

Juvenile hormone: action in regulation of sexual maturity in Caribbean fruit flies and potential use in improving efficacy of sterile insect control technique for tephritid fruit flies

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Abstract: Tephritid fruit flies, including the Caribbean fruit fly, pose a serious invasive threat to citrus production. These invasive species are quarantine pests and strict monitoring protocols are in place to detect introductions. The Sterile Insect Technique (SIT) is ideally suited to control tephritid fruit fly outbreaks and provides an environmentally safe and species specific method to eradicate tephritid fruit flies of agricultural importance world wide. Control is achieved in SIT by mass release of sterile males who mate with wild females. Females, mated with sterile males, do not produce offspring and rarely mate more than once. Optimization of SIT requires that sterile males compete with wild males for mates. We have discovered that loss of virginity enhances the sexual prowess of young males of the Caribbean fruit fly. After mating for the first time, males release twice as much sex pheromone, and they acquire another mate in less than half the time required by virgins. Additionally, we discovered that hemolymph of mated males contains significantly more juvenile hormone (JH) than that present in hemolymph of virgin males of the same age. Application of JH or the potent JH mimic, methoprene, to males on the day of adult eclosion induced precocious release of pheromone and mating by males. Thus, males, treated with JH, mated 4days earlier than control treated males. This discovery has the potential to improve efficacy of the SIT method because incorporating hormone supplement therapy into mass rearing of sterile males will allow for release of sterile males that are more competitive than those currently in use.

Key words: Caribbean fruit fly, Mediterranean fruit fly, juvenile hormone, sterile insect technique

Introduction

The Tephritid fruit flies, like the Caribbean fruit fly (*Anastrepha suspensa* (Loew)), have evolved complicated sexual communication systems. These systems rely on male produced auditory, visual and chemical signals for attraction of females and mating (see Sivinski and Burk, 1989). The signaling modalities are coordinated so that optimized signaling occurs during daily periods (Burk, 1983; Hendrichs, 1986; Hendrichs and Hendrichs, 1990; Landolt and Sivinski, 1992; Epsky and Heath,

1993). Pheromones appear to be responsible for long distance attraction (Perdomo *et al.*, 1975, 1976; Webb *et al.*, 1983; Heath *et al.*, 1993; Landolt *et al.*, 1992; Sivinski *et al.*, 1994) and, when coupled with auditory signals, maximize the probability of females finding and landing in the vicinity of male leks. The complexity of the signaling system is even more evident when considering the individual signaling modalities. For example, initial studies on pheromone communication indicated that, although only males produced and released pheromones (Feron, 1959; 1962; Nation, 1972), both sexes responded to male pheromone in the field (Perdomo *et al.*, 1976; Ohinata *et al.*, 1977). This suggests that the pheromone serves as both an intra- and intersexual function, particularly given the lek forming behavior of males (Dodson, 1982; Burk, 1983; Sivinski, 1984; McDonald, 1987; Kaspi and Yuval, 1999). To date nine chemicals have been identified from volatiles released by Caribbean fruit fly males. These include: (Z)-3-nonen-1-ol and (Z,Z)-3,6-nonadien-1-ol (Nation 1983), anastrephin and epianastrephin (Battiste *et al.*, 1983), suspensolide (Chuman *et al.*, 1988), b-bisabolene (Tumlinson, 1988; Nation, 1991; Rocca *et al.*, 1992), E,E-a-farnesene and a-trans-bergamotene (Rocca *et al.*, 1992), and (Z)-b-ocimene (Tumlinson, 1988; Nation, 1991; Rocca *et al.*, 1992).

Age is also a major factor that regulates the sexual signaling system. Studies on mating of wild flies showed that insects do not engage in mating until they are 9-10 days old while laboratory reared insects begin mating several days earlier (Mazomenos *et al.*, 1977; Wong and Nakahara, 1978; Dodson, 1982). Additionally, female reproductive behavior, including response to pheromone, is directly correlated with ovarian maturity (Nation, 1972). Similarly, males undergo a period of maturation before they engage in sexual signaling (Nation, 1972; Landolt and Davis-Hernandez, 1993). Evidence from feeding studies has indicated that both sexes require protein sources for reproductive maturity (Galun *et al.*, 1985; Landolt and Davis-Hernandez, 1993) because correlations have been found among protein consumption, ovarian development and male calling behavior. However, although flies consume more protein during the maturation period, the amount consumed declines when they are sexually mature (Landolt and Davis-Hernandez, 1993). The fact that dietary protein is not required for sexual signaling by mature flies (Landolt and Sivinski, 1992; Epsky and Heath, 1993) suggests that the flies undergo a period of hormonally regulated adolescence during which time gametes mature and secondary sexual characters, including the ability to produce pheromone, develop. This apparent requirement for a period of hormonally regulated sexual development led us to explore the factor(s) responsible for coordination of reproductive maturity with sexual signaling in the Caribbean fruit fly.

