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Methoprene and protein supplements accelerate reproductive development and improve mating success of male tephritid flies

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Abstract

We have been studying the behavioural and physiological mechanisms associated with coordination of reproductive maturity and sex pheromone communication in male tephritid flies in order to develop methods for acceleration of reproductive maturity among sterilized males. Our studies revealed that exposure to the juvenile hormone analogue methoprene can accelerate the rate of sexual maturity in some but not all tephritid species tested. Additionally, we have determined that incorporation of protein hydrolysate into the adult diet improves sexual performance of sterile males. Coupling a diet enriched with protein hydrolysate for adult food and application of methoprene to adult males or pupae was found to advance significantly the age at which males of a number of species of flies from the genus *Anastrepha* and *Bactrocera cucurbitae* (Coquillet) become sexually mature and improve reproductive success of the males. These results have led to the development of a novel strategy to accelerate the reproductive development and increased competitiveness of mass-reared fruit flies for use in the sterile insect technique by incorporating methoprene treatment and protein hydrolysate diets into protocols for fruit fly emergence and release facilities. The following reviews research conducted to develop the system and our suggestions for use in improving efficacy of mating by sterile males destined for release in programmes to control some species of tephritid flies using the sterile insect technique.

Introduction

Tephritid fruit flies have evolved complicated sexual communication systems (Thornhill and Alcock 1983). For many species males are the signalling sex and form aggregations (leks) on plants for mating purposes. Females visiting these leks rely on chemical, auditory, and visual signals emitted by males to select mates (Nation 1972; Sivinski and Burk 1989). In this male signalling system, pheromones are important factors responsible for female attraction over at least 2 m (Perdomo et al. 1975, 1976; Webb et al. 1983; Heath et al. 1993; Sivinski et al. 1994) and, when coupled with auditory signals (Webb et al. 1984), maximize the probability of females landing in the vicinity of male leks. Visual displays most likely function in concert with pheromones and sound for close range mate selection (see Sivinski and Burk 1989).

Several factors affect sexual signalling by tephritid flies. For example age is a major factor that regulates the sexual signalling system of numerous *Anastrepha*

and *Bactrocera* species. In nature *Anastrepha* spp. generally require between 1 and 2 weeks to become sexually mature, but for some *Bactrocera* the maturation phase may take as long as three or more weeks (Sivinski et al. 2000). This period of sexual maturation coincides with the development of male secondary sexual characters, such as salivary glands of *Anastrepha* spp. and rectal glands of *Bactrocera* spp. (Fletcher 1969; Nation 1974). Similarly, females undergo a period of maturation before they engage in sexual behaviour when ovaries mature (Nation 1972). Maximal pheromone production by laboratory-reared males of *Anastrepha suspensa* (Loew) is not achieved until the males are more than a week old, and the levels remain relatively constant for at least the next 2 weeks (Nation 1990). Females require somewhat longer. Thus, both sexes require a period of maturation prior to engaging in sexual behaviour, and we have shown that the coordination of sexual signalling and reproductive maturity in males is regulated by juvenile hormone (Teal et al. 2000).

Diet has also been implicated as a regulator of sexual communication. Nation (1989) reported that males of the Caribbean fruit fly, *A. suspensa*, denied access to a protein source from the time of emergence, released 40–70% less pheromone than males having access to protein in addition to sugar in their food. Landolt and Sivinski (1992) showed that the degree of sexual activity during the afternoon was dependent on the type of food present, and flies deprived of all food during the day did not exhibit sexual activity during the afternoon. Results of their studies indicated that males provided with sugar or sugar plus protein (hydrolysed torula yeast) exhibited higher rates of sexual activity than males provided with only protein hydrolysate (Landolt and Sivinski 1992). Studies on *Anastrepha ludens* (Loew) and *Anastrepha obliqua* (Macquart) have demonstrated that females produce more eggs when fed sugar plus protein than when fed only sugar (Aluja et al. 2001a) and males of *A. obliqua*, *Anastrepha striata* Schiner and *Anastrepha serpentina* (Wiedemann) have higher copulatory success when fed a diet also containing protein hydrolysate (Aluja et al. 2001b). Results of studies on the Queensland fruit fly, *Bactrocera tryoni* Froggatt, have shown that providing protein to young adults significantly improves sexually activity of both sexes (Perez-Staples et al. 2007, 2009). Additionally, some studies on the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), have shown that calling behaviour, sexual competitiveness and reproductive success are enhanced significantly when adult males from either wild or laboratory-reared colonies are provided with protein in the adult diet (Warburg and Yuval 1996; Blay and Yuval 1997; Papadopoulous et al. 1998; Kaspi and Yuval 2000; Kaspi et al. 2000; Yuval et al. 2002).

