

Influence of a Juvenile Hormone Analog and Dietary Protein on Male *Anastrepha suspensa* (Diptera: Tephritidae) Sexual Success

RUI PEREIRA,^{1,2} JOHN SIVINSKI,² AND PETER E. A. TEAL²

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ABSTRACT Juvenile hormone levels and adult diet have important effects on the attractiveness and competitiveness of male *Anastrepha suspensa* (Loew) (Caribbean fruit fly). Because the success of the sterile insect technique requires the release of males that can compete in the wild, these effects are of crucial importance. Laboratory and field cage experiments were conducted to compare male sexual performance on a lifetime basis and daily basis when submitted to four different treatments: (M^+P^+) application of the 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. On a daily basis, M^+P^+ males always performed better sexually, and 10% of these individuals were able to mate three consecutive times in the same day. However, the copula duration decreased with the increased number of matings on same day. In addition, M caused earlier maturation. On a lifetime basis, M^+P^+ males had significantly greater sexual success than other flies. The substantial improvement in male sexual performance because of the hormone application, protein supply, and interaction of hormone and protein has the potential of producing more efficacious sterile males.

KEY WORDS Caribbean fruit fly, hydrolyzed yeast, male competitiveness, methoprene, sexual performance

Polyphagous tephritid fruit flies often have complex mating systems in which aggregated males occupy individual territories from which they emit chemical, acoustic, and visual signals (Prokopy 1980, Burk 1981, Sivinski and Burk 1989). Females arrive at these leks to choose mates, and the variance in male reproductive success is typically high, i.e., relatively few males obtain the majority of copulations (Shelly and Whittier 1997). Differences among males in terms of competitiveness and attractiveness play major roles in generating this variance. Because the success of the sterile insect technique (SIT) requires the release of males that can compete in these arenas (Knipling 1955), it is important to fully understand the target pest's mating behavior and to incorporate the best possible sexual qualities into the mass-reared insects (Hendrichs et al. 2002).

Exposure to juvenile hormones, analogs of juvenile hormones, and increased protein consumption during the adult presexual maturation period accelerate male tephritid development and may lead to greater sexual success through increased pheromone production (Teal et al. 2000, Teal and Gomez-Simuta 2002b).

Similar phenomena have been reported in other insects, such as the German cockroach, *Blattella germanica* L. (Schal et al. 1994), and a social wasp, *Polybia occidentalis* (Olivier) (O'Donnell and Jeanne 1993). Greater sexual ability is a valuable characteristic, but more rapid maturation can lead to cost reductions at fly handling facilities in SIT programs because of space savings and release of sexual mature males. In fruit fly species with long adult precopulatory periods, like *Anastrepha* species (Aluja 1994), early maturation is particularly important.

Obtaining nutritional resources before engaging in territorial or courtship behavior is essential for reproductive success in many tephritids. Blay and Yuval (1997) reported the improvement of sexual performance of the Mediterranean fruit fly, *Ceratitidis capitata* (Wied.), caused by additional protein in diets. Accelerated maturity caused by hormone manipulation may have particularly serious nutritional consequences because there is less time for flies to acquire reserves. Thus, the addition of a protein rich adult diet could be critical to methods used to accelerate male development by delivering exogenous hormone. For example, in the mountain spiny lizard, *Sceloporus jarrovi*, males with testosterone implants required additional food to survive at rates similar to untreated males (Marler and Moore 1991).

The goal of this study was to determine the effects of a juvenile hormone analog (methoprene) application, of protein in the adult diet, and their interactions

¹ Corresponding author: Entomology and Nematology Department, University of Florida, Gainesville, FL 32611. (Current address: Insect Pest Control Section, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, A-1400 Vienna, Austria [e-mail: R. Cardoso-Pereira@iaea.org]).

² Center for Medical, Agricultural and Veterinary Entomology, USDA-ARS, Gainesville, FL.

on the sexual performance of males of the Caribbean fruit fly, *Anastrepha suspensa* (Loew). Specifically, we measured male competitiveness in agonistic interactions with competing mates, attractiveness to females, and mating success. Experiments were conducted in laboratory and field cages and on daily and lifetime bases. The implications of the results for SIT are discussed.