Methods

Behavioral and mating observations. Pupae, obtained at least seven days prior to adult eclosion from laboratory cultures maintained by the Florida Division of Plant

Industry, Gainesville Fl, were housed in a greenhouse. On the day of eclosion, adults were segregated by sex, transferred to separate 30x30x30cm cages and provided with water and a 3:1 mixture of sugar and hydrolyzed brewers yeast. Experiments were conducted during the reproductive period that extended from between 12:00-18:00h (Heath *et al.*, 1993). We observed groups of five males for calling (exposure of the lateral abdominal, anal glands and other behavioral criteria associated male sexual signaling behavior) throughout the reproductive period on each day until flies were 10-days old. Five virgin males and virgin females were caged together each day after adult eclosion and observed for mating throughout the reproductive period. Mating pairs were removed from cages. New males and females were used each day. Thus, all flies had not been exposed to the opposite sex prior to pairing. Additionally, groups of five males, mated on the 5th day after emergence were caged with five sexually mature females on the next day and the time each took to mate was recorded. These times were compared to times it took for virgin males 6-days-old to mate. In other experiments groups of five males were combined with females on the 5th day. We removed mating pairs and held them separately. All males mated by the end of the 6th reproductive period. We then caged the males who mated on either the 5th or 6th day with virgin females for a second time on the 7th day. We compared the time it took for these mated males to acquire mates with the time it took for virgin males of the same age to mate. The experiment was repeated with groups of 8-day-old virgins or males mated on either the 6th or 7th day caged with females for a second time on the 8th day.

Collection and analysis of pheromone. We collected pheromone from groups of five males, placed in volatile collection chambers prior to the daily commencement of sexual signaling (Teal *et al.*, 1999, 2000a). The system was purged with air for 1h prior to collection of pheromone during the first 4h of the reproductive period. Pheromone was collected from virgin males on each day after emergence. Pheromone was collected on the day after mating from groups of five males mated on days 5,6 and 8. At the same time we collected pheromone from groups of virgin males who were 6,7 and 9-days-old. The amounts of pheromone released by males were determined using capillary gas-liquid chromatography (Teal *et al.*, 1999).

Hormone supplement therapy and identification of JH. We used synthetic JH III and the JH agonist, methoprene, for hormone supplement therapy and applied a dose of 5µg of one or the other to the thorax of virgin 5-day-old males in a 1µl drop of acetone (Teal *et al.*, 2000a). This dose was selected because it has been used effectively by others (Yin *et al.*, 1995). Control males were treated with only acetone. Pheromone was collected from treated and control males on day 6. In other tests we applied either hormone or just acetone to males on the day of adult eclosion and collected pheromone and observed mating on each day.

We synthesized mixed diastereomers of JH IIIB as described by Richard *et al.* (1989) and purchased JH III from Sigma Chemical Company for use as analytical standards in mass spectral analyses. To identify both JH III and JH III bisepoxide we

collected hemolymph separately from 12-day-old males and mated and virgin 7-day-old males and extracted with hexane containing farnesyl acetate as a quantitative internal standard (Teal *et al.*, 2000b). The hexane extract was subjected to GC-chemical ionization (isobutane) mass spectral analysis using a Finnigan-Matt ITS 407 ion trap MS interfaced to a Varian Star 34007 GC. The GC was equipped with a cool-on-column injector. The 30m x 0.25 mm (id) analytical column used in the GC, a DB5-MS7 (J&W), was interfaced to a 10 m x 0.25 mm (id) uncoated, deactivated fused silica retention gap. Conditions of chromatography were initial injector temperature= 40° for 30 sec; injector temperature increased at 170°/ min to 270°; initial column temperature= 40° for 5 min; column temperature increased at 5°/ min to 210°; He carrier gas linear flow velocity= 24 cm/sec; GC-MS transfer line temperature= 230°. Diagnostic ions used for identification and quantification of JH III included m/e = 267 (M+1), 235 (M+1-CH₃OH), 217 (M+1-CH₃OH-HOH), 189 (M+1-CH₃OH-HOH-CO), 147 (M+1-C₂H₄O₂-C₃H₈O) (see Teal *et al.*, 2000b). Diagnostic ions used for identification and quantification of JH IIIB included m/e = 283 (M+1), 265 (M+1-HOH), 251 (M+1-CH₃OH), 233 (M+1-CH₃OH-HOH), 205 (M+1-CH₃OH-HOH-CO), 187 (M+1-CH₃OH-CO-2(HOH)).

