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Influence of methoprene and dietary protein on male *Anastrepha suspensa* (Diptera:Tephritidae) mating aggregations

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ABSTRACT

The Caribbean fruit fly, *Anastrepha suspensa* (Loew), like many polyphagous tephritids, exhibits a lek polygyny mating system, and juvenile hormone levels and adult diet are known to have important positive effects on male sexual success. Among the potential components of this success are male lek tenure and female response to the sexual signals of lekking males. Male *A. suspensa* where submitted to four different treatments: (M^+P^+) application of juvenile hormone analog, methoprene (M) and sugar and hydrolyzed yeast as adult food; (M^+P^-) application of M and sugar as adult food; (M^-P^+) no application of M and sugar and hydrolyzed yeast as adult food; and (M^-P^-) no application of M and sugar as adult food. M^+P^+ males initiated and participated more in aggregations, mated more frequently, and occupied the lek centers more often. They also had fewer unsuccessful mounting attempts than males in all the other treatments. M^+P^+ males also emitted pheromones and acoustically signaled more often and attracted more females than males in other treatments. Male sexual performance was improved due to methoprene, protein supply, and the interaction of methoprene and protein for most of the parameters. Since the success of the sterile insect technique (SIT), a commonly employed technique to control pest tephritids, requires the release of males that can form leks, engage in agonistic sexual interactions, and attract females, these positive effects of protein and methoprene may substantially improve SIT programs.

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1. Introduction

Polyphagous tephritid fruit flies often have complex mating systems in which aggregated males defend individual territories from which they emit chemical, acoustic, and visual signals that presumably function in male–male aggression and to attract and court females (Prokopy, 1980; Burk, 1981; Thornhill and Alcock, 1983). Females visit these male aggregations, or "leks", for mating, and as in the leks of other species, the variance in male reproductive success on tephritid leks is typically high, i.e., relatively few males obtain the majority of copulations (Sivinski and Burk, 1989; Shelly and Whittier, 1997; Sivinski and Petersson, 1997). This high variance could be due to differences among males in their attractiveness to females, their success in male–male agonistic interactions, or both.

Among the signal-channels employed by males, visual and acoustic signals probably act at close range (Sivinski et al., 1984; Sivinski and Pereira, 2005), but pheromones may have both short- and long-range effects (Nation, 1972; Webb et al., 1983; Sivinski et al., 1994). In addition to attracting females, male Caribbean fruit fly, (*Anastrepha suspensa* (Loew)), respond to pheromones as well, presumably to locate lekking sites (Burk, 1983; Kaspi and Yuval, 2000). In contrast to Shelly (2000), Yuval's group found that in another tephritid, the Mediterranean fruit fly, *Ceratitis capitata* (Wied.), males are attracted to other lekking males in a field cage situation.

Exposure to the juvenile hormone analog, methoprene, at emergence accelerates male *A. suspensa* development (Teal et al., 2000) and may lead to greater sexual success through increased pheromone production (Teal and Gomez-Simuta, 2002). However, accelerated maturity and increased pheromone production may have nutritional consequences, since there is less time for young flies to acquire reserves, and these nutrients may be used at a higher rate (Pereira, 2005). Thus, the addition of a protein-rich adult diet may have particularly important consequences in the nutritional status when juvenile hormones titers are manipulated.

Protein is an important component of the adult diet of some fruit fly species, and consumption during the adult stage can contribute to male gonadal and accessory gland development and influence sexual success (Yuval et al., 1998). Greater sexual success of protein-fed males has been documented in *A. obliqua* (Macquart), *A. serpentina* (Wied.) and *A. striata* Schiner (Aluja et al., 2001). However, Aluja et al. (2001) found no effect of

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additional protein on sexual performance in *A. ludens* (Loew). In *C. capitata*, protein-fed males call and mate more frequently than protein-deprived males in both wild (Kaspi et al., 2000) and mass-reared, sterile flies (Kaspi and Yuval, 2000). However, Shelly and McInnis (2003), Shelly and Edu (2008) and Faria et al. (2008) found no influence of protein in the adult diet on mating success of sterile males.

Evaluation of methoprene application, adult protein-enriched diet and their interactions on male *A. suspensa* performance within leks and male attractiveness to females is the main goal of this study. Experiments in laboratory, field cages and under semi-natural conditions in a greenhouse were conducted with males treated with different methoprene and protein regimens. Male attractiveness to females, initiation and participation in leks, male pheromone-calling, male position in the lek, male–male and male–female interactions, and sexual success were observed. Success of the sterile insect technique (SIT) (Knipling, 1955), a commonly used means of tephritid control (Hendrichs et al., 2002), requires the release of males that can form leks, engage in agonistic and sexual interactions, and attract wild females. We discuss whether or not materials, such as methoprene and protein, that may substantially improve male sexual success can be economic additions to SIT programs.