Materials and Methods

Insects. Caribbean fruit flies used in the study had been in a laboratory colony at the Center for Medical, Agricultural, and Veterinary Entomology (CMAVE)–USDA–ARS, at Gainesville, FL, for <2 yr and were reared as described elsewhere (FDACS 1995). The flies were maintained under low stress condition (≈ 100 flies in 20 by 20 by 20-cm adult cages and one larva per 4 g of diet), which results 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 used in experiments came from a size class whose average weight was 10.7 ± 0.91 mg ($n = 40$). Females were obtained from the next larger class size, whose average weight was 11.9 ± 0.90 mg ($n = 30$). In the field, males are typically 80% of the female's size (Sivinski and Calkins 1990, Sivinski 1993). These pupal weights were in the middle range of *A. suspensa* pupae collected from infested fruits in nature (Hendrichs 1986).

After emergence, the flies were maintained in a laboratory room with a photoperiod of 13 L:11 D (light from 0700 to 2000 hours), with light intensity of 550 ± 50 lux, temperature of $25 \pm 1^\circ\text{C}$, and relative humidity of $55 \pm 5\%$. All laboratory experiments were conducted in this same room under the same environmental conditions.

Treatments. The study compared sexual performance of male *A. suspensa* subjected to the following treatments: (M^+P^+) application of the juvenile hormone analog methoprene (M) and sugar and hydrolyzed yeast (protein source) as adult food (P); (M^+P^-) methoprene application and sugar only as adult food; (M^-P^+) no methoprene application and sugar and hydrolyzed yeast as adult food; and (M^-P^-) no methoprene application and sugar only as adult food. The methoprene was applied topically in the first 24 h after adult emergence at a rate of $5 \mu\text{g}$ in $1 \mu\text{l}$ acetone solution per male. In M^- treatments, $1 \mu\text{l}$ of acetone was applied to serve as a control. Males were immobilized in a net bag (as in standard marking techniques; FAO/IAEA/USDA 2003), and the solution was applied by pipette through the net onto the dorsal surface of the thorax. No anesthesia was used to immobilize the flies. Two different net bags and pipettes were used (one for M^+ treatments and the other for M^- treatments) to prevent methoprene con-

tamination. Males from each treatment were maintained in separate 30 by 30 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 P^- treatments, only water and sugar ad libitum were supplied to the flies. In the P^+ treatments, hydrolyzed protein was added to the sugar diet in a proportion of three parts of sugar and one part of hydrolyzed yeast, and water was supplied ad libitum. This mixture is considered a high-quality diet for *Anastrepha* species (Jácome et al. 1995, Aluja et al. 2001).

Females used in the experiments were sexed on the first day of adult life and maintained in 20 by 20 by 20-cm screen cages without exposure to males. They were provided with a P^+ diet, i.e., sugar plus hydrolyzed yeast (3:1) and water ad libitum.

Sexual Success in the Laboratory. The experiment was conducted in 20 by 20 by 20-cm screen cages. There were 12 replications (different days) with 15 cages per replication for a total of 180 cages. In each cage, four males (one per treatment) were released in midafternoon (1500 hours). These 13- to 16-d-old, virgin sexually mature males were marked on the day before the experiment with a dot of water-based paint (different color per treatment and rotated among treatments) on the dorsal surface of the thorax (FAO/IAEA/USDA 2003). At 1700 hours, a 20- to 23-d-old, sexually mature virgin female was released into each cage and observed until 1900 hours. This afternoon period coincides with the peak of sexual activity in *A. suspensa* (Dodson 1982, Burk 1983, Hendrichs 1986, Landolt and Sivinski 1992). When mating occurred, the pair was removed and all the flies, including the three males that did not mate, were killed in a freezer. Male wing lengths (right wing of each fly) were measured at the end of the experiments to quantify male size (Landolt and Sivinski 1992, Yuval et al. 1998).

Sexual Success in Field Cages. The experiment was conducted in standard field cages used for the study of male compatibility and sexual performance in tephritids (FAO/IAEA/USDA 2003). Cages are cylindrical, with flat floors and ceilings, 2.9 m diameter and 2.0 m high (Calkins and Webb 1983). In this experiment, two cages were observed per day for 6 d (4, 5, 6, 10, 12, and 13 October 2004) for a total of 12 replications. In each cage, a 1.8-m-high potted guava plant (*Psidium guajava* L.), a preferred host of Caribbean fruit fly (Dodson 1982, Hendrichs 1986, Landolt and Sivinski 1992, Sivinski 1989), served as a substrate for calling males and sexual interactions. Sixty virgin males (15 per treatment), 13–16 d old, and color marked as above, were released at 1650 hours. Ten minutes later, 30 virgin females, 20–23 d old, were added. The experiment ran until 1900 hours to coincide with *A. suspensa*'s sexual activity peak (Dodson 1982, Burk 1983, Hendrichs 1986, Landolt and Sivinski 1992). During these 2 h, temperature, relative humidity, and light intensity were measured every 30 min. Mating pairs were removed to 10-ml individual vials, and mating duration was recorded, as was position inside the cage (cage or tree), plant part, elevation within the