The use of the Sterile Insect Technique (SIT) provides an environmentally safe and species-specific method to prevent, contain, suppress, or eradicate tephritid fruit flies of agricultural importance worldwide (Enkerlin 2005; Hendrichs et al. 2005) as part of area-wide integrated pest management programmes (Klassen 2000). However, there are drawbacks associated with use of SIT for control of species having long pre-reproductive periods. These include the requirement of holding adults until they become sexually mature, which is costly, or more commonly releasing immature sterile flies into the field, where they suffer high mortality due to predation and other losses, resulting in a significant reduction in the number of males that reach sexual maturity. Developing techniques that accelerate reproductive development and sexual signalling in sterile tephritid males would reduce these negative effects, because males could be released earlier in life and closer to sexual activity.

The following reviews our efforts to understand factors affecting reproductive development among species of tephritid flies that have a long pre-reproductive period, the interactions between these factors, and our efforts to reduce the pre-reproductive period by incorporating our results into sterile fruit fly pre-release protocols.

Juvenile Hormone and Male Sexual Development

Reproductive success among insects requires the use of efficient sexual signalling systems. Mating, in most cases, is correlated with adult reproductive maturity to ensure that reproductive effort and energy are not wasted by mating when fertilization is not possible. Therefore, the endogenous mechanisms that regulate the signalling systems are closely coordinated with the factors responsible for controlling reproductive maturity (Teal et al. 2000). When we began our studies, there was little information on the hormonal regulation of sexual maturity in tephritid fruit flies. Studies on *C. capitata* indicated that application of juvenile hormone (JH) accelerated ovarian maturity (Chang et al. 1984; Hsu et al. 1989). Also, the attractiveness of females to males was found to be reduced among males treated with precocene II, which inhibits synthesis and release of

JH by killing cells of the *corpora allata* (Chang and Hsu 1982; Chang et al. 1984). Application of JH III reversed the effect of precocene II (Hsu and Chang 1982) and strongly indicated that sexual signalling was affected by JH in *C. capitata*.

Although the effects of JH III on reproductive behaviour of the Mediterranean fruit fly indicated that JH was associated with coordination and regulation of sexual signalling and reproductive development in tephritids, there was no chemical information supporting its presence as a circulating hormone in these flies. Indeed, no information was available on amounts of JH in haemolymph of adult males of any dipteran species. Therefore, we began our studies by looking for the presence of JH III and related compounds in haemolymph of mature *A. suspensa* males. When we analysed haemolymph from mated 6-day-old males we identified JH III and its bisepoxide homologue (JH IIIB) in a ratio of 1 : 3 (Teal et al. 2000). Interestingly, the amounts of these compounds in similarly aged virgin male flies were lower than those found in mated flies. Consequently, we decided to determine the effect of topical application of JH III to newly emerged males in terms of their sexual development. When we compared ages at which all males engaged in calling and mating we found that JH-treated males engaged in these activities about 3 days earlier than those receiving only acetone (Teal et al. 2000). Treatment with stable analogues of JH III, methoprene and fenoxycarb, yielded better results probably due to their resistance to enzymatic degradation in the haemolymph. Subsequently, we conducted similar studies using males from laboratory strains of other *Anastrepha* species, including the Mexican (*A. ludens*), West Indian (*A. obliqua*) (Teal et al. 2007), and South American flies (*A. fraterculus*) (Segura et al. 2009) as well as for the melon fly (*Bactrocera cucurbitae* Coquillett) (Haq et al. 2010). In all cases there was a significant decrease in the age at which males became sexually mature when methoprene was applied topically to newly emerged males, which were fed on a diet rich in sugar and hydrolysed protein.