2. Methods

2.1. Insects

The Caribbean fruit flies used in this study had been in a laboratory colony at the Center for Medical, Agricultural and Veterinary Entomology (CMAVE) USDA-ARS, at Gainesville, FL, for about 2.5 years and were produced according to a specific mass rearing protocol (FDACS, 1995). The flies were maintained under low stress conditions (~100 flies in 20 cm by 20 cm by 20 cm adult cages and one larva per 4 g of diet), which result in low selection pressure for characteristics associated with domestication (Liedo et al., 2002; Mangan, 2003).

Flies to be used in experiments were obtained from pupae sorted into size classes with a sorting machine (FAO/IAEA/USDA, 2003). This was done to eliminate any impact of size on male competitiveness (Burk and Webb, 1983; Burk, 1984; Webb et al., 1984; Sivinski and Dodson, 1992; Sivinski, 1993). Males for the experiment came from the size class whose average pupal weight was 10.8 ± 0.71 mg. Females were obtained from the next larger class size, with an average pupal weight of 11.8 ± 0.74 mg. In the field, males are typically 80% of the female size (Sivinski and Calkins, 1990; Sivinski, 1993). These pupal weights were in the middle range of *A. suspensa* pupae collected from infested guava (*Psidium guajava* L.) in nature (Hendrichs, 1986).

After emergence the flies were maintained in a laboratory room with a photoperiod of 13L:11D (light from 7:00 to 20:00), a light intensity of 550 ± 50 lx, a temperature of 25 ± 1 °C, and a relative humidity of 55 ± 5%.

2.2. Treatments

The study compared sexual performance of male *A. suspensa* subjected to the following four treatments:

- M^+P^+: application of juvenile hormone analogue, methoprene (provided gratis by Zoecon Professional Products, Schaumburg, IL 60173), and sucrose and hydrolyzed yeast (protein source) as adult food.
- M^+P^-: methoprene application and sugar as adult food.
- M^-P^+: no methoprene application and sugar and hydrolyzed yeast as adult food.
- M^-P^-: no methoprene application and sugar as adult food.

Methoprene was applied topically within 24 h of adult emergence at a rate of 5 μg in 1 μl acetone solution per male in M^+ treatments. In M^- treatments, 1 μl of acetone was applied to serve as control. Males were immobilized in a net bag (as used in standard marking techniques, FAO/IAEA/USDA (2003)), and the solution was applied via micropipette through the net onto the dorsal surface of the thorax. No anesthesia was used to immobilize the flies. Precautions were taken to avoid cross contaminations between experimental subjects. Males from each treatment were maintained in independent 30 cm by 30 cm by 30 cm screen cages with a maximum male density of 200 flies/cage and with the type of food assigned for each treatment.

In the P^- treatments only water and sugar *ad libitum* were supplied to the flies. In the P^+ treatments hydrolyzed yeast was added to the adult diet as protein source (mixed with sugar in a proportion of three parts of sugar and one part of hydrolyzed yeast). This mixture is considered a high quality diet for *Anastrepha* species (Jácome et al., 1995; Aluja et al., 2001).

Females to be used in the experiments (a maximum of 200) were maintained following eclosion in 20 cm by 20 cm by 20 cm screen cages without direct exposure to males. They were provided with a P^+ diet, i.e., sugar plus hydrolyzed yeast (3:1) and water *ad libitum*.

2.3. Lek tenure in field cages

The experiment was conducted in standard field cages used for the study of strain compatibility and male sexual performance in tephritids (FAO/IAEA/USDA, 2003). These screen cages are cylindrical, 2.9 m diameter and 2.0 m high with a flat floor and ceiling (Calkins and Webb, 1983). Twelve replications were run (one per day) from 7 to 19 June, 2005. In each cage, a 1.8 m high potted guava was placed to serve as a substrate for sexual interactions. Guava is considered a key host of *A. suspensa* and a common substrate for lekking (Dodson, 1982; Hendrichs, 1986; Landolt and Sivinski, 1992; Sivinski, 1989). A different potted guava was used every day to prevent the influence of male pheromones deposited on leaves the previous day (Sivinski et al., 1994).

In the cage, 40 sexually mature, 13–16 days old, virgin males (10 per treatment) were released at 16:50. They were previously marked with a dot of water-based paint on the dorsal surface of the thorax to identify the males from each treatment according the FAO/IAEA/USDA (2003) manual. The colors were rotated among treatments. Ten minutes later, 20 sexually mature virgin females, 20–23 days old, were released inside the cage. The experiment was run until 19:00 to coincide with the sexual activity peak (Dodson, 1982; Burk, 1983; Hendrichs, 1986; Landolt and Sivinski, 1992). During these 2 h (17:00–19:00), temperature, relative humidity and light intensity were measured every 30 min. During the field cage experiment, the temperature ranged from 24 °C to 32 °C, with a daily variation of 1–6 °C. Relative humidity varied from 40% to 94%. Light intensity varied from 3540 lx to 12,090 lx. Rapidly developing clouds contributed to these variations. Sunsets occurred between 20:28 and 20:32.