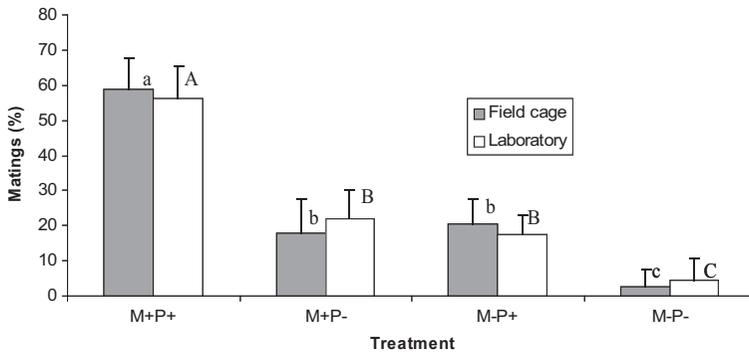


Fig. 1. Percentage of total matings, in both laboratory and field cage experiments, per treatment of male Caribbean fruit flies, when treated or not with methoprene (M^+/M^-) and fed or not with protein (P^+/P^-) (mean \pm SD). Data were obtained from 12 replications in both in laboratory and field cage experiments. Different lowercase and capital letters represent significant differences among treatments for field cage and laboratory tests, respectively (Tukey's test, $P = 0.05$).

tree canopy (high, middle, or low), and side of leaves on which mating occurred (under or over).

The abiotic conditions during the 6 d of the field cage experiment were similar. The temperature at 1700 hours was similar on the 6 d of experiments, 28–30°C, and varied from 24 to 30°C throughout the 2-h period of the study (1700–1900 hours). Daily fluctuations were on the order of 2–4°C. Relative humidity varied from 48 to 79%. Light intensity dropped from $1,112 \pm 135$ lux at 1700 hours to 291 ± 71 lux at 1900 hours. The maximum light intensity was registered on 12 October at 1700 hours (1,345 lux). Sunset occurred at 1911 hours on 4 October (first day of experiment) and at 1900 hours on 13 October (last day of experiment).

Sexual Performance Over Life. This experiment was conducted in the laboratory in individual cylindrical cages (10 cm high and 7 cm diameter). Cages were placed over a container that supplied water ad libitum to the flies through a cotton wick. Eighty cages (20 per treatment) were examined over a period of 35 d. Virgin males in cages were provided with the adult food appropriate to each treatment (sugar in P^- treatments and sugar and hydrolyzed yeast [3:1] in P^+ treatments). Daily, at 1700 hours, one virgin female (20–23 d) was released in to each cage and observed until 1900 hours. The adult females were maintained with optimal adult diet (sugar plus hydrolyzed yeast in 3:1 proportion) and water ad libitum. Mating and copulation duration were recorded. If mating occurred, females were removed at the end of copulation (copulations lasted \approx 30 min on average). All females that had not mated were removed at 1900 hours. The ones that were still in copula were removed as soon as they finished. The same procedures were repeated daily until males were 35 d of age.

Sexual Performance on a Daily Basis. This experiment was conducted in the laboratory in individual cylindrical cages (10 cm high and 7 cm diameter) as described above. Eighty cages (20 per treatment) were examined with males that were 5, 10, 15, 20, 25, 30, and 35 d of age. Males were maintained before transfer in a 30 by 30 by 30-cm cage (one cage per

treatment). At each of the abovementioned ages, males were transferred to individual cages at 1500 hours. One 20- to 23-d-old virgin female per cage was released at 1600 hours. After a 1-h interval (at 1700 hours), the resident female was replaced by another female whether or not the original female had mated. If a mating was still in progress, the female was replaced immediately after the pair separated, and another female was introduced. The procedure was repeated at 1800 hours. The experiment finished at 1900 hours, with the exception of the pairs still in copula (continued until pair separated). Mating and copulation duration were recorded.