Protein and Male Sexual Development

Although sugar is an absolute necessity for survival of adult Caribbean fruit flies, protein hydrolysate is not (Teal et al. 2004). Nonetheless, it has long been recognized that protein is of importance for achievement of sexual maturity by the adults of tephritids (Bateman 1972 and references therein). Our results indicated that removal of protein hydrolysate from the adult diet after 11 days has a minor negative effect on pheromone production of *A. suspensa* after a 3-day withdrawal period (Teal et al. 2007). However, switching flies from sugar-only to the diet containing a 3 : 1 ratio of sugar to hydrolysed yeast at day 11 resulted in a rapid increase in the ability to produce and release pheromone. We consider this to be a rapid increase in the ability to produce pheromone, because it takes 8 days from the time of adult emergence for flies fed the optimal diet to release this amount of pheromone (Teal et al. 2000). If protein deprivation alone inhibited the development of cells responsible for the production and release of pheromone, then it might be expected that development of competent pheromone production machinery would be delayed by this same 8-day period when males were switched from sugar-only to the sugar-protein hydrolysate diet. However, this was clearly not the case. Consequently, we believe that cells responsible for producing pheromone are competent at 11 days of age when males are fed only sugar but that protein is required to switch on key regulatory genes or enzymes required for synthesis of pheromone by the cells.

Protein is a more limited resource than carbohydrate in the natural environment of these flies (Bateman 1972; Hendrichs et al. 1991), and the processes of pheromone biosynthesis, lekking, and engaging in courtship behaviour are energetically expensive. Studies have also shown that protein hydrolysate supplements improve male sexual performance in *A. suspensa* (Pereira et al. 2009, 2010), *A. ludens* (Pereira et al. 2011), *A. fraterculus* (Segura et al. 2009), *B. cucurbitae* (Haq et al. 2010), *B. tryoni* (Perez-Staples et al. 2007, 2009) suggesting that the importance of protein hydrolysate for sexual development is widespread among the tephritid flies.

Combining Protein Hydrolysate and Hormone Supplements to Accelerate Reproductive Development

Application of methoprene to flies provided with only sugar as a source of nutrient did accelerate reproductive development and improved sexual signalling by males. However, application of methoprene to flies provided with sugar and protein hydrolysate as a nutrient source was even more effective (Pereira et al. 2009, 2010, 2011; Segura et al. 2009; Haq et al. 2010). Indeed, the improvement resulting from application of methoprene to sugar-fed flies was, in general, no better than when

flies were provided with sugar plus protein hydrolysate, but no methoprene, rather than just sugar. The fact that coupling methoprene application with a diet rich in protein hydrolysate effectively doubled the effect of either individual treatment indicates that neither hormone nor food are solely responsible for accelerating sexual maturity. Therefore, we believe that neither hormone therapy nor a protein hydrolysate enriched diet alone would be cost-effective for mass-rearing of sterile flies due to the increased cost and limited effect. Thus, the combination of a protein hydrolysate rich diet with hormone therapy is required to optimize reproductive potential of sterile males competing with wild males for wild females.

Adding protein hydrolysate to the adult diet is not difficult, and we have shown that addition of 5–10% to sugar is sufficient to supply *A. suspensa* with the necessary proteinaceous resources (Teal et al. 2007). The problem lies in developing methods for incorporating methoprene into the pre-release adult holding system. The most efficient way to deliver the combination is in a single treatment. An agar-based gel diet containing sugar is the standard diet used for providing water and feeding sugar to adult sterile fruit flies at fly emergence and release centres in many fruit fly SIT programmes (Animal and Plant Health Inspection Service-United States Department of Agriculture [APHIS-USDA], 2009). So it seemed logical that this would be a good starting point for development of diets containing both protein hydrolysate and hormone. As a first step in development, we conducted studies using *A. suspensa* to determine the minimum amount of protein hydrolysate required in the agar to optimize male pheromone production, calling behaviour and the attraction of females to males in flight tunnel studies (Teal et al. 2007). Results of our studies showed that addition of between 5% and 10% protein hydrolysate to this agar-sugar diet yielded the same results as the optimal diet, a dry diet containing a 3 : 1 mixture of sugar and protein hydrolysate. Subsequently, we conducted studies to assess the effects of adding methoprene to the agar-sugar diet containing 10% protein hydrolysate. We found that adding 0.025, 0.05 and 0.1% methoprene to the agar-sugar-protein hydrolysate diet increased production of pheromone in proportion to the amount added in 5- to 8-day-old flies (Teal et al. 2007). However, flight tunnel studies showed no significant difference in number of females attracted to pheromones released by males treated with 0.05% and 0.1% methoprene. Given this result and the doubled cost of adding 0.1% methoprene to the diet, we used 0.05% methoprene in other tests. Males fed the agar-sugar diet containing both protein hydrolysate and methoprene attracted significantly more females than did the diet containing no protein hydrolysate or methoprene (Teal et al. 2007), which supported our contention that both protein hydrolysate and methoprene are necessary to optimize sexual signalling by males.