Results and Discussion

Male Caribbean fruit flies form mating leks (Nation, 1972; 1989; Sivinski and Burk, 1989; Webb *et al.*, 1983). Females, visiting leks rely on male size and elaborate interactions between auditory, visual and chemical signals emitted by males to select mates ((Nation, 1972; 1989; Sivinski and Burk, 1989; Webb *et al.*, 1983). The expression of male and female sexual behavior is closely coordinated with physiological development of reproductive maturity (Nation, 1972; 1974). In our laboratory strain of Caribbean fruit flies we found that all males engaged in calling behavior by the eighth day after emergence and that by day 9 all males released the maximum amount of pheromone and mated (Fig. 1). Interestingly, we also found that if we introduced virgin males to females on the fifth day and left them together, all males mated by the end of the sixth day (Fig. 2). However, only 37% of the six-day old virgin males mated on that day (Fig. 2). Similarly, males caged with females continuously during days six and seven all mated where as only ca 51% of the seven-day old virgin males paired with females, for the first time on day seven, mated (Fig.2). These results indicated that the experience of being in proximity to virgin females over a 24h period induced males to mate. Another feature of our study was the discovery that, once mated, males engage in mating much more readily than do their virgin counterparts of the same age. When we allowed males to mate, for the first time, on day 5 and 6 and then paired them with virgin females for a second time on day seven we found that all males mated with females for a second time within 2h after being combined with females (Fig. 3). However, only 30% of the naïve virgin

seven-day old males mated on that day (Fig. 3). Similarly, when we caged males together over the sixth and seventh days and allowed all to mate and then re-caged the mated males for a second time on day eight all of them mated within 90 min. However, only 60% of the virgin 8-day-old males mated with females on day eight. The remaining 8-day old virgin males required a second day to complete the initial mating. Results of these studies indicated that prior mating experience, and not size, was the key factor that influenced the male’s ability to successfully attract, court and mate with females because males were selected at random, without regard for size, in all of these experiments.

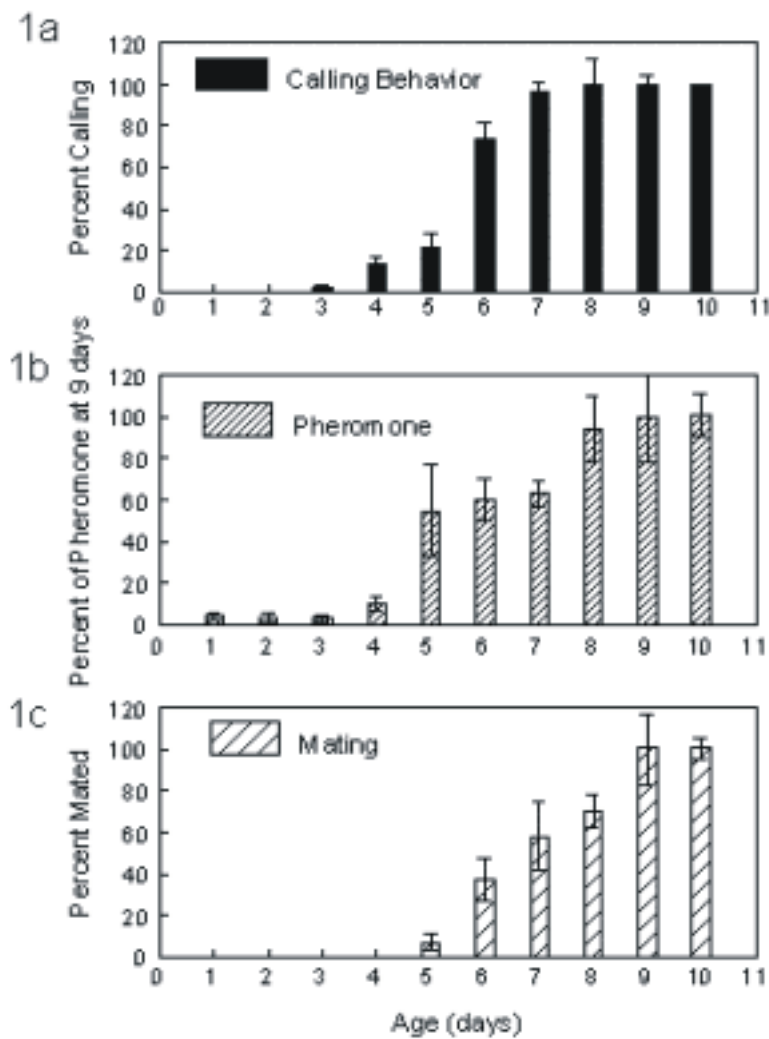


Figure 1. Effect of age on calling behavior, amount of pheromone released and mating by virgin males of the Caribbean Fruit fly. Data for calling behavior (12 groups of five males) and mating (17 replications of five males and females) represent the cumulative percentages of the total number of animals observed over 10 days. Data on pheromone release (10 groups of five males) represents the percentage of the average amount of pheromone released by males who were 10-days old.

