Improving the Efficacy of the Sterile Insect Technique for Fruit Flies by Incorporation of Hormone and Dietary Supplements into Adult Holding Protocols

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ABSTRACT The sterile insect technique (SIT) is a universally accepted method of control for tephritid flies. Improving efficacy of mating by sterile males would reduce costs significantly. This paper describes studies of the physiological mechanisms responsible for coordination of reproductive maturity and sex pheromone communication in males of the genus *Anastrepha* in order to develop methods for acceleration of reproductive maturity among sterilized males. These show that juvenile hormone III and its bisepoxide homologue are key hormones involved in coordination of reproductive development and pheromone-calling in both males and females. Additionally, incorporation of protein into the diet fed to sterile adults prior to release is critically important to improve pheromone calling, attraction of females and mating by sterile males. These results have led to development of a novel strategy to accelerate reproductive development of laboratory-reared sterile flies by incorporating hormone supplement therapy using mimics of juvenile hormone including methoprene and fenoxycarb and protein diets for use in mass-rearing protocols. This strategy resulted in accelerating reproductive development in males of the Mexican fruit fly *Anastrepha ludens* (Loew), the West Indian fruit fly *Anastrepha obliqua* (Macquart), the Caribbean fruit fly *Anastrepha suspensa* (Loew), and the Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) fruit flies by 3-7 days. Incorporating the technology into mass-rearing will significantly improve the efficacy of area-wide integrated pest management progammes with an SIT component to control these pests.

KEY WORDS *Ceratitis capitata*, *Anastrepha ludens*, *Anastrepha obliqua*, *Anastrepha suspensa*, juvenile hormone, protein diet supplements, sexual maturation, sexual signalling

1. Introduction

The interventionist approach of directly killing insect pests with toxic chemicals has been the prevailing control strategy for over 50 years. This has led to environmental contamination and pest resistance to the toxins while tolls due to pests grow higher, costs of treatment increase and profits decrease. Truly satisfactory and lasting solutions to pest problems will require a shift to understanding and promoting other means of control, such as the sterile insect technique (SIT). Indeed the SIT is a universally accepted and environment-friendly method for controlling a variety of insect pests. Better knowledge of the basic mechanisms that govern insect reproduction and a more thorough understanding of the

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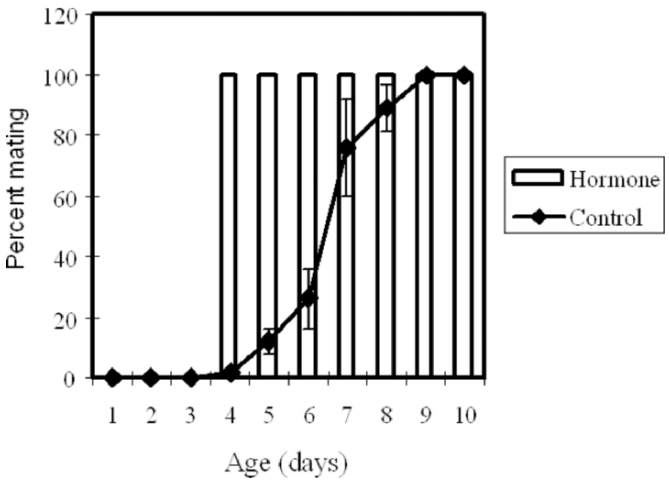

Figure 1. *Effects of hormone supplement therapy on mating by males of the Caribbean fruit fly* Anastrepha suspensa*. Males were either treated with just one microlitre of acetone or treated with five micrograms of methoprene on the day of eclosion. Six replicates of ten males per each treatment and age. Significantly more males treated with hormone mated on each of days 4-6. Data for fenoxycarb were identical to those of methoprene.*

physiology and chemical ecology of pests are necessary to develop improved methods to increase efficacy of the technique. To this end, research has been conducted in two areas: (1) determining the hormonal mechanisms regulating sexual maturity, and (2) determining the dietary requirements of adults to optimize pheromone calling and performance of males. The following describes the work conducted using tephritid fruit flies as model species.