Although incorporating both methoprene and protein hydrolysate into the agar diet clearly accelerated reproductive maturity of males and improved their ability to attract females, this method has two disadvantages for operational use at fly emergence and release facilities: adding protein hydrolysate to the wet agar-sugar diet makes the diet sticky for sterile flies (and it eventually becomes mouldy), causing significant mortality. Secondly, such a diet would waste a considerable amount of methoprene, because the amount of agar provided to flies in production facilities, used as a carrier for the water, far exceeds the amount consumed by the flies. To minimize waste of methoprene we explored the potential of treating large numbers of pupae topically with methoprene. We began by using the highly purified methoprene we used in earlier studies (Teal et al. 2000). This required us to dissolve the methoprene in acetone, because the compound is not appreciably soluble in water. Using irradiated pupae of *A. suspensa*, 3 days prior to adult emergence we first tested the effect of bathing batches of 100 pupae in acetone for different times to determine if acetone impacted survival. There were no differences in survival between groups soaked in acetone for as long as 10 min (93.30 $\pm$ 0.54%, SE, n = 3) compared with untreated flies (93.70 $\pm$ 0.27%, SE, n = 3). Then we tested the impact of soaking pupae for 5 min in acetone solutions containing 0.005%, 0.05%, or 0.5% methoprene. We conducted flight tunnel trapping studies and determined that males dipped in a 0.005% solution of methoprene attracted as many females as did males topically treated with 5 μg of methoprene and fed the optimal dry diet. Therefore, a 0.005% solution of methoprene was used in additional studies to determine the effects of bathing pupae of both *A. ludens* and *A. fraterculus* (Pereira et al. 2011; Segura et al. 2011). For both species, the acetone bath was effective in significantly accelerating reproductive development of males. However, acetone is not ideal for routine large scale exposure of irradiated pupae at fly emergence and release facilities, because it volatilizes and has been associated with health hazards.

To overcome the problems associated with using acetone, we developed an alternative system in which pupae are bathed in a methoprene emulsion prepared using a commercially available emulsifiable concentrate containing 5% methoprene (Wellmark International, Dallas, TX, USA.). The concentrate was mixed with sufficient water to yield a 0.005% emulsion of methoprene in which *A. ludens* male pupae were bathed for 5 min immediately following irradiation. Results of studies conducted by Pereira et al. (2011) demonstrated that males treated in this way became sexually mature and mated at the same age as males treated topically with methoprene and fed the optimal dry diet. Overall, this approach to treating flies is superior to using acetone because any health risks associated with acetone are reduced. Additionally, because water is less volatile than acetone, larger numbers of pupae can be bathed before it becomes necessary to replenish the bath, resulting in less wastage of methoprene. This system is also superior to incorporating methoprene into agar blocks because the hormone analogue is not wasted when disposing of the spent agar. Nonetheless it is still necessary to provide adults with a protein hydrolysate enriched diet.