Marked males were observed as they moved about inside the cage. Initiation (first male that started to emit pheromone in a certain area of the plant canopy) and participation in leks (males that join the first male to create an aggregation, calling or not), male calling (indicated by the expansion of pleural regions of the abdomen and eversion of glistening rectal tissue), male position in the lek, male–male and male–female interactions, and matings were observed. Mating pairs were removed to 10 ml individual vials, and copulation duration was recorded.

Males that landed within 20 cm of another calling male and eventually called were considered participants in a lek (Sivinski,

1989). Positions in leks were divided into three tiers: central – the male is located on the middle leaves of the aggregation or in the case of paired flies the first male to call was considered central; surrounding – males that land on the leaves adjacent to the center (within a 20 cm radius of the edge of the center area of the aggregation); and satellites – males that are adjacent and peripheral to the surrounding males. Male–male interactions took place when two males occupied the same leaf (the resident occupied the leaf, and the intruder arrived and attempted to displace the resident). Typically, this resulted in an agonistic interaction with wing waving that lasted for several seconds, although physical contact was rare. Losers left and the winners stayed on the leaf. Male–female interactions occurred when males attempted to mate. Males could succeed or be rejected when females flew away or moved to the upper side of the leaf. A female rejection index was calculated according the following formula:

$$\text{Female rejection index} = \frac{\text{Number of male attempts to mate}}{\text{Number of successful matings}}$$

2.4. Male attractiveness in laboratory

Thirteen- to 16-day-old males were transferred to individual cylindrical screen cages (10 cm high and 7 cm diameter) with water supplied by a cotton wick situated in a separate cup of water and penetrating the cage bottom. Each replicate included one male from each of the four treatment groups. Four individual cages (each containing one male from a treatment) were placed inside a larger 30 cm by 30 cm by 30 cm screen cage. Four of these large cages, placed 30 cm apart, were observed at any one time. A single male was released into each of the four individual cylindrical cages at 16:00. A 20–23-day-old fully mature female was then released into the outer, larger cage 1 h later (17:00), and her movements and approaches to the male-containing inner cages were observed until 19:00. No food or water was supplied in the outer cage in order not to complicate female responses, and no food (only water) was supplied to the male inner cages so as not to interfere with the male treatments.

The time spent by males calling when females were in the outer cage was recorded (from 17:00 to 19:00), as was the number of female visitations (landing on cylindrical male cages) and the time they spent on the male cages. The position of each male cage (treatment) was rotated inside the outer screen cage between replicates. Four large screen cages were run daily during a total of 12 days so that a total of 48 females were observed. Each day new individual cylindrical screen cages were used to eliminate the possibility that differential male pheromone deposition on the previous day might influence female response (Sivinski et al., 1994). Laboratory conditions were the same as those used to maintain flies in the laboratory before the experiments (light intensity of 550 ± 50 lx, temperature of 25 ± 1 °C and relative humidity of 55 ± 5%).

2.5. Male attractiveness in a greenhouse

The experiment was conducted in a 8.6 m by 6.0 m greenhouse containing 24 potted guavas (1.8–2.0 m high). The potted trees were distributed in four rows of six trees each (Fig. 1).

Four artificial leks were hung within particular tree canopies each test day. These were placed on the second and fifth tree of the second line (on the side of the canopy facing line one) and on the second and fifth tree of the third line (on the side of the canopy facing line four) at 2.0 m from the greenhouse sidewalls and 2.2 m from the end walls. Males from each treatment occupied one of the four positions, and treatments were rotated every day.

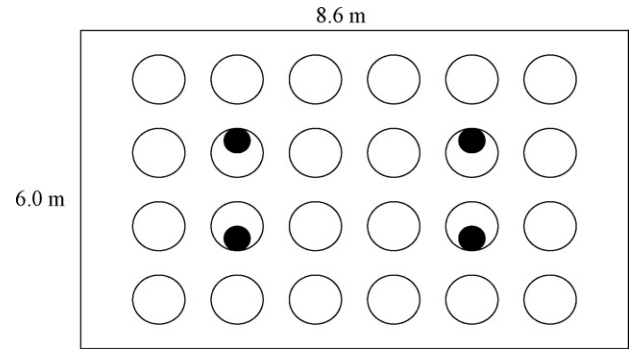

Fig. 1. Spatial arrangement of artificial leks in the greenhouse experiment examining male attractiveness. Open circles represent tree canopy, and filled circles represent the location of the artificial leks (with six males each in each and one artificial lek per treatment).

