ECOLOGY, BEHAVIOR AND BIONOMICS

Sexual Behavior of the Navel Orangeworm, *Amyelois transitella* (Walker) (Lepidoptera: Pyralidae)

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Comportamento Sexual de *Amyelois transitella* (Walker) (Lepidoptera: Pyralidae)

RESUMO - Com o objetivo de se estudar o comportamento sexual de *Amyelois transitella* (Walker), desenvolveu-se uma metodologia de criação da mariposa em condições de laboratório, utilizando-se como substrato alimentar pistaches secos e torrados. A criação foi desenvolvida a partir de lagartas coletadas em Bakersfield, Califórnia. A atividade de acasalamento foi observada principalmente durante a última hora da escotofase e os primeiros 30 min. da fotofase, em laboratório mantido a 25ºC. O comportamento de chamamento das fêmeas foi caracterizado pela projeção do abdome entre as asas, com os segmentos distais mantidos perpendicularmente ao corpo, pela exposição da glândula produtora de feromônio e pela contínua antenação. Os machos aproximaram-se das fêmeas "chamando" a uma curta distância, batendo fortemente as asas e movimentando as antenas. Após o macho tocar o abdome da fêmea com a antena, esta o aceita abaixando a ponta do abdome, ou não o aceita caminhando em direção oposta a ele. O macho que foi aceito pela fêmea aproxima-se, permanecendo paralelamente ao corpo dela e, nesta posição, introduz o edeago na porção final do abdome e rotaciona o corpo 180º, permanecendo em sentido linear e oposto à fêmea por mais de 3h em média. Os machos emergem antes das fêmeas, provavelmente como estratégia para aumentar as chances de acasalamento. Cerca de 80% dos acasalamentos ocorreram nos dois primeiros dias de vida. As fêmeas acasalaram uma única vez, entretanto, 55% dos machos acasalaram somente uma vez, 40% duas vezes e 5% três vezes.

PALAVRAS-CHAVE: Comportamento de cópula, comportamento de corte, protandria, técnica de criação

ABSTRACT - To get a better understanding of the mating behavior of the navel orangeworm, *Amyelois transitella* (Walker), we developed a robust laboratory colony derived from larvae collected in Bakersfield, California and fed on dried, roasted pistachio. In the lab at 25ºC, most of the mating activity was observed during the last hour of the scotophase and for the first 30 min of the photophase. Female calling was characterized by the abdomen being protruded between the wings with the distal segments perpendicular to the body and exposing a pheromone gland, as well as by continuous antennation. Males approached calling females from a short distance by displaying wing fanning and antennation. When a male antennated on a calling female's abdomen, she either accepted the male and lowered the abdomen, or walked away. The accepted male made a final approach parallel to the female's body, but after coupling he rotated 180º with male and female remaining in a linear, abdomen-to-abdomen position for over 3 h in average. In a possible strategy to maximize the chances of mating, the sex ratio was significantly skewed towards males in the first two days of emergence. Almost 80% of mating took place in the first two days after adult emergence, with females mating only once. About 55% of males mated only once and approximately 40% of the observed males mated twice and 5% tree times.

KEY WORDS: Mating behavior, courtship behavior, protandry, rearing protocol

The Navel OrangeWorm (NOW), *Amyelois transitella* (Walker), is a commercial pest of a number of crops (e.g.: walnut, *Juglans regia* L.; figs, *Ficus carica* L.) and the most serious insect pest of almond, *Prunus dulcis* (Miller), and pistachios, *Pistacia vera* L., in California. NOW is native of the southwestern United States and Mexico and was first described in Arizona in 1899. In 1921 it was found infesting damaged and rotting navel oranges in Arizona, and so its common name originated (Rice *et al.* 1996). In almond, NOW is controlled by thorough postharvest orchard sanitation (Zalom *et al.* 1984) along with applications of organophospate (OP) and pyrethroid insecticides. Given the regulations regarding applications of OPs and secondary pest problem caused by pyrethroids (Bentley *et al.* 1987),

alternative methods of control are highly desirable.

Pheromones offer an environmentally-friendly alternative to control insect populations. Indeed, a number of economically important lepidoteran insect pests have been successfully controlled by using pheromones for mating disruption. Pheromones may also be employed in IPM strategies to monitor populations and determine treatment timing. In general, female moths produce a mixture of pheromone constituents and the complete bouquet is important for attraction. Control by mating disruption may be achieved even with a single major constituent or a partial mixture. The potential of pheromones for controlling NOW populations has prompted a cadre of chemical ecologists over the years to investigate the pheromone system of this economically important agricultural pest. The major constituent of the sex pheromone, Z11Z13-16Ald, was identified earlier (Coffelt *et al.* 1979), but other essential constituents remained elusive for almost three decades.

Earlier attempts to control NOW populations with this major constituent alone have generated mixed results (Landolt *et al.* 1981, Curtis *et al.* 1985). Disruption of pheromone communication could be improved by dispensing puffs of pheromones from pressurized canisters (Shorey & Gerber 1996), probably because the pheromone (Z11Z13-16Ald) was protected from sunlight. However, use of a single component in mating disruption is less effective than a mixture. In addition, it was demonstrated with another lepidopteran species that continuous use of a single pheromone constituent in mating disruption may lead to "resistance", which can be avoided with a multi-component pheromone system (Mochizuki *et al.* 2002).

Employing a multi-disciplinary approach, including but not limited to a non-conventional molecular-based approach, sensory physiology, and state-of-the-art analytical techniques, we discovered eight additional constituents of the pheromone blend of the navel orangeworm (Leal *et al.* 2005), including two novel highly unsaturated hydrocarbons (pentaenes). The discovery of the complete pheromone system produced by the navel orangeworm opened new opportunities for effective monitoring and control of NOW populations. The present work was aimed at studying the sexual behavior of the navel orangeworm to get a better understanding of chemical communication in this species and consequently lay the foundation for improving mating disruption strategies.