2. Tephritid Fruit Flies of Economic Importance

Tephritid flies, including members of the *Ceratitis*, *Anastrepha*, and *Bactrocera* genera, pose a serious threat to agriculture in the USA. More than 260 different hosts, including stone fruits like plums and peaches, and cash crops like tomatoes and peppers, have been recorded for the Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) alone. All agriculturally important tephritids are under both national and international quarantine restrictions which, in the case of the Caribbean fruit fly *Anastrepha suspensa* (Loew), an established pest in Florida, have very significant impacts on the USD 1500 million citrus industry due to loss of world markets. The economic impacts of established populations of any quarantine tephritid in California or other citrus-producing states would be devastating. The cost of control of an established population of a single quarantined species of tephritid fruit fly to the USD 6800 million per year California citrus and vegetable industries has been estimated at USD 1000 million, and quarantine of California fruit and vegetables from foreign markets would result in the loss of 35 000 jobs and decrease output by USD 3600 million per year (CDFA 2006a,b).

Increased importation of fruit and vegetables from other countries and between states has magnified considerably the probability of populations becoming established. For example, in 2000 the California Department of Food and Agriculture declared an exterior quarantine for 100 fruits, vegetables and berries (including such common items as blackberries, figs, citrus, bell pepper, and tomato) from the areas in the State of Florida

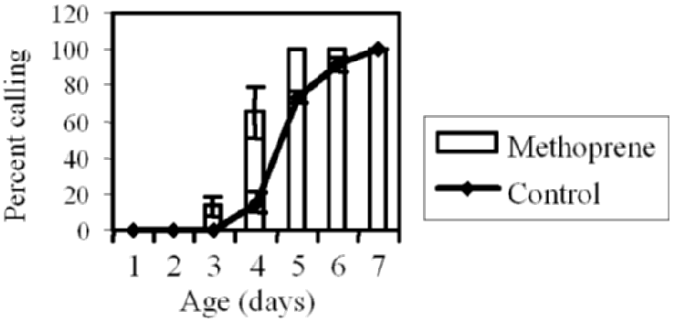

Figure 2. *Comparison of age (cumulative percentage) at which males of the West Indian fruit fly* Anastrepha obliqua *engage in pheromone calling when treated with methoprene or with just solvent (control) on the day of adult emergence (six replicates of ten males per each age).*

south of and including Hernando, Sumter, Lake and Volusia Counties, the major production areas for these commodities in Florida.

Strict monitoring protocols are in place to detect introductions of the pests. For example, the State of Florida deploys more than 13 000 attractant traps to detect potential invasions of the Mediterranean fruit fly alone. Once an outbreak is detected, the infested areas are subjected to immediate quarantine to eliminate movement of fruit to other areas, fruit from host plants are stripped from infested sites, and ground and aerial pesticide application control protocols are initiated. In the past, fumigant treatment of imported fruit using ethylene dibromide significantly limited invasions of these flies. However, registration for ethylene dibromide use has been withdrawn and no substitute has been found. Thus, three practical control methods remain: (1) stripping and destroying fruit from infested areas, (2) bait sprays using 10% malathion and more recently Spinosad®, and (3) release of sterilized males. The use of malathion bait sprays has come under constant criticism due to environmental and health related problems and several law suites have been filed to stop application.

Use of the SIT provides an environmentally safe and species-specific method to suppress or eradicate tephritid fruit flies of agricultural importance worldwide. In fact, California employs the technique on a routine basis for preventing and eradicating both Mediterranean and Mexican fruit fly outbreaks (CDFA 2006a) and also in Florida the technique is being used as a preventive method to ensure that the Mediterranean fruit fly does not become established. While the basic protocols associated with the SIT are well established, the technique is expensive in terms of time and money. An important goal is therefore to dramatically improve the efficacy of the SIT by developing methods to improve mating efficiency of sterile males and to reduce costs associated with implementation of control programmes.