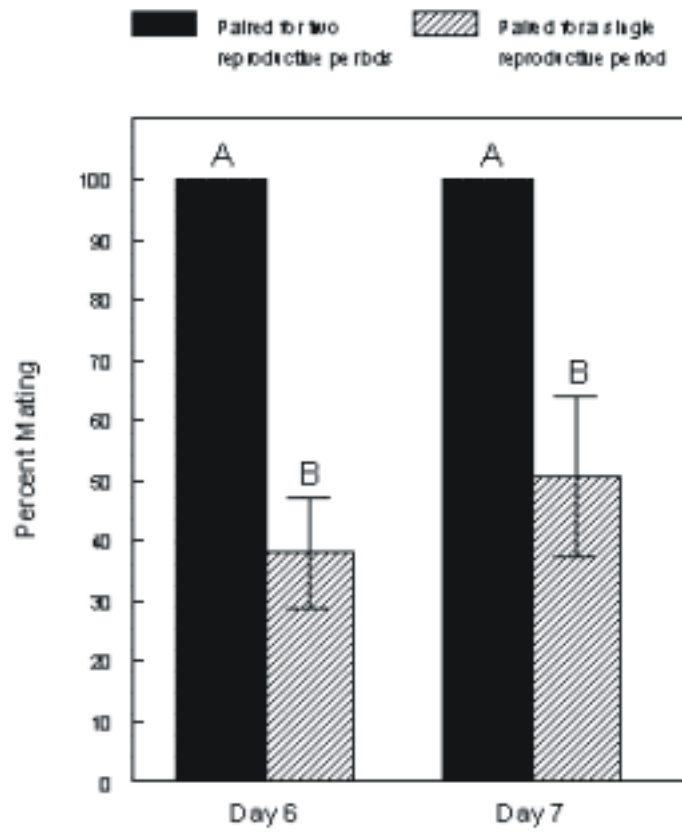


Figure 2. Comparison of percent of virgin males mating when paired continuously sexually mature virgin females for two reproductive periods, day five and day 6 or day six and day seven, with percent of virgin males mating when paired with sexually mature females for a single reproductive period on either day six or day seven. When mating occurred the mating pair was removed from the cage. All males paired with females for 2 successive days mated by the end of the second reproductive period whereas significantly fewer males mated when paired with females for a single day (t-test, $p=0.05$, 6 replicates).

Taken collectively, the results of our mating experience studies suggest that males have evolved an adaptive mating strategy that allows them to take advantage of mating opportunities even if they have not reached the peak of sexual maturity. Such a strategy could have evolved because the probability that tropical species of Tephritid fruit flies, like the Caribbean fruit fly, will find mates when oviposition sites are available is limited because host plants are widely distributed both in time and space. If few females were available early in a male's life it would be more efficient to wait until greater numbers of females arrive before engaging in sexual behavior. This would allow for accumulation of sufficient energy reserves to maintain sexual signaling and territory defense. Alternatively, if large numbers of receptive females are

available, then younger males have mating opportunities. Indeed, females avoid recently mated males and select naïve, less competitive, virgins for mating (Sivinski, 1984). Thus, if a male has an opportunity to mate early in life he may need to take it in order to achieve reproductive success and use the experience as an indication that other females are available. Thus, he may contribute proportionally more to the gene pool.

