed in an identical cage (20 × 20 × 20 cm) containing six wild males and 30 sterile males, either exposed or non-exposed to GRO or 36 wild males (for a total of 38 insects/cage, in a ratio of 1 wild female : 3 wild males : 15 sterile males or 1 wild female : 18 wild males respectively) with free access to water, food and grape berries. The cages were observed until 3:30 PM to detect mating couples. Once a couple formed, the female was identified, the couple was allowed to complete the copulation, and the mating duration was recorded. The couple remained in the cage, and the female was able to mate freely over the entire observation period. At the end of the observation period, the two females were removed from the cage and kept together in a similar cage but without males until the next morning. The same procedure was repeated over six consecutive days. This experiment was repeated twice to get at least 30 repetitions for each mating scenario, with each female alive until the end of the experiment being considered a repetition.

Data analysis

Data were first analyzed by combining all the females within each of the three mating scenarios (wild males only; wild and untreated sterile males; and wild and GRO-treated sterile males), regardless of the male type involved in the first mating, to evaluate the remating behaviour of females in a wild Mediterranean fruit fly population without or with SIT suppression. Differences in remating rate were evaluated by a homogeneity chi-squared test followed by pair-wise comparisons with Bonferroni's correction. The number of matings per female and the refractory period between the first and the second mating were analyzed by a nonparametric one-way ANOVA (Kruskal and Wallis test), and the mean values were compared by the nonparametric test of multiple comparisons. To evaluate the influence of the first male on both of these parameters, a nonparametric one-way ANOVA (Kruskal and Wallis test with comparison by Wilcoxon two-sample test) was performed within each scenario. As the mating duration data follow normal distribution ($P = 0.5878$, D'Agostino–Pearson normality test), independence and homocedasticity ($P > 0.05$, Bartlett's test of

homoscedasticity), the duration of the first mating was analysed by one-way ANOVA with the mean values compared by Tukey's Studentized Range (HSD) Test at P = 0.05. The duration of the second matings among the three different male types was compared according to the first mating by one-way ANOVA. All statistical analyses were performed by means of the SAS 9.2 Statistical Package (SAS Institute Inc., Cary, NC, USA).

Results

The remating rate varied significantly among the three scenarios ($\chi^2 = 17.30$; $P < 0.001$) (table 1). In the cages with sterile males without GRO treatment + wild males (normal SIT situation), the remating rate was significantly higher and reached 62.5% within the six observation days. In the case of sterile males treated with GRO + wild males, female remating frequency was significantly lower (32.2%) and resembled the value obtained with wild males only (31.11%) ($\chi^2 = 0.07$; P = 1.00 after Bonferroni's correction). Combining all the females from the three scenarios revealed that remating rate depended on the identity of the first male ($\chi^2 = 18.31$; $P < 0.001$). The remating rate of females first mated with GRO-exposed sterile males was equal to those that first mated with wild males ($\chi^2 = 0.07$; P = 1.00), while females first mated to sterile non-exposed males had a higher remating rate, which differed significantly from the other two types of males.

In terms of number of matings, the female population showed a significantly higher number of matings under a typical SIT scenario with sterile males non-treated with GRO + wild males (Kruskall Wallis nonparametric ANOVA, $H = 22.05$, d.f. = 2, $P < 0.0001$), were one female could mate until 13 times. Females from cages either with wild males only or sterile males treated with GRO + wild males had a similar number of matings (P = 1.00) (table 1), where the maximum numbers obtained were eight and five matings respectively.

When the number of matings per female was compared according to the identity of the first male, within each of the two scenarios involving wild and sterile males, there was no difference in the number of matings achieved by females first mated with wild or with sterile males, either treated or not with GRO ($H = 0.859$, d.f. = 1, P = 0.354 for wild and sterile males; $H = 0.549$, d.f. = 1, P = 0.457 for wild and GRO males) (table 1).

Refractory periods were similar in remating females from the three different scenarios ($H = 2.92$, d.f. = 2, P = 0.232) (table 1). This was also evident in the comparison according to the identity of the first male within the two mating scenarios involving wild males + sterile males, either treated or not with GRO ($H = 1.274$, d.f. = 1, P = 0.259 for wild and sterile males; $H = 1.099$, d.f. = 1, P = 0.294 for wild and GRO males). Combining all the females from the three scenarios revealed that the refractory period did not differ among females first mated with either wild, sterile or GRO-treated sterile males ($H = 2.12$, d.f. = 2, P = 0.346).