Statistical Analyses. Data were analyzed by two-way analysis of variance (ANOVA) with interactions on effects of methoprene and protein. These analyses were followed by an ANOVA to detect differences between means in the treatments. Tukey's mean separation test ($P = 0.05$) was used for significant factors in the ANOVA (Ott and Longnecker 2001). In the sexual performance on a daily basis experiment, a two-way ANOVA was performed to detect treatment and age effects. Statistical analyses were performed using R software (version 2.1.0, www.r-project.org).

Results

Sexual Success in the Laboratory. From a total of 180 cages (12 replications with 15 cages each), 131 successful matings were recorded in the laboratory so that 73% of all females mated. There was a significant interaction between effects of methoprene application and protein supply ($F_{1,44} = 16.81$, $P < 0.001$) on mating. Therefore, data were reanalyzed using one-way ANOVA on all four treatments (Fig. 1). Of the total matings, 55% were performed by M^+P^+ males, which was significantly higher compared with males from all the other treatments ($F_{3,44} = 75.17$, $P < 0.001$). M^-P^- males had significantly fewer matings (5%) than other treatments in this competitive arena. Wing lengths (mean \pm SD) of males that did and did not mate were not significantly different (mated: 4.7 ± 0.30 mm, $n = 131$; unmated: 4.7 ± 0.24 mm, $n = 393$).

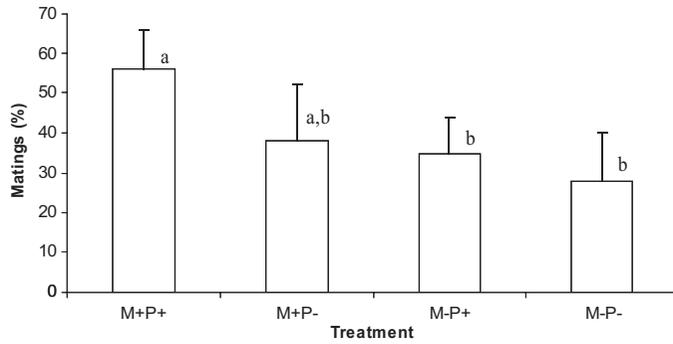


Fig. 2. Percentage of total matings obtained per treatment of male Caribbean fruit flies from day 4 to the end of experiment (day 35), when treated or not with methoprene (M^+/M^-) and fed or not with protein (P^+/P^-) (mean \pm SD). Bars with the same letter were not significantly different (Tukey's test, $P = 0.05$).

Sexual Success in Field Cages. A total of 104 matings (29% of females) were recorded in the 12 field cage replicates. There was a significant interaction between effects of methoprene application and protein supply ($F_{1,44} = 20.04$, $P < 0.001$) on mating. Therefore, data were reanalyzed using one-way ANOVA on all four treatments. The M^+P^+ males with 61 matings (59%) had significantly higher mating success ($F_{3,44} = 84.84$, $P < 0.001$) compared with other treatments (Fig. 1). M^-P^- males obtained only three matings (3%) and had a significantly lower male competitive ability compared with the other treatments.

Of the 102 matings that occurred on the tree, all were on leaves, and 94 (92%) occurred on the undersides of leaves. Seventy-five matings (73%) occurred in the highest part of the canopy, 22 (22%) in the middle part, and only 5 (5%) in the lower part.

Sexual Performance Over Life. There was a significant interaction between methoprene and protein ($F_{1,124} = 5.98$, $P = 0.016$). During the period we monitored, M^+P^+ males obtained significantly more copulations ($F_{3,124} = 15.57$, $P < 0.001$) than males with no methoprene application (Fig. 2). The earliest mating occurred on day 4 for M^+P^+ males, on day 5 for M^+P^- males, and on day 6 for males that did not receive methoprene. In addition, M^+P^+ males mated consecutively over ≥ 3 d significantly more than males in other treatments.

Sexual Performance on a Daily Basis. There was a significant effect of both treatment ($F_{3,18} = 49.56$, $P < 0.001$) and adult age ($F_{6,18} = 12.63$, $P < 0.001$) on mating on a daily basis. At each of the examined ages, M^+P^+ males obtained more copulations (Fig. 3). As in the previous experiment, the M^+ males began to mate earlier (see adult age 5 d in Fig. 3). At 5 d, only one M^- male mated. M^+P^+ males were not only more likely to mate, but 10% (14 males) were able to mate three times on the same day. One M^+P^- , one M^-P^+ , and none of the M^-P^- males were able to mate so often (Fig. 4).