3. Hormone Supplement Therapy

Until recently, few studies had been conducted on the endogenous regulation of sexual maturity and pheromone production in tephritid fruit flies. Studies on the Mediterranean fruit fly indicated that application of juvenile hormone accelerates ovarian maturity (Chang and Hsu 1982, Chang et al. 1988, Hsu et al. 1989). Also, the attractiveness of males to females was found to be reduced among males treated with precocene II (Chang and Hsu 1982, Chang et al. 1984). Application of juvenile hormone III reversed the effect of precocene II (Chang and Hsu 1982) suggesting that pheromone calling was affected by juvenile hormone in some, as yet unknown,

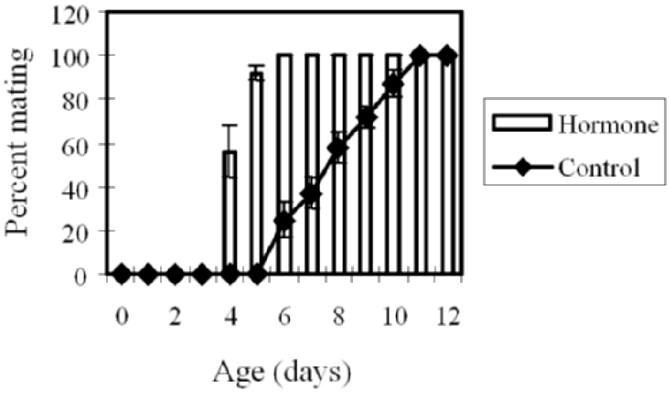

Figure 3. *Comparison of age at which male sterile Mexican fruit flies* Anastrepha ludens *mate when they are treated with hormone or with just solvent (control) on the day of adult emergence. All hormone-treated males (six replicate-treated groups of ten males per each age) mated by day five whereas only 86% of control males mated by day ten.*

fashion.

Research on the effects of juvenile hormone in regulation and acceleration of sexual maturity in tephritid flies has followed protocols developed for the Caribbean fruit fly (Teal et al. 2000). Results of studies on this fly have documented conclusively that juvenile hormone is a crucial hormone regulating development of reproductive competence and pheromone calling since topical application of juvenile hormone or the juvenile hormone mimics, methoprene and fenoxycarb, to newly eclosed adults or late stage pupae of the Caribbean fruit fly induced precocious reproductive development. Thus, Caribbean fruit fly males treated with hormone on the day of adult emergence mate 4-5 days earlier than untreated males (Fig. 1).

The same methods were followed to conduct studies on the effects of hormone supplement therapy on acceleration of reproductive development as indicated by age at which pheromone calling occurred using both the Mexican fruit fly *Anastrepha ludens* (Loew) and the West Indian fruit fly *Anastrepha obliqua* (Macquart) (Fig. 2). In both cases, application of methoprene or fenoxycarb induced a significant acceleration in reproductive development so that treated males mated earlier than their solvent-treated controls. These studies were conducted using flies that had not been irradiated. To determine if irradiated flies underwent the same acceleration in reproductive development, males of the Mexican fruit fly were treated with hormone on the day of adult eclosion and the same mating studies performed using fertile females. As shown in Fig. 3 essentially all hormone-treated flies mated by day 5, but even at day 10 only 86% of the solvent-treated sterile males had mated.

Field cage studies were conducted using the Mexican fruit fly to determine the competitiveness in mating with 12-day-old wild females of 5-day-old hormone-treated sterile males with 5-day-old untreated sterile males and 12-day-old wild males to mate with wild females. These studies were conducted using published standard protocols (FAO/IAEA/USDA 2003), except that each male and female released into flight cages was marked with a number so that the mating status of each individual could be assessed. The results of the study indicate that 6-day-old sterile males treated with hormone are fully competitive with both 12-day-old untreated sterile males (that matured naturally), and 14-day-old wild males in mating with wild females (Fig. 4).