Each artificial lek consisted of six 13–16-day-old males, one male in each of six (4 cm high and 2 cm diameter) cylindrical screen cages, which were bound together in a bundle. The cage ends were closed with cotton wicks. Males were caged individually to prevent intense male–male interaction when in a confined area. No food or water was supplied to the males so as to not to confound female responses. At 16:50, 100 virgin 20–23-day-old females were evenly distributed in the greenhouse (four females in each tree canopy plus four in the center). Ten minutes later, male lek-cages were deployed. The experiment was conducted over 12 days (28 June to 13 July 2005) with new males in fresh individual cages on each day (replicate) in order to prevent previous pheromone depositions from influencing female behavior (Sivinski et al., 1994).

Females from the previous replicate were removed from the greenhouse the following morning, and new ones released at 16:50 on the test day. Temperature, relative humidity, and light intensity were measured every 30 min during the experiment (from 17:00 to 19:00). The numbers of males calling and the number of females in the immediate vicinity of each lek (within a radius of 25 cm) were recorded every 10 min, and values were averaged across one replicate.

The abiotic conditions during the 12 days of the greenhouse experiment (17:00 to 19:00) varied according to outside temperature, relative humidity, and light intensity. Inside the greenhouse, the temperature ranged from 25.0 °C to 35.6 °C, with a daily variation of 1 °C to 3 °C over the observation period. Relative humidity ranged from 46% to 99%, and light intensities from 656 lx to 13,700 lx. Sunset occurred between 20:31 and 20:33 over the course of the experiment.

2.6. Statistical analyses

Lek initiation, lek participation, males calling, matings in field cage tests and male calling and female visitation, both in laboratory and greenhouse experiments were analyzed using a two-way analysis of variance (ANOVA) to detect methoprene application effect, protein supply effect, and the interactions between methoprene and protein. These analyses were followed by an ANOVA to detect differences between means in the treatments, although data in the figures are presented as percentages. Female acceptance index data was analyzed by ANOVA. Tukey's mean separation test ($P = 0.05$) was used for significant factors in the ANOVA (Ott and Longneaker, 2001). Linear regression was used to correlate calling males with female visitation in the greenhouse experiment. Statistical analyses were performed using R software (version 2.1.0, www.r-project.org).

3. Results

3.1. Initiation and participation in the aggregation

The number of leks per replicate averaged 3.4 ± 0.8 and varied between 2 and 5. The number of males in an aggregation (lek) varied between 2 and 6 with an average of 3.6 ± 1.1 males per aggregation.

Significant positive effects of methoprene application ($F_{1,44}$ = 63.14, $P < 0.001$), protein supply ($F_{1,44}$ = 72.84, $P < 0.001$), and the interaction of the methoprene application and protein supply ($F_{1,44}$ = 19.49, $P < 0.001$) were found in lek initiation. The percentage of males, by treatment, initiating aggregations is presented in Fig. 2. Of a total of 41 leks observed, 28 (68%) were initiated by M^+P^+ males, which was significantly higher compared with all the other treatments ($F_{3,44}$ = 51.80, $P < 0.001$). M^-P^- males did not initiate any leks.

Significant positive effects of methoprene application ($F_{1,44}$ = 137.84, $P < 0.001$), protein supply ($F_{1,44}$ = 199.99, $P < 0.001$), and interaction of the methoprene application and protein supply ($F_{1,44}$ = 31.40, $P < 0.001$) were found in lek participation. Significant effects of methoprene application ($F_{1,44}$ = 64.57, $P < 0.001$), protein supply ($F_{1,44}$ = 84.34, $P < 0.001$), and interaction of the methoprene application and protein supply ($F_{1,44}$ = 8.23, P = 0.006) were found for male calling as well.

The percentages of males, by treatment, participating in aggregations and calling, are presented on Fig. 2. The data show a similar pattern for both participation and calling. Of a total of 212 males participating, 53% (112) were M^+P^+ males, which was significantly higher than all the other treatments ($F_{3,44}$ = 123.10, $P < 0.001$). M^-P^- males had significantly fewer males participating in aggregations (7%). One hundred and seventy eight males called (84% of total participating in leks). Of these, 55% were M^+P^+ males, which was significantly higher than all the other treatments ($F_{3,44}$ = 52.40, $P < 0.001$). M^-P^- males had significantly fewer males calling (4%). Calling duration of those males that called was not significantly different among treatments ($F_{3,174}$ = 1.40, P = 0.245). On average, males spent 18.8 ± 9.1 min calling in an aggregation.

3.2. Matings

Significant positive effects for methoprene application ($F_{1,44}$ = 65.32, $P < 0.001$), protein supply ($F_{1,44}$ = 83.90, $P < 0.001$), and interaction of the methoprene application and protein supply ($F_{1,44}$ = 25.52, $P < 0.001$) were found. Of a total of 73 matings observed in the 12 replicates (6.3 ± 1.0 per replication), 67% were performed by M^+P^+ males, significantly more than all other treatments ($F_{3,44}$ = 57.60, $P < 0.001$). M^-P^- males had significantly fewer copulations (only 1%) than all other treatments (Fig. 2). All matings observed were performed by males that were participating in aggregations. Copulation duration was not different among treatments ($F_{3,69}$ = 0.351, P = 0.789) and averaged 27.4 ± 5.7 min (range of 15–40 min). Among all treatments, a high percentage of males (78%) that initiated an aggregation subsequently copulated.