The duration of first mating was longer for females that mated with wild males than those that mated to sterile males, irrespective of whether they were exposed to GRO or not ($F = 22.83$, d.f. = 2, $P < 0.01$) (table 2). However, the duration of the second matings did not vary significantly among the three

Table 1 Number of remated females, remating rate, number of matings/female (mean ± SE) and refractory period (RFP) between the first and second mating (mean ± SE) of *Ceratitis capitata*, assessed in laboratory cages in three mating scenarios (wild males only, or wild males competing for wild females with sterile males, either exposed or not to GRO

	Mating scenarios				
	Wild	Wild + sterile		Wild + sterile GRO-exposed	
Identity first male		Wild	Sterile	Wild	GRO
Females evaluated	45	5	75	5	54
Remated females	14	2	48	1	18
Remating rate (%)	31.11b	62.50a		32.20b	
		40.00	64.00	20.00	33.33
Matings/female	1.60 ± 0.20b	3.11 ± 0.33a		1.56 ± 0.13b	
		2.6 ± 1.36	3.15 ± 0.34	1.2 ± 0.2	1.59 ± 0.14
RFP (days)	1.85 ± 0.42a	2.04 ± 0.19a		1.44 ± 0.14a	
		2.5 ± 0.5	2.02 ± 0.2	2.0	1.44 ± 0.14

Mean values within rows followed by the same letter were not significantly different (P > 0.05, Kruskal and Wallis test).

Table 2 Duration (minutes) of first and second matings of wild *Ceratitis capitata* females mated in laboratory cages to wild, sterile or GRO-exposed sterile males

First mating by type of male		Second mating by type of male		
		Wild	Sterile	Sterile GRO-exposed
Wild	114.89 ± 1.56 (419) A	95.21 ± 9.23 (14) a	93.50 ± 0.50 (2) a	96.00 (1) a
Sterile	94.10 ± 3.99 (86) B	112.83 ± 25.63 (7) a	85.52 ± 7.69 (41) a	–
Sterile GRO-exposed	96.14 ± 3.00 (82) B	73.00 ± 17.74 (4) a	–	65.46 ± 8.65 (14) a

Values are given as mean ± SE (N).
Mean values within a column (upper case) or row (lower case) followed by the same letter were not significantly different (P > 0.05, Tukey's test).

male types within each first mating category (F = 0.002, d.f. = 1, P = 0.99; F = 1.53, d.f. = 1, P = 0.22; F = 0.16, d.f. = 1, P = 0.68 for first wild, sterile and GRO male matings respectively) (table 2).

Discussion

This study analysed the effect of exposing the males to GRO volatiles on the tendency of wild *C. capitata* females to remate. We found under small cage conditions that female remating rates were influenced by the identity of the first male partner. Sterile males exposed to GRO were as effective as wild males in reducing female receptivity to remating. By contrast, females first mated with sterile males non-exposed to GRO had a higher remating rate and mated more times than the other females. In the future, this work should be extended to the standard field cage tests under semi-natural conditions.

When the sex ratio in a cage is male biased and the cost of refusing a courtship is higher than accepting it, female remating frequency is expected to increase (Vera et al. 2002; Kraaijeveld et al. 2005). The greatly male-biased sex ratio in this study (18 males : 1 female in remating cages) might have favoured high female remating more than the high insect density, as other studies (Shelly and Kennelly 2002; Kraaijeveld and Chapman 2004) were conducted with similar to larger fly densities inside the remating cages than in this one (0.0047 flies/cm^3), and presented lower remating rates. Vera *et al.* (2002) considered crowded and relaxed conditions in mating cages as 0.0083 and 0.0016 flies/cm^3 respectively. In addition, the constant availability of oviposition substrate during the experiment may have increased the frequency of remating among females due to the need to replenish sperm. The insect strain is another relevant trait in remating frequency. Remating is more common in laboratory females than in wild ones (Robinson et al. 2002), and the tendency to remate shows heritability and acts as an adaptation in various strains (Saul and McCombs 1993). Paternity analysis performed on the progeny of wild females collected from the field also revealed that remating frequency varied considerably among natural populations from different localities (Bonizzoni et al 2007).