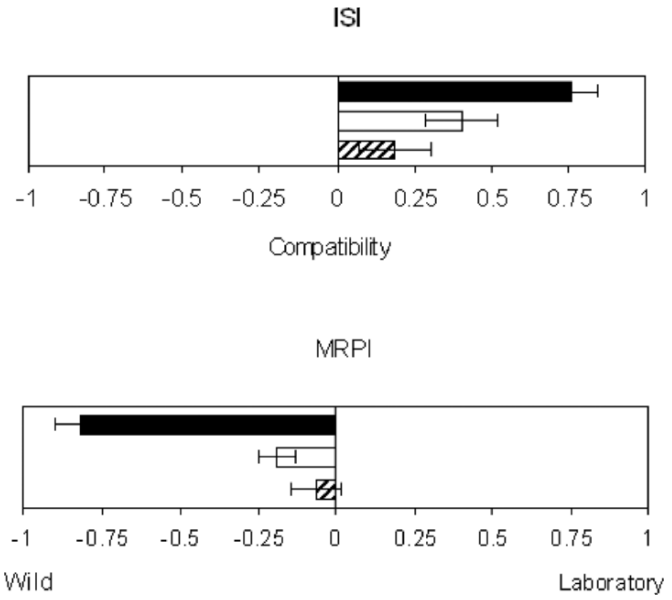

Figure 4. *Mating compatibility tests using Mexican fruit flies* Anastrepha ludens *comparing hormone-treated 6-day-old sterile males (open bars), control 6-day-old sterile males (hatched bars) and wild 14-day-old males (black bars). In all cases 6-day-old hormone-treated sterile males performed as well as 12-day-old untreated sterile males. Descriptions of tests conducted and of each of the performance indexes used are given in "Mating Compatibility Tests" in FAO/IAEA/USDA (2003).*

Preliminary studies were made on the effects of juvenile hormone supplement therapy on bisexual sex strain irradiated Mediterranean fruit flies obtained from shipments sent from Guatemala to Florida during 1998. In these experiments, the effects of application of juvenile hormone to newly eclosed males on pheromone production were monitored. It was found that 2-day-old males treated with hormone released twice as much pheromone as the solvent-treated controls.

Also monitored was the calling (pheromone release) period of treated and solvent-treated control sterile Mediterranean fruit flies. The pheromone release period is directly correlated with the mating period in this species. From this it was determined that flies treated with hormone began calling significantly earlier in the day than did untreated control sterile males. In fact the calling period of the hormone-treated flies occurred during the same period that has been determined as the reproductive period for wild Mediterranean fruit flies in Guatemala (data on natural calling period was obtained from data reported by Landolt et al. (1992)). If the trends for production and release of more pheromone and shifts to calling times that coincide with the wild flies are confirmed when sterile males are treated with hormone, then hormone therapy will significantly improve the mating performance of Mediterranean fruit flies.

Individual sterile males are often considered to have the potential to remove a single wild female from the reproductive population. Compounds in the accessory glands of the Mediterranean fruit fly have been shown to

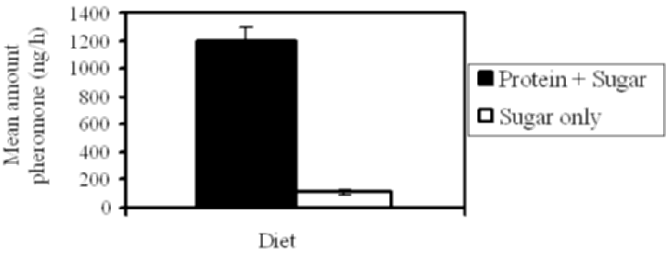

Figure 5. *Comparison of pheromone released by 14-day-old male Caribbean fruit flies* Anastrepha suspensa *fed either the dry diet composed of 1:3 protein plus sugar or sugar alone (N=6 replicates of each treatment).*

inhibit remating and to induce females to engage in searching for fruit and oviposition (Jang 1995, Jang et al. 1998, Miyatake et al. 1999). It is very possible that sterile males fail to replenish the secretions from the accessory glands in the reproductive system after mating. Wild males replenish these secretions within a few hours after mating. Juvenile hormone is known to induce replenishment of accessory gland secretions in other fruit flies. Thus, hormone supplement therapy with juvenile hormone should allow sterile males to rapidly replenish the accessory gland secretions after the initial mating. Therefore, sterile males could be capable of effectively mating more than once with wild females. Multiple mating by released sterile males will make the SIT even more cost effective.