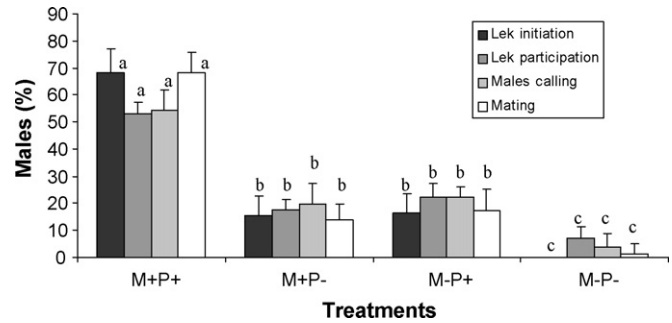

Fig. 2. Lek parameters and sexual success of male *Anastrepha suspensa* presented as percentages of males (mean plus standard deviation) when treated or not with methoprene (M^+/M^-) and fed or not with protein (P^+/P^-). Bars with the same letter for each parameter were not significantly different (Tukey's test, P = 0.05). Observations were based on 41 leks (lek initiation), where 212 males participated, 178 called, and 73 matings occurred.

3.3. Position in the lek

Sixty four percent of matings were obtained by males in the center of the aggregation, 36% by surrounding males, and none by the satellites. Numbers of males in each category differed so that of the 94 center males 50.0% copulated, while 24.8% of the 105 males on surrounding territories mated. None of the 13 satellite males copulated. Sexual success was correlated with treatment (Table 1). Fifty-seven of the 94 center males (60.6%) were M^+P^+, and of those, more than half copulated (54.4%).

3.4. Male–male and male–female interactions

In general, residents had an advantage over intruders (Table 2), although M^-P^- resident males lost nearly half, 42%, of their contests.

Unsuccessful mating attempts were common; 224 male–female interactions without copulation were observed. On

Table 1
Number of male *Anastrepha suspensa* that occupied the various positions within leks, when treated or not with methoprene (M^+/M^-) and fed or not with protein (P^+/P^-). Percentages of males that copulated are inside parenthesis.

Position on the lek	M^+P^+	M^+P^-	M^-P^+	M^-P^-	Total per position
Central	57 (54.4%)	14 (42.9%)	21 (47.6%)	2 (0.0%)	94 (50.0%)
Surrounding	49 (36.7%)	22 (18.2%)	22 (13.6%)	12 (8.3%)	105 (24.8%)
Satellites	6 (0.0%)	3 (0.0%)	3 (0.0%)	1 (0.0%)	13 (0.0%)
Total per treatment	112 (43.8%)	39 (25.6%)	46 (28.3%)	15 (6.7%)	212 (34.4%)

Table 2
Percentages of resident male *Anastrepha suspensa* in leks that won contests against intruders, when treated or not with methoprene (M^+/M^-) and fed or not with protein (P^+/P^-). Number of interactions is in parenthesis. Totals with different letter are significantly different (Tukey's test, P = 0.05).

Residents	Intruders				Total wins
	M^+P^+	M^+P^-	M^-P^+	M^-P^-	
M^+P^+	94.4 (n = 36)	88.2 (n = 17)	92.0 (n = 25)	100.0 (n = 22)	94.0 (n = 100) a
M^+P^-	92.9 (n = 14)	100.0 (n = 5)	100.0 (n = 9)	100.0 (n = 2)	96.7 (n = 30) a
M^-P^+	72.2 (n = 18)	100.0 (n = 5)	100.0 (n = 2)	100.0 (n = 7)	84.4 (n = 32) a
M^-P^-	50.0 (n = 6)	66.7 (n = 3)	66.7 (n = 3)	(–) (n = 0)	58.3 (n = 12) b

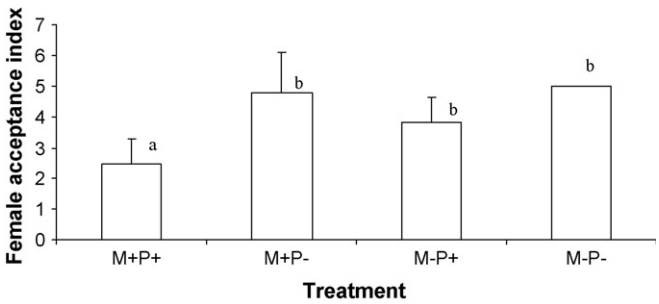

Fig. 3. Female *Anastrepha suspensa* rejection index when males were treated or not with methoprene (M^+/M^-) and fed or not with protein (P^+/P^-). Bars with the same letter were not significantly different (Tukey's test, $P = 0.05$).

average, and across all treatments, males obtained copulation after 3.1 ± 0.8 attempts. However, when the female rejection index was analyzed by treatment (Fig. 3), M^+P^+ males made significantly fewer unsuccessful attempts (2.5 ± 0.4) than males from other treatments ($F_{3,24} = 26.56$, $P < 0.001$).