Male exposure to GRO aroma resulted in a reduction of female receptivity after mating comparable to matings involving wild males, which was, in turn, greater than that found for females mated with sterile non-exposed males. This was evident for both the remating rate and the mean number of mating achieved by the females. However, Shelly *et al.* (2002), working with wild-like flies (four generations in laboratory) and *tsl* sterile males, found that females initially mated to sterile males treated with GRO displayed a similar tendency to remate as females first mated to non-exposed sterile males or wild males (10%, 9% and 11% respectively). These authors also observed that remating propensity of females first mated to sterile males was equal to those initially mated to wild ones. In this study, the fly density inside the remating cages varied from 0.0003 to 0.0018 flies/cm^3, being the largest density lower than in this study. The insect density might have favoured lower remating rates but not the remating propensity according to the identity of the first male. The discrepancy between the results may be due to the use of wild-like flies, whose rearing process for four consecutive generations in laboratory conditions might have selected for less-discriminating females. Other publications on remating (Katiyar and Ramirez 1970; Vera et al. 2002) are in agreement with our studies given that females mated to sterile non-exposed males displayed a higher remating rate than females first mated to wild males.

Results found here suggest that the effect of GRO exposure can extend beyond mating initiation and hence is somehow also acting at a post-copulatory

level. However, the possible mechanisms by which GRO exposure may affect female receptivity are not clear. At the age the males are treated with GRO (4 days old), the testis are completely developed with free sperm in the seminal vesicle (Anwar et al. 1971), and the accessory glands contain a large amount of secretion (Marchini et al. 2003). Thus, it appears unlikely that the exposure to the aroma of GRO for 20 h increases the sex peptide amount of the accessory glands fluid or increases the transfer of both sperm and accessory fluid to inhibit remating.

Previous studies have failed to find an explanation for the increased sexual success of GRO-exposed males given that GRO exposure does not enhance male pheromone attractiveness (Papadopoulos et al. 2006) nor produce consistent differences in male courtship behaviour of sterile males (Briceño et al. 2007) and does not confer direct reproductive benefits to the females (Shelly 2005).

The fact that GRO aromatherapy apparently alters the male's cuticular odour perceptible to females at short distances (Papadopoulos et al. 2006; Briceño et al. 2007; Shelly et al. 2007) may account for the enhanced mating success of GRO-exposed sterile males. GRO contains α-copaene, which is a hydrocarbon found in many Mediterranean fruit fly host species (Nishida et al. 2000). It attracts *C. capitata* males and confers mating advantages to males that have contacted it (Shelly and Villalobos 2004). The odour emitted by the GRO-exposed males may indicate to females a greater ability by these males to find natural sources of α-copene (Papadopoulos et al. 2006), i.e. a host plant. Choosing a male able to find natural sources of α-copaene might increase the probability that a female's sons will likewise have this capability and thus enjoy high mating success. It may be the case that short distance chemical signals are also involved in post-mating decisions, i.e. perhaps the scent of GRO sterile males 'satisfied' some female requirements to the same extent as wild males and somehow reduced subsequent female receptivity. Beyond the sexual performance of the males and the ejaculate transferred to females, the odour emitted by the male might play some role in the female tendency to remate. Nevertheless, this should be left in the field of speculation and further research is needed.

As shown by Paranhos *et al*. (2008), we found that mating duration was longer when the male was wild. However, this was evident only for the first mating. In addition, in this study the exposure to GRO did not increase the mating duration of the sterile males, and this result is in agreement with other studies (Shelly et al. 2003; Paranhos et al. 2008).

Overall, the most important finding in our study is that the exposure to GRO can give to sterile males a wild-type capacity to prevent remating of their wild female partner, which will promote SIT's effectiveness in area-wide Mediterranean fruit fly control programmes.

Acknowledgements

We would like to thank Teresa Vera and Todd E. Shelly for reviewing the early version of the manuscript, the São Paulo Research Foundation for the fellowship and financial support (2007/02679-0) and to Biofábrica Moscamed Brasil for providing the sterile males. This research was also supported by the Food and Agriculture Organization/International Atomic Energy Agency (FAO/IAEA) through research contract No.13495.

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