4. Effects of Diet on Sexual Communication and Mating

Current protocols used for holding adult flies prior to release include feeding flies a sugar agar diet composed of 15% sucrose, 0.8% agar (a mixture of polysaccharides) and 84.2% water. No protein is included in the diet. The exclusion of protein from the adult diet was justified for three reasons: (1) it increased costs, (2) it was hypothesized that the protein benefited females greatly but had minimum effects on male survival (Galun et al. 1985), and (3) protein supposedly increased microbial growth in the diet which could lead to sickness. As a consequence the sugar agar diet is used to feed adult sterile flies prior to release. However, it has long been recognized that protein is of importance for achievement of sexual maturity by the adults of tephritid fruit flies (Bateman 1972, and references therein). Indeed, recent work on the Mediterranean fruit fly has shown that calling behaviour, sexual competitiveness and reproductive success are enhanced significantly when adult males from either wild stocks or laboratory-reared colonies are provided with protein in the adult diet (Warburg and Yuval 1996, Blay and Yuval 1997, Papadopoulos et al. 1998, Kaspi et al. 2000, Kaspi and Yuval 2000, Yuval et al. 2002). Similarly, males of the West Indian fruit fly and its relatives, the guava fruit fly *Anastrepha striata* Schiner and the sapote fruit fly *Anastrepha serpentina* (Wiedemann) have higher copulatory success when fed a diet containing protein hydrolysate (Aluja et al. 2001a,b).

Male Caribbean fruit flies will survive for at least 20 days when fed only sugar and water (Teal et al. 2004). However, the amount of pheromone produced and released by males fed only sugar is less than 10% of that produced and released by males fed both protein and sugar (Fig. 5). These authors also conducted studies in which flies were fed either on sugar alone or sugar plus protein for 11 days and their diets then changed so that sugar-fed flies were provided with protein plus sugar on days 12-14 and protein plus sugar-fed flies were fed only sugar for days

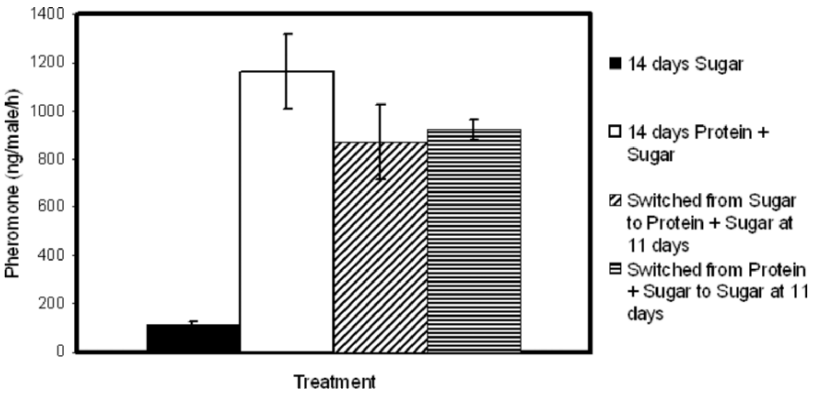

Figure 6. *Pheromone production by male Caribbean fruit flies* Anastrepha suspensa *fed sugar alone or sugar plus protein for 14 days, or by males switched from one diet to another on day 11 and allowed to feed until day 14 (N=8 replicates per treatment).*

12-14. On day 14, volatile pheromone released by these flies was collected and the amount released compared to that released by flies fed only sugar or sugar plus protein for 14 days. The results were of significance because adding protein to the sugar on day 11 caused flies fed only sugar for the first 11 days to produce as much pheromone as was produced by flies fed protein plus sugar for 14 days (Fig. 6). Additionally, flies switched from protein plus sugar to only sugar on day 11 produced as much pheromone as was produced by flies fed protein and sugar for 14 days. Clearly, providing protein is critical for optimizing pheromone release.