3.5. Male attractiveness in the laboratory

Significant positive effects of methoprene application ($F_{1,44} = 72.00$, $P < 0.001$), protein supply ($F_{1,44} = 81.31$, $P < 0.001$), and the interaction of the methoprene application and protein supply ($F_{1,44} = 7.51$, $P = 0.009$) on male calling duration were found. The duration of female visits was significantly effected by methoprene application ($F_{1,44} = 120.53$, $P < 0.001$), protein supply ($F_{1,44} = 104.30$, $P < 0.001$), and interaction of the methoprene application and protein supply ($F_{1,44} = 28.75$, $P < 0.001$). M^+P^+ males spent more time calling, and females spent more time visiting these males than those of any of the other treatments. M^-P^- males called significantly less often and females spent less time with them than with males of other treatments (Fig. 4; males calling: $F_{3,44} = 53.7$, $P < 0.001$; and duration of female visitations: $F_{3,44} = 84.5$, $P < 0.001$).

Similarly, there were significant effects of methoprene application ($F_{1,44} = 120.14$, $P < 0.001$), protein supply ($F_{1,44} = 106.23$, $P < 0.001$), and interaction of the methoprene application and protein supply ($F_{1,44} = 22.51$, $P < 0.001$) on the number of female visits. M^+P^+ males received a significantly higher number of female visits (1.7 ± 0.28) when compared with the other treatments ($F_{3,44} = 83.0$, $P < 0.001$). M^-P^- males had significantly fewer female visits (0.4 ± 0.17) than males of the other treatments ($M^+P^- = 0.8 \pm 0.24$; $M^+P^- = 0.7 \pm 0.18$).

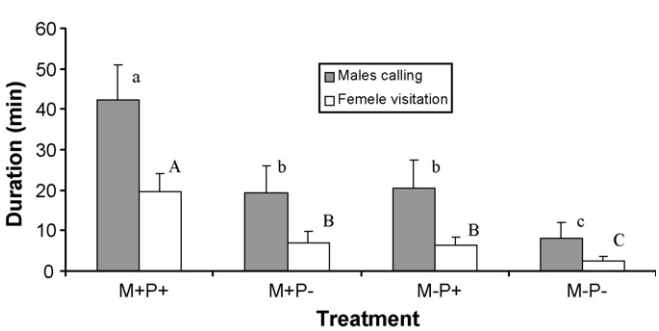

Fig. 4. Time spent by male *Anastrepha suspensa* calling and time spent by females visiting males (mean plus standard deviation), when treated or not with methoprene (M^+/M^-) and fed or not with protein (P^+/P^-). Bars with the same letter (lowercase for males calling and uppercase for female visitations) are not significantly different (Tukey's test, $P = 0.05$).

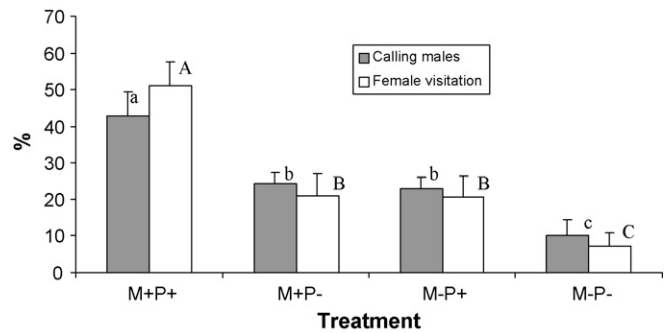

Fig. 5. Percentage of male *Anastrepha suspensa* in greenhouse calling and percentage of females approaching males (mean plus standard deviation), when treated or not with methoprene (M^+/M^-) and fed or not with protein (P^+/P^-). Bars with the same letter (lowercase for males calling and capital for female visitation) are not significantly different (Tukey's test, $P = 0.05$).

3.6. Male attractiveness in the greenhouse

Significant positive effects of methoprene application ($F_{1,44} = 106.76$, $P < 0.001$) and protein supply ($F_{1,44} = 93.59$, $P < 0.001$) were found on male calling. However, no interaction was found between methoprene application and protein supply ($F_{1,44} = 3.13$, $P = 0.084$). In terms of female visitation, there were significant effects of methoprene application ($F_{1,44} = 88.11$, $P < 0.001$), protein supply ($F_{1,44} = 85.97$, $P < 0.001$), and the interaction of methoprene application and protein supply ($F_{1,44} = 12.59$, $P < 0.001$).