Copulation Duration. The 104 matings performed in the field cages averaged 30.8 ± 8.6 min, and there were no significant differences among treatments ($F_{3,100} = 0.176$, $P = 0.912$). Similarly there were no

differences in duration of copulation among treatments on a lifetime basis ($F_{3,577} = 1.69$, $P = 0.304$) or in the durations of matings by number of matings during their lifetimes (1, 2, 3, and >3) within each treatment ($F_{3,577} = 0.854$, $P = 0.465$). The total of 581 matings observed in the lifetime experiment averaged 26.9 ± 9.8 min.

On a daily basis, no differences in mating duration were found among treatments ($F_{3,415} = 1.001$, $P = 0.392$). However, in males that mated for a third time within a single day, the duration of the third mating was significantly shorter ($F_{3,415} = 40.30$, $P < 0.001$; Fig. 5).

Discussion

Both laboratory and field cage tests found a clear increase in male sexual performance caused by methoprene application, the addition of protein in the diet, and the interaction of methoprene and protein. The effect of acetone was not studied. The proportions of females simultaneously exposed to males of different treatments and that subsequently copulated differed in the laboratory and in field cages (73 versus 29%). This may be because of the greater ratio of males to females in the laboratory tests (4:1 in laboratory versus 2:1 in field cages). However, the larger space available

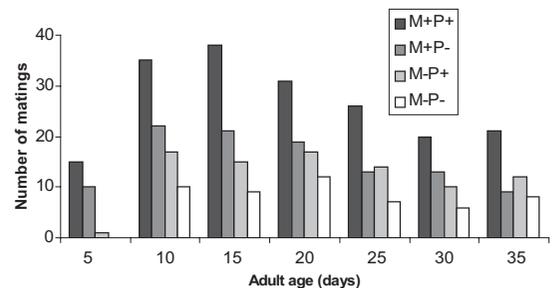


Fig. 3. Number of matings obtained per treatment at various ages by male Caribbean fruit flies when treated or not with methoprene (M^+/M^-) and fed or not with protein (P^+/P^-).

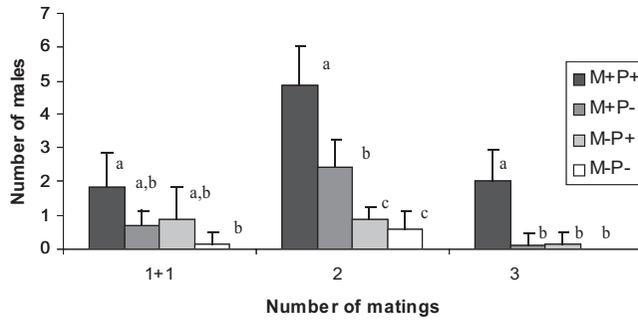


Fig. 4. Average number of male Caribbean fruit flies that mated twice (with two consecutive females provided or 1 + 1 when mate with first and third female provided) or three times on the same day (mean \pm SD). Data derived from a total of 140 males (20 per treatment of seven different ages) when treated or not with methoprene (M^+/M^-) and fed or not with protein (P^+/P^-). Bars with the same letter for each number of matings were not significantly different (Tukey's test, $P = 0.05$). ANOVA values: 1 + 1 matings ($F_{3,24} = 3.56$, $P = 0.038$); 2 consecutive matings ($F_{3,24} = 15.74$, $P < 0.001$); 3 matings ($F_{3,24} = 9.74$, $P < 0.001$).

inside the field cages might have given females more opportunities to exercise mate choice and more easily escape the attentions of unwanted suitors.

Sugar is a basic nutritional requirement for sexual activities of male *A. suspensa* (Landolt and Sivinski 1992, Teal et al. 2004). Incorporation of protein in adult diet improves male sexual success in tephritids such as *C. capitata* (Blay and Yuval 1997, Yuval et al. 1998, Kaspi et al. 2000), *Anastrepha obliqua* (Macquart), *Anastrepha serpentina* (Wied.), *Anastrepha striata* Schiner (Aluja et al. 2001), and *A. suspensa* (Landolt and Sivinski 1992). Aluja et al. (2001) found no effect of additional protein on male *Anastrepha ludens* (Loew) sexual success, and Mangan (2003) found no effect of protein-fed males on the female egg production, when studying the transferred products by trophallaxis.