Combining Methoprene and Protein Hydrolysate is not Effective for all Tephritids

Despite the successes in accelerating reproductive development and improving mating potential for the species discussed above, there are species for which these treatments seem to have little or no effect. In earlier work we found that more sterile females of the Mediterranean fruit fly were attracted by males fed agar-sugar diet containing protein hydrolysate and methoprene than to males fed agar diet that did not (Teal et al. 2007). Based on these results we (Faria et al. 2008) and others (Shelly et al. 2009) conducted more detailed laboratory and field trials to determine if methoprene and protein hydrolysate would cause a significant improvement in mating of sterile males. Although the results of these studies indicated that both protein hydrolysate and methoprene had positive effects on males in the laboratory neither treatment improved male performance in field cage studies. We cannot explain the differences observed between laboratory and field trials for this species. However, differences in environmental conditions between the laboratory and field may affect the response of the males. Additionally, mass-reared strains of *C. capitata* that are inadvertently selected for rapid maturation (2–5 days after adult emergence).become sexually mature so quickly, that any effect of methoprene may not be manifest. Thus, adding methoprene to pre-release diets for use in SIT programmes for the Mediterranean fruit fly is not warranted. On the other hand, adding protein hydrolysate may be warranted for the Mediterranean fruit fly in view of the enhanced calling behaviour, sexual competitiveness and reproductive success (Faria et al. 2008). However, the positive effects of adding protein to adult diet for Mediterranean fruit flies are variable between strains (Yuval et al. 2002, 2007), and the added expense may not warrant incorporation of protein into diets.

Bactrocera dorsalis (Hendel) is another species which does not appear to respond to treatment with methoprene (Shelly et al. 2009). No differences in mating frequency between males treated with methoprene and control males treated with acetone were found although the effects of and interaction with protein were not studied (Shelly et al. 2009). A possible explanation for the absence of effect of methoprene on mating frequency for this species is that *B. dorsalis* is a methyl eugenol feeding fly and uses this compound as a precursor in the production of pheromone (Wee et al. 2002). Thus, in this species, as well as other *Bactrocera* which respond to methyl eugenol, the effects of methoprene may only be manifest after feeding on methyl eugenol because of the inability of flies to produce pheromone in the absence of the precursor. Alternatively, methyl eugenol, rather than JH, may be the key chemical required for optimizing reproductive success. Clearly, additional studies are required.

Conclusions

Results of the numerous studies conducted on the impact of providing protein hydrolysate and methoprene supplements to sterile tephritid males destined for use in SIT programmes have demonstrated that for some, but not all species, there is an improvement in male sexual performance. Indeed the facts that: (i) irradiated males of *A. suspensa* fed both methoprene and protein hydrolysate outperform wild males in attracting wild females in flight tunnel studies (Teal et al. 2007); and (ii) treated males of *A. fraterculus*, *A. ludens* and *A. suspensa* and *B. cucurbitae* effectively outcompete untreated males in mating, are capable of engaging in multiple fertile matings, and do not have increased mortality (Segura et al. 2009; Pereira et al. 2009, 2010, 2011, this issue; Haq et al. 2010) strongly support incorporating these treatments into pre-release holding protocols for these species.

Important factors associated with incorporation of protein hydrolysate and methoprene into pre-release holding systems are the form of administration, the interaction with access to protein, and the cost. Therefore, it is critical that, prior to employing the technology, the impact on acceleration of reproductive development and improvement of sexual performance be studied for each species. Additionally, the benefits must be weighed against cost for each species and simple cost-effective approaches to application must be developed. We have employed three methods to provide methoprene to sterile flies: (i) addition to sugar; (ii) incorporation into agar-sugar blocks; and (iii) dipping flies in an emulsifiable concentrate containing methoprene and providing protein hydrolysate as part of the adult diet. All three resulted in significant improvements in sexual performance. Nevertheless, providing males with a dry non-agar based diet containing a sugar and protein to which 0.05% methoprene is added seems the easiest and most cost efficient. An effective modification of this formulation has recently been developed for pre-release holding systems for mass-reared *A. ludens* used for SIT purposes in Mexico. This modification is based on mixing protein hydrolysate and methoprene with sugar and water. The resulting paste is then painted on 15×15 cm pieces of paper and allowed to dry. The diet is then placed inside paper bags used to hold pupae and maintain sterile adults before chilled aerial release. MoscaFrut (Mexico) is currently employing the technology for releases of sterile *A. ludens* in the area of Rio Verde in San Luis Potosi, Mexico. The data collected to date by the Mexican National Campaign against Fruit Flies indicates the population of feral flies has been reduced to below 0.01 flies/trap/day and it has been proposed that it be declared an area of low pest prevalence for the Mexican fruit fly (Gómez-Simuta and Teal 2010). Not only is this an example of effective extension of the technology reported here but also the cost associated with the method is reported to be only $13.00/million flies released, making it cost-effective.

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