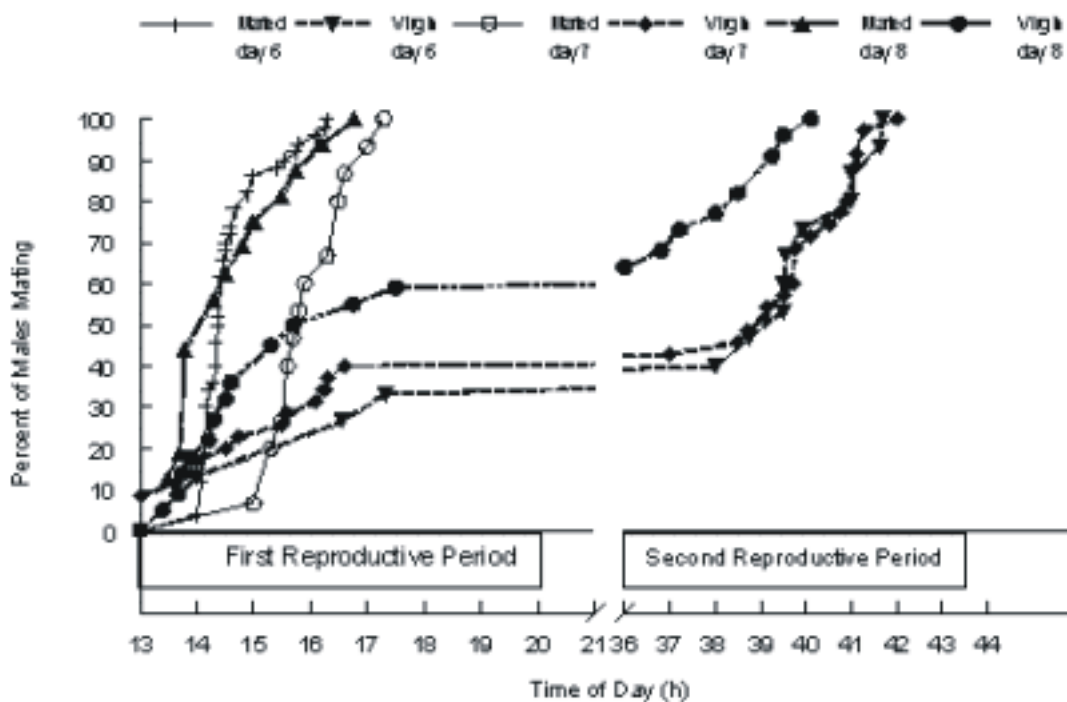


Figure 3. Comparison of the times required for mating by mated or virgin individuals after initiation of the 6th, 7th or 8th reproductive periods. Each data point represents the cumulative percent of the total population of a given treatment mated at that time. N=50 mated day six, 15 virgin day six, 15 mated day seven, 20 virgin day 7, 20 mated day eight, 20 virgin day 8.

Our research on mating by virgin and mated males suggested strongly that the act of mating induced physiological changes in males that caused them to engage in effective sexual signaling much more readily than their virgin counterparts. If this were so then we hypothesized that quantifiable differences in sexual behavior would be obvious between virgin and mated males of the same age. Male produced sex pheromones are key elements in the sexual communication system (Perdomo *et al.*, 1975; 1976; Nation, 1989; Sivinski and Burk, 1989; Heath *et al.*, 1993) and, in combination with visual and auditory cues, affect all aspects of sexual behavior. Therefore, we considered that release of sex pheromones would be a good diagnostic tool to monitor reproductive behavior and sexual competence. If this were so then we

hypothesized that mated males should release more pheromone than virgins of the same age. When we measured the amount of pheromone released by six- and seven-day old virgin and mated males we discovered that mated males released at least twice as much pheromone as did their virgin counterparts (Table 1). In fact, the amounts of pheromone released by these mated males were no different from that released by virgins on day nine, the age at which pheromone production peaks. Thus, the act of mating caused a physiological change in males enabling them to produce as much pheromone as males, either mated or virgin, who were at the peak of their sexual prowess. The benefit of this is clear if one considers that males must compete directly for the affections of females because if a mated 6-day-old male is to compete effectively in leks containing males who are nine or more days old, and at their sexual peak, then he must present the same qualities that render mature males attractive to females.

Table 1. Comparison of amount of pheromone released by mated and virgin 6-, 7- and 9-day old males. Five replications per treatment. Means are significantly different in a Fisher's least significant difference test ($p = 0.05$) if the letter is different in the significance column.

Male age and mating status	Mean amount pheromone (\pm SE)	Statistical significance
Day 6 Mated	412 (\pm 69.1)	A
Day 6 Virgin	180 (\pm 64)	B
Day 7 Mated	637 (\pm 63.2)	A
Day 7 Virgin	375 (\pm 66.5)	B
Day 9 Mated	643 (\pm 64.9)	A
Day 9 Virgin	683 (\pm 128)	A

Juvenile hormone (JH) coordinates development of sexual signaling with gamete maturity in many insects (Blomquist and Dillwith, 1983) and we had previously identified JH III from sexually mature (9-day old) males but not from immature 1-day old males (Teal *et al.*, 2000b). Analysis of extracts of hemolymph from 12-day old virgin and mated males resulted in identification of JH III and for the first time the bisepoxide homologue of JH III (JH IIIB). However, no differences in the total amount of JH in extracts from either mated or virgin males were found. When we analyzed extracts of the hemolymph from 7-day old mated males we also identified JH III and JH IIIB (Fig.4) in a ratio of 1:3 (Fig. 5). The identification of JH IIIB was important because this JH homologue had only been identified from extracts obtained from tissue culture media in which the corpora allata had been incubated (Richard *et al.*, 1989; Yin *et al.*, 1995; Yin and Stoffolano, 1997). Thus, although JH IIIB was synthesized by the corpora allata of Diptera, it had not been found in the circulatory system and

skepticism existed about the validity of the assumption that JH IIIB was, in fact, a functional hormone. We determined that each μl of hemolymph from a mated 7-day old male contained an average of 16pg (± 2.0) of JH. This was significantly more (4.5fold) JH than was present in hemolymph from virgins of the same age (Fig. 5).