In the greenhouse, M^+P^+ males called more often and were approached by females more frequently than males in other treatments (Fig. 5; males calling: $F_{3,44} = 67.1$, $P < 0.001$; and female visitation: $F_{3,44} = 62.20$, $P < 0.001$). M^-P^- males had significantly fewer males calling and attracted fewer females.

Female visitation, regardless of treatment, was correlated with male calling frequency (Fig. 6).

4. Discussion

Methoprene application, dietary protein, and the combination of methoprene and protein significantly improved most parameters of male lekking, attractiveness, and sexual success (Table 3). Among the males that initiated a lek, 78% went on to copulate. Lek initiation in various tephritids can be dependent on the nutritional status of the male (Yuval et al., 1998) or perhaps by the male's hormonal state (Teal et al., 2000). In the case of the *A. suspensa*, either methoprene or protein resulted in higher rates of lek initiation, attractiveness, and ultimately greater sexual success.

Additionally, laboratory and greenhouse experiments, using individual and aggregated males respectively, obtained similar

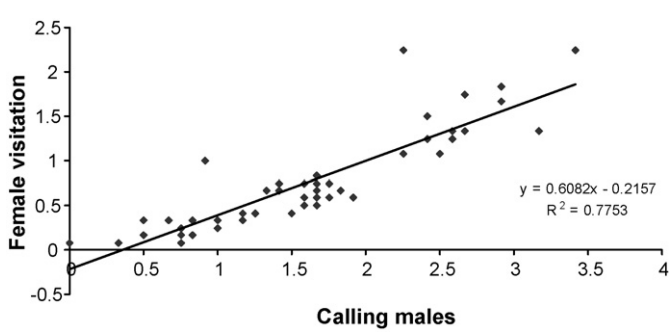

Fig. 6. Correlation between the number of male *Anastrepha suspensa* calling within an artificial lek and the number of female visiting in the greenhouse environment.

Table 3
Summary of sexual success parameters in *Anastrepha suspensa* when treated or not with methoprene (M^{+}/M^{-}) and fed or not with protein (P^{+}/P^{-}).

Parameters	$M^{+}P^{+}$	$M^{+}P^{+}$	$M^{+}P^{+}$	$M^{+}P^{+}$
Initiation of aggregation	a	b	b	c
Male participation	a	b	b	c
Males calling	a	b	b	c
Calling duration	ns	ns	ns	ns
Matings	a	b	b	c
Copula duration	ns	ns	ns	ns
Male–male interaction	a	a	a	b
Male–female interaction	a	b	b	b
Calling duration in laboratory	a	b	b	c
Female visitation in laboratory	a	b	b	c
Calling duration in greenhouse	a	b	b	c
Female visitation in greenhouse	a	b	b	c

ns: non significant differences; a > b > c significant different ($P = 0.05$).

results. Overall, the immediate effects of both methoprene and protein were to increase male signaling and enhance sexual success, and the interaction of these two factors was additive.

What are the physiological and/or behavioral consequences of methoprene and protein that resulted in this improved sexual performance? Some possibilities include longer calling durations, more pheromone produced per unit of calling time, and more time spent in leks (or in better locations within leks) due to favorable outcomes in agonistic encounters. The proportion of $M^{+}P^{+}$ males that participated and called in leks was significantly higher than in other treatments. However, calling duration was not significantly different among treatments. That is, although other-treatment males were less likely to participate in leks those that did so spent as much time calling. On the other hand, $M^{+}P^{+}$ males are known to produce more pheromone per unit time of calling (Teal et al., 2000), and this could produce a more powerful signal that might attract more females to their positions within aggregations. The greater success of $M^{+}P^{+}$ males in agonistic encounters, particularly in the role of resident, might allow them to spend more time in the aggregation, and all other things being equal, be available longer to visiting females. In addition, certain territories may be particularly valuable within the lek, because they serve as a superior signaling platform, because females prefer from predation or because they indicate male quality (Field et al., 2002; Kaspi and Yuval, 1999a,b). Males may fight to establish calling stations in favorable locations or to keep other males at a distance (Dodson, 1982). In the Mexican fruit fly, *A. ludens*, male mating success is influenced by the propensity to engage in fights with other males and fighting ability (Robacker et al., 1991).

While it is difficult to separate some of these possibilities, it does seem unlikely that increased female encounter rates alone are sufficient to explain the sexual success of $M^{+}P^{+}$ males. For one thing, $M^{+}P^{+}$ males had a lower female rejection index, evidence that they required fewer encounters, on average, to successfully obtain copulations. Thus, female preference, either for a more powerful signal(s) or for the male's position within the aggregation, seems to be an important component of the variance in male reproductive success.