Flight tunnel studies have also been conducted in which 14-day-old females Caribbean fruit flies were released into the down-wind end of a 1.5-metre long flight tunnel and allowed to choose between the volatiles released by males fed on protein plus sugar or by males fed just sugar. All males were at least 11 days old. Volatiles were piped into the tunnel from holding cages held outside the tunnels. The outport of the volatile release tubes was attached to insect isolation traps lined with sticky paper to capture the female flies (Heath et al. 1993). Females were released early in the reproductive period (15:00 hours) and trap captures were recorded 23 hours later. The data showed that females were much more attracted to males fed protein plus sugar than to males fed only sugar.

5. Development of Hormone and Protein Delivery Techniques for the SIT

Although the results described above demonstrated that both hormone therapy and provision of protein supplements to male adults improved reproductive competence in experimental conditions, transfer of the technology to tephritid fruit fly factories requires that the methods are incorporated into practical systems. As indicated above, the diet currently used to feed adults prior to field release is a gel diet composed of 15% sucrose, 0.8% agar and 84.2% water. Therefore, it was critical that treatments developed could be incorporated into this agar-based diet.

The studies began by comparing the pheromone calling abilities in a flight tunnel and determining the amount of pheromone released by male Caribbean fruit flies fed the agar diet containing no protein or males fed the optimal diet for adult development, a dry diet composed of 3:1 sucrose and protein. For flight tunnel studies, females were released 1.5 metres downwind from traps releasing

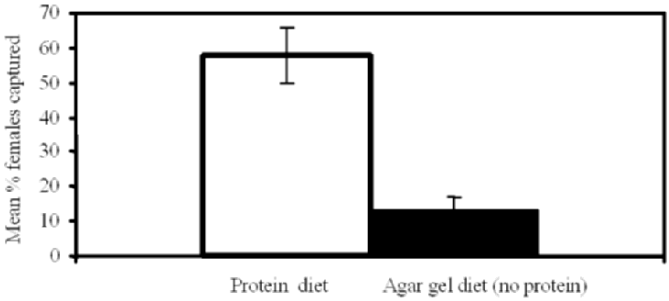

Figure 7. *Comparison of female Caribbean fruit flies* Anastrepha suspensa *responding to volatiles released by males fed the optimal dry diet or the agar gel diet as indicated by males captured in traps releasing pheromone from caged males held outside of the flight tunnel (N=8 replicates; 20 females released per trial).*

volatiles emitted from males fed either of the diets. As indicated in Fig. 7, females overwhelmingly chose traps releasing pheromone from males fed the dry diet. Additionally, males fed the dry diet released six times more pheromone than did males fed the agar diet. To determine if adding protein into the diet caused a change in pheromone calling, different amounts of protein were incorporated into the agar diets. Choice flight tunnel tests were conducted and the volatile pheromones collected from males. The results showed that addition of between 5-10% protein to the agar diet enabled males to compete equally with males fed the dry diet in attracting females and in release of pheromone (Fig. 8).

The effect of incorporation of hormone into the agar diet was then determined by incorporating a water-soluble formulation of methoprene at doses of 0.025, 0.05 and 0.1% active ingredient into the diet. Attraction of females to either the agar diet containing 10% protein or the agar diet containing 10% protein + either 0.025, 0.05 or 0.1% methoprene was compared in flight tunnel experiments. The data showed that females responded more

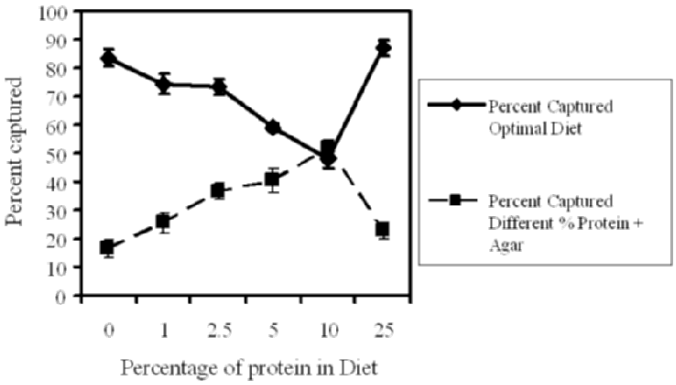

Figure 8. *Comparison of trap captures in flight tunnel studies where female Carribean fruit flies* Anastrepha suspensa *were offered the option of pheromones released by males fed the optimal dry diet or the agar gel diet to which various amounts of protein had been added (N = 6 replicates of each treatment).*