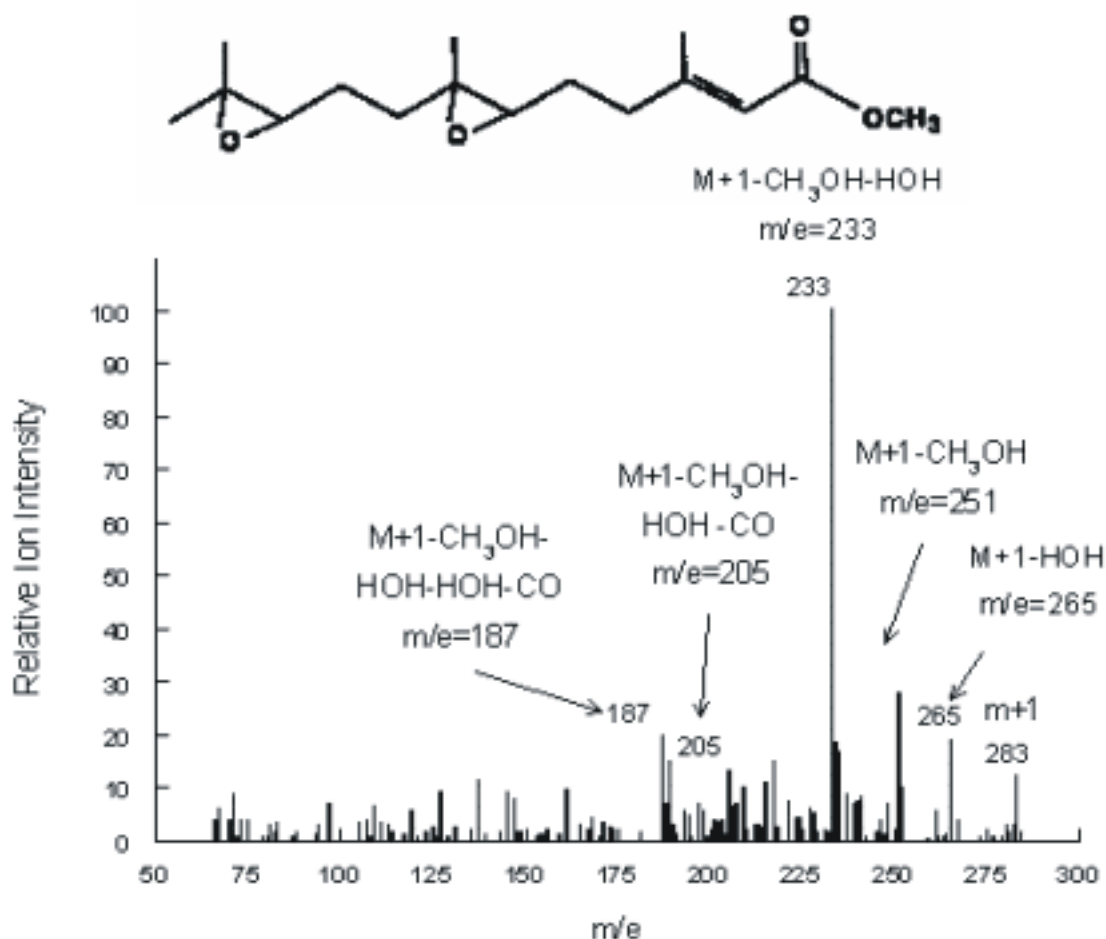


Figure 4. Chemical ionization (isobutane reagent gas) mass spectrum of naturally occurring JH IIIB isolated from 15:1 of hemolymph from 7-day old mated male Caribbean fruit flies. The structure of JH IIIB is shown above the spectrum and major diagnostic fragments are indicated along with the fragment losses. The structure was elucidated by comparison of retention time and fragmentation pattern of the natural compound with that of synthetic JH IIIB.

Knowing that circulating amounts of JH were higher in mated than in virgin males we hypothesized that JH was a pivotal hormone in regulating all aspects of sexual signaling and reproductive competence in these flies. If this were so then we believed that we could induce precocious expression of sexual behaviors and accelerate reproductive development times by application of JH to male flies. When we applied hormone to 5-day old virgin males we found that these males released three

times more pheromone than did the control group of virgins on day six. Indeed, application of hormone to males on the day of emergence caused precocious reproductive development and expression of sexual signaling when compared to control males (Fig. 6). In fact, all treated males mated on the fourth day after emergence. Results of these experiments showed that JH levels increased after mating and that this hormone alone can stimulate young virgin males to increase pheromone production and mate.