Taylor and Yuval (1999) reported that female *C. capitata* store more sperm when they copulate with protein-fed males, which reduces subsequent female remating. In our study, protein diets improved sexual performance. This was true whether methoprene was applied or not, although the addition of methoprene with protein resulted in the highest levels of male sexual success. Protein diet might also influence suc-

cess in agonistic encounters, a frequent occurrence in lekking *A. suspensa* (Burk 1984). Males that win territorial contests may obtain more suitable and attractive locations within leks (Burk 1983, Thornhill and Alcock 1983, Hendrichs 1986, Sivinski and Heath 1988, Sivinski 1989).

The combination of methoprene application with a supply of protein-enriched food resulted in greater male sexual success than when either was provided separately. This effect was consistent in all the tests performed. For example, basically only M^+P^+ males were able to perform the sexual feat of mating three consecutive times in a 3-h window. It seems likely that insects with an optimal diet would have the energetic capacity to exploit the physiological effects of methoprene application.

Average mating duration was similar in field cage (coincident with the data of Hendrichs 1986 under a similar protocol) and in laboratory studies (coincident with data from Fritz 2004). No effect on duration was found among treatments. However, in males that performed a third mating on the same day, the final copulation was significantly shorter.

Aluja et al. (2001) found no differences in the durations of copulations performed by males fed on dif-

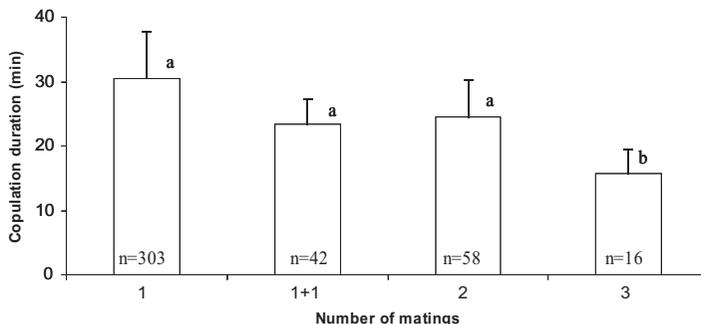


Fig. 5. Copulation duration (mean \pm SD) of male Caribbean fruit flies that mated one, two (with two consecutive females provided or 1 + 1 when mate with first and third female provided), and three consecutive times on the same day. Bars with the same letter were not significantly different (Tukey's test, $P = 0.05$).

ferent diets in *A. ludens*, *A. obliqua*, and *A. serpentina*. However, Pérez-Staples and Aluja (2004) found that protein-fed males of *A. striata* have significantly shorter copulation durations, but these males are able to mate more often. Inconsistent results were found for diet effects on copulation duration in *C. capitata*. Protein-deprived, mass-reared males had longer copulations than protein-fed males (Blay and Yuval 1997, Field and Yuval 1999); however, no such differences were found in wild males (Taylor et al. 2000, Shelly and Kennelly 2002). The causes of this sort of variability are presently not understood but could be caused by either male capacity or female perception of male quality. In either case, Fritz (2004) found that copulation duration was positively correlated with the quantity of sperm stored by the female *A. suspensa*.

The improved sexual performance of males and their earlier maturation caused by methoprene application could contribute enormously to the success of SIT. Ultimately, this technique is based on release in the field of competitive sterile males (Knipling 1955). Increased male signaling and pheromone production, although not physiologically well understood (Teal and Gomez-Simuta 2002a), seem to lead to both significantly greater daily and lifetime sexual success (Teal et al. 2000).

Additionally, earlier maturation can significantly reduce the costs of fly handling operations. This is particularly the case in tephritids with long adult pre-copulatory periods like the *Anastrepha* species (Aluja 1994) but less so for species like *C. capitata* with shorter precopulatory periods (Liedo et al. 2002). Sterile *A. suspensa* flies treated with methoprene might be ready to release 2–3 d earlier than untreated males, and this means that space for storage of adults could be considerably reduced. At the same time, the release of sexually mature males could represent a significant increase in SIT efficacy.

The combination of protein diet and methoprene resulted in greater sexual success than either treatment alone. Dietary protein also increases adult lifespan in flies both treated and untreated with methoprene (Pereira 2005). For these reasons, the incorporation of protein in adult diets in SIT programs together with methoprene is highly recommended. In addition, the release of well-fed flies would allow sterile males to immediately search for mates rather than spend time foraging for food.

The transfer of this research to SIT action programs is now in progress. However, the further development of means of mass methoprene application is necessary. This may be accomplished through incorporation into along diets, along with additional protein. The ultimate technology must address the safety of the methoprene application, alone or with acetone, the cost-effectiveness of the technology, and its applicability to sterilized insects.

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