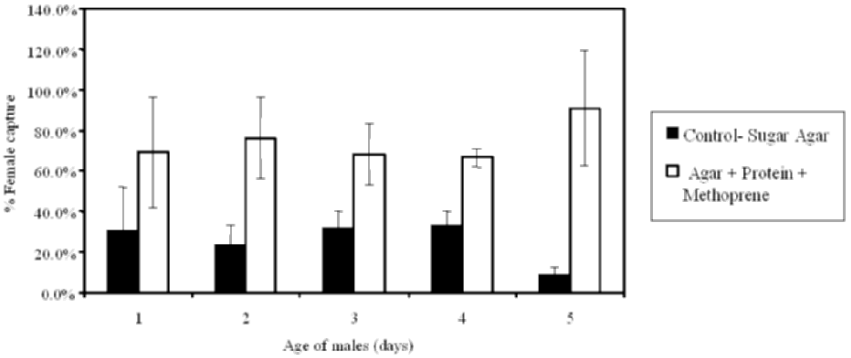

Figure 9. *Capture of sterile female Mediterranean fruit flies* Ceratitis capitata *in flight tunnel studies where females were given the choice of responding to males fed the control diet (sugar/agar gel) or the sugar/agar diet containing both 10% protein and methoprene. The numbers captured are significantly different on days 2-5 in a* t*-test (8 replicates).*

effectively when males had been fed any of the diets containing methoprene. Also, methoprene-fed males attracted females at much earlier ages. Analysis of pheromone released by males fed these diets showed that the amounts of pheromone were greater for all treatments that contained the methoprene.

Of course the most critical component of the SIT is effective mating. Therefore, mating studies were conducted using fertile females and sterile males fed the agar/protein plus methoprene diet or fed the agar/sugar diet that did not contain hormone or protein. Significantly more males that fed the agar diet containing protein plus hormone (48%, t = 3.16, d.f. = 8) mated. Only 30% of the males fed the agar only diet mated.

As with the Caribbean fruit fly, incorporating 10% protein into the agar diet fed to males belonging to the strain of Mediterranean fruit fly that carry a *temperature sensitive lethal* (*tsl*) mutation significantly improved the ability of males to attract sterile female flies on the second day after emergence, and these males produced approximately five times the amount of pheromone produced by males fed the agar diet alone. Adding methoprene to the agar/protein diet resulted in production of twice the amount of pheromone that flies fed the agar/protein diet produced (eight times more than flies fed the agar diet without protein), and this was reflected in flight tunnel studies indicating that females were far more likely to move to volatiles released by males fed the agar/protein plus methoprene diet than to males fed just the agar diet (Fig. 9). From this it was concluded that adding methoprene and protein to the agar diet fed to *tsl* male Mediterranean fruit flies improves their sexual competitiveness as was the case for the Caribbean fruit fly.

6. Conclusions

Although this research has demonstrated the need for protein in the diets fed to adult sterile males, and that incorporation of methoprene into the adult diet results in improved and accelerated development of pheromone calling, the technology has not yet been incorporated into mass-rearing systems. Currently, the cost effect of incorporating the technologies into sterile fly rearing programmes for both the Mediterranean and Mexican fruit flies is being assessed by the authors while numerous other research groups are examining the feasibility of the technology for different genera of tephritid flies. While it is believed that the technology will greatly improve the efficacy of the SIT for the Mediterranean, Caribbean, Mexican and West Indian fruit flies, a complete understanding of

the effects of hormone therapy and a benefit/cost analysis data are needed prior to employing the technology with other species of tephritids.

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