Material and Methods

Insects. A lab colony was initiated from larvae collected in Bakersfield, CA. The larvae were kept on dried and roasted pistachio at 28 ± 2°C, 75 ± 10% relative humidity, and a 16:8h (light:dark) photoregime. To allow copulation, adults were transferred to aluminum cages (30 x 30 x 30 cm^3, BioQuip, Rancho Dominguez, CA; Fig. 1a) and kept at 25 ± 2°C, 70 ± 10% relative humidity, and a 16:8h (light:dark) photoregime. After 48h, 5-8 couples were transferred to oviposition boxes (13 x 13 cm^2; height, 4.5 cm) covered with paper towel (Thirsty Ultra Absorbent, 27.9 x 27.9 cm^2; Safeway) (Fig. 1b).

Orange-colored fertilized eggs laid in the ditches of the paper towel were washed with a 0.1% solution cupric sulfate and transferred to petri dishes (14 cm i.d x 1.5 cm) covered with a moistened filter paper (Millipore, FP1041500), sealed with Parafilm (America National Can) and kept at 28 ± 2°C until eclosion. Groups of 300 eclosed larvae were transferred with an artist brush (Round #2, A383/LJ800, Linzer) to the surface of dried pistachios placed in plastic developmental cages (30 x 19 cm^2; 20 cm height). To increase the substrate area for wandering larvae, a sheet of tissue paper was placed on the surface of pitscahios. Pupation took place inside pistachios, on the sides of cages, or under the cover of the cages.

After the first generation, 20% of the emerged adults were used to maintain the colony. The remainder of the pupae were kept individually in capped culture tubes (17 mm i.d x 10 cm long; Fisher Scientific) containing a piece of filter paper (1 cm x 1 cm) soaked with a 0.1% solution of cupric sulfate. These tubes (Fig. 1c) were kept in vertical position at 25±2°C, 65±10% relative humidity, and a 16:8 h (light:dark) photoregime, with the dark period starting at midnight (Pacific Standard Time, U.S.A.; hereafter referred to as PST). Emerged adults were kept in the individual tubes

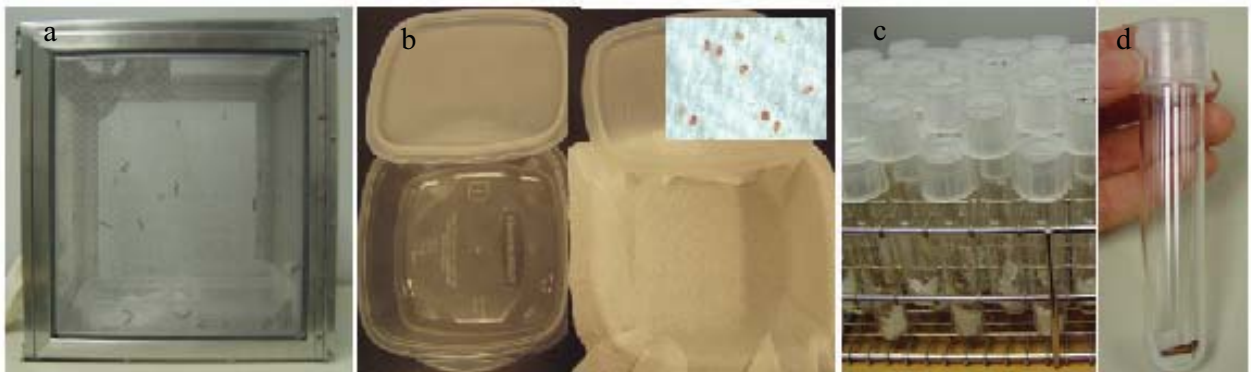

Fig. 1. Some of the materials used for rearing the navel orangeworm. (a) Mating cage; (b) Oviposition boxes with detail of the paper towel covering and eggs laid on ditches of the paper towel (inset); (c, d) Segregated pupae to produce unmated adults.

(Fig. 1d) until use. Adults were sexed according to Husseiny & Madsen (1964).

Time, age, duration and number of copulations. To determine a broad peak of mating activity, we observed for 24h a group (16 couples) of 24- and 48-h-old adults kept at 25 ± 2ºC at 20 min interval. A flash light with a red filter was employed for observations during the scotophase. Adult moths were placed inside observation chambers (aluminum boxes; 30 x 30 x 30 cm) and fed on 10% honey and water. When the broad peak of activity was determined, more refined observations were recorded for a group of 10 couples every day at the time of activity for eight consecutive days. Data were recorded for each couple, including age, time and duration of the first mating, and number of copulations per individual.

After the first mating, couples were removed from the original observation chamber and placed in separated male and female chambers. For each mated female transferred, one unmated male was added to the same observation chamber, whereas a virgin female was added to the chamber where mated males were placed. These experiments were repeated eight times, with age and time of the first mating being analyzed by Tukey's test at $P < 0.05$.

Calling behavior. Forty 24- or 48-h-old unmated couples were observed inside observation chambers. Direct observations of female calling, courtship events and time of mating were complemented with analysis of video obtained with a Sony digital handycam (DCR-PC101-NSTC) with Super Night Shot. The events were also recorded for individual couples, which were removed from the observation chamber after mating.

Daily rhythm of adult emergence. To determine whether the emergence of males and females was synchronized, we placed 100 couples in an aluminum chamber (60 x 60 x 60 cm) for 24h to allow mating and then transferred females to oviposition boxes where they