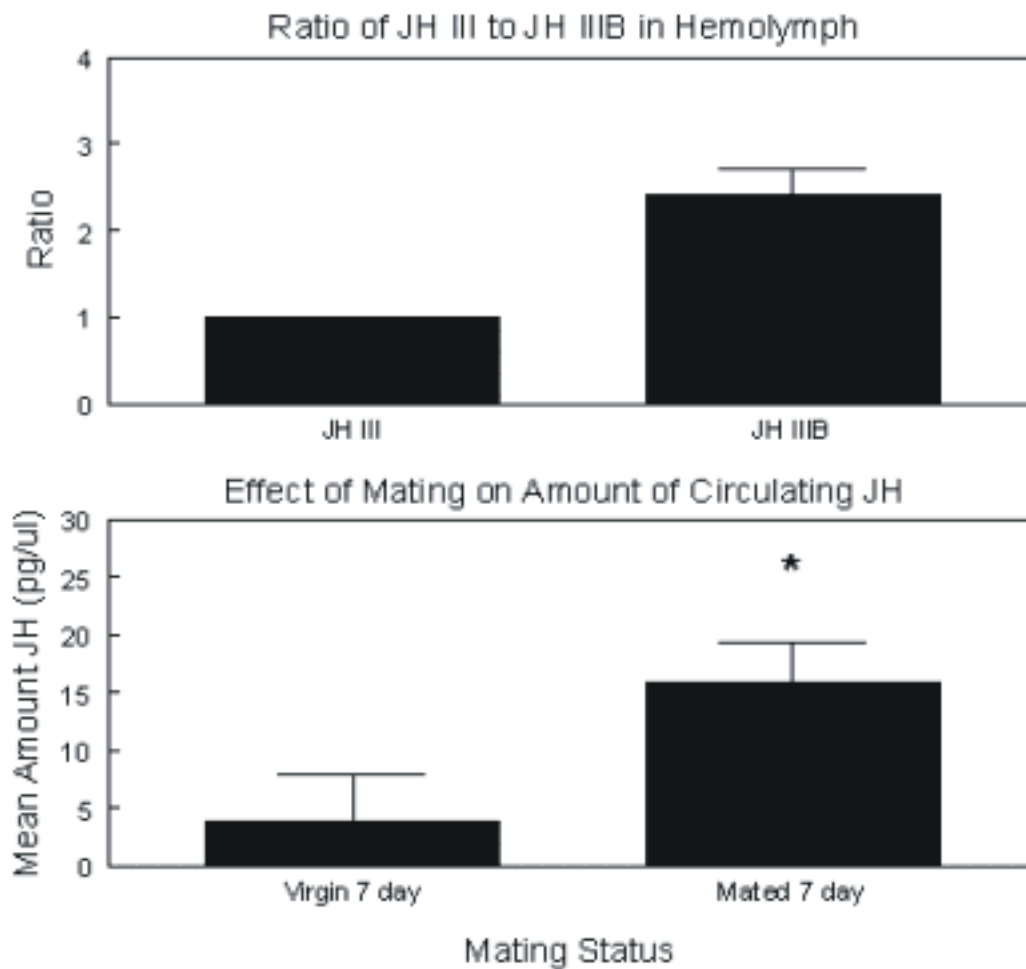


Figure 5. Ratio and amounts of JH III and JH IIIB found in extracts of hemolymph. Top: Ratio of JH III to JH IIIB obtained from samples from 12-day old virgins ($n=5$). The ratio was no different in samples obtained from 7-day old virgin or mated males. Bottom: Comparison of total amount of JH (JH III plus JH IIIB) in samples obtained from 7-day old virgin or mated males. The amount of JH in mated males was significantly greater in mated than in virgin 7-day old males (T test, $p=0.05$, 4 replicates).

Tephritid fruit flies including the Caribbean, Mediterranean and Mexican fruit flies pose the most serious invasive threat to citrus produced in the United States. Damage by these pests is not limited to citrus. 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 alone.

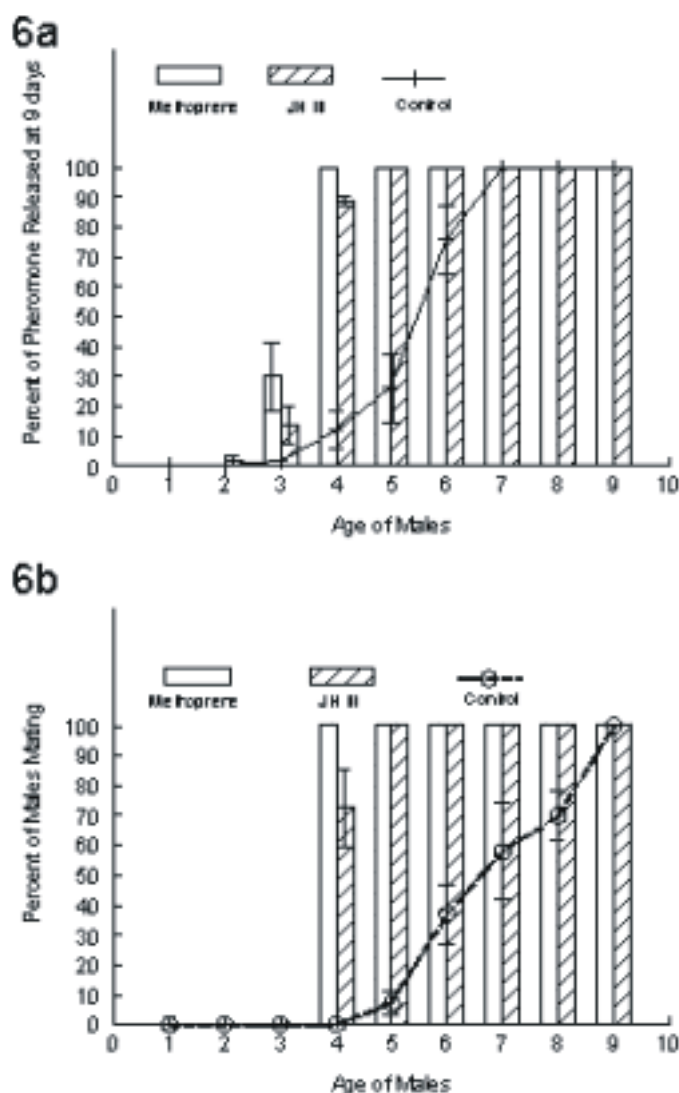


Figure 6. Effect of application of the JH agonist, methoprene, to newly eclosed males on amount of pheromone released and mating by males on each day after emergence compared with amount of pheromone released and mating by control males. 5a: Amount of pheromone released as a percentage of that released by virgin males at their sexual peak (9-days old) (n=6 replicates per treatment). 5b: Percent of males mating each day after emergence (n=4 replicates per treatment).

These invasive species are quarantine pests and strict monitoring protocols are in place to detect introductions. Once detected, the infested areas are subjected to immediate quarantine to eliminate movement of infested 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 (EDB) significantly limited invasions of these flies. However, registration for EDB use has been withdrawn and no substitute has been found. Thus, we are left with three practical alternatives for control: 1) stripping and destroying fruit from infested areas; 2) bait sprays using Malathion and more recently Spinosad; and 3) release of sterilized males in Sterile Insect Technique (SIT) protocols. The use of bait sprays has come under constant criticism due to perceived environmental and health related problems and several law suites have been filed to stop application. The use of SIT provides an environmentally safe and species specific method to eradicate Tephritid fruit flies of agricultural importance throughout the world. Control is achieved in SIT by mass release of sterile males who mate with wild females. Females, mated with sterile males, do not produce offspring and rarely mate more than once. Thus, optimization of SIT requires that sterile males compete with wild males for mates. We believe that incorporating hormone supplement therapy into the rearing protocols used to mass produce sterile males of Tephritid fruit flies for use in SIT will improve efficacy of the technique significantly. Currently, protocols for release of sterile males of both Caribbean and Mexican fruit flies indicate that males need to be held for several days prior to release in order for the males to become sexually mature. Accelerating reproductive maturity by inclusion of hormone supplement therapy into mass rearing protocols would allow for release of sexually mature insects several days earlier than prescribed by the current protocols. This would reduce significantly the cost of holding adult flies prior to release and reduce the negative effects of holding large numbers of males in small cages, which results in physical damage to the flies. Additionally, sterile flies have a much shorter life span than do non-irradiated flies. Thus, when flies are held, as adults, prior to release many die. The ability to release sexually mature flies at a much earlier age would minimize this negative effect of mortality on mating and more sterile flies would mate prior to death. Finally, sterile males are considered to be effective for only a single mating. Thus, each male released is considered to remove only a single wild female from the reproductive population. It is very possible that the reason that sterile males fail to function effectively in more than one mating is that they fail to replenish the secretions from the accessory glands in the reproductive system after mating. Compounds in the accessory glands of the Mediterranean fruit fly have been shown to inhibit remating and to induce females to engage in searching for fruit and oviposition (Miyatake *et al.*, 1999; Jang, 1995; Jang *et al.*, 1998). Wild males replenish the secretions within a few hours after mating. In other fruit flies, secretions from the accessory glands are responsible for inhibiting females from remating. JH has been implicated in inducing replenishment of accessory gland secretions. If this is so for Tephritid fruit flies then hormone supplement therapy could allow sterile males to 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 add even more to the cost effectiveness of the SIT technique.

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