Can the relative importance of signal and residence quality to female mate choice be determined? Hendrichs (1986) examined the sexual behavior of *A. suspensa* in a field cage and found that males compete for leaves in the centers of aggregations and females usually mate in the center as well, which was substantiated by this study. Territory location within leks figures in two prominent theories of lek evolution: the "hotspot" and "hotshot" models (Höglund and Alatalo, 1995), both of which are consistent with the results of this study. In the first, females choose males on the basis of location within the aggregation either because male–male competition for a particular site acts as a "filter" that guarantees male quality, or because certain locations provide more protection from predators during periods when insects might be distracted by courtship and copulation.

In the second model ("hotshot"), males accumulate around unusually attractive males attempting to intercept females as they with move toward the "hotshot". There is an unusual permutation of this model in the case of many tephritids. Calling *A. suspensa* males deposit pheromones on leaf-surfaces while calling, probably to enlarge the surface area for evaporation, and some of these compounds persist for at least 24 h (Sivinski et al., 1994). Thus, prior occupation could make a territory valuable as a signaling site, and by boosting chemical signals, turn subsequent residents into relative "hotshots".

With the present data it is difficult to completely eliminate one or the other explanation. Certainly the chain of events that leads from males that initiate leks to be more likely to occupy the lek center, to being better able to defend their territories, and then to subsequently mate more often would seem to favor the "hotshot" model; i.e., an attractive (high-output signaling) male is quickly surrounded by less capable satellites but still manages to copulate. However, lek sites can be consistent over time (Sivinski, 1989) and it could be that $M^{+}P^{+}$ and other successful males are more competent at locating "hotspots" and thus being the first in to signal from incipient leks.

Female visitation was linearly related to increased calling, however the slope of this relationship is less than 1; i.e., doubling the number of calling males did not double the number of female visits. Certain theories of lek evolution argue that females prefer to compare males in close proximity and thus are disproportionately attracted to male aggregations (Field et al., 2002). While our data did not support this argument, there are methodological difficulties in periodically counting the numbers of signaling males. The observations of these behaviors were not continuous, and the behaviors associated with pheromone signaling may not accurately predict the intensity of the signal itself. We suggest that the response of *A. suspensa* females to different numbers of males be explored with formulated pheromones released at different rates. In *C. capitata* (Shelly, 2000) and Oriental fruit fly (*Bactrocera dorsalis* (Hendel)) (Shelly, 2001), female attraction was a direct reflection of male signal output. In both situations, the relationship between female responses and calling activity among lekking males suggest that a difference in signal production by itself accounted for inter-lek variation in female visitation. However, in later studies Shelly (2001) found that while female medfly sightings per calling male were similar between the 18- and 36-male leks, the number of males for these larger leks were significantly greater than those noted for the six-male leks. However, unlike the artificial leks in the present study, the artificial aggregations studied by Shelly (2001) were larger than those found in the field (Prokopy and Hendrichs, 1979; Arita and Kaneshiro, 1989).

Regardless of how *A. suspensa* leks evolved, it is clear from the present study that $M^{+}P^{+}$ males, and to a lesser extent those that receive either methoprene or protein, are more likely to initiate leks, more likely to occupy lek centers, better able to defend their territories and ultimately enjoy greater sexual success. Since the capacity of mass-reared males to compete with wild males is the foundation of SIT, these findings have important implications for fruit fly area-wide control. Additionally, male *Anastrepha* species often have an extended, often week long or more, pre-copulatory period (Aluja, 1994; Pereira et al., 2007), and under these conditions methoprene treatment has an additional advantage for mass rearing through acceleration of sexual maturation. As a consequence space is saved in fly handling facilities and costs are reduced. In addition, reducing the pre-copulatory period means males can be released already sexual mature or are more likely to survive to sexual maturity.

Protein-rich adult diets have been previously shown to increase male sexual success in *C. capitata* (Kaspi and Yuval, 2000), but the associated cost of hydrolyzed yeast or other protein sources inhibits their use in SIT programs. However, the data presented here suggest that avoiding the costs of protein (or methoprene) might seriously undercut the potential of SIT. Detailed studies involving effectiveness of protein-fed compared to protein-deprived males, particularly sterile males, need to be done on a larger scale to support the addition of protein in adult diet and its concomitant costs. *A. suspensa* males with neither methoprene nor protein, i.e., those most resembling mass-reared males at present, were sexually incompetent in comparison to those receiving either food or hormone additives.

Two further studies are immediately suggested by this work. The first is to identify the most economic source of protein for adult mass-reared flies. Incorporation of bacteria in diets to optimize the microbial symbiont flora (Lauzon et al., 2000) might offer one avenue. The second is to repeat these experiments using radiation-sterilized flies. It is ultimately sterilized flies that must compete in the field, and sterilization often results in decreased sexual performance (Heath et al., 1994; Lux et al., 2002; Barry et al., 2003). Methoprene and protein may prove to be even more critical given the expected loss of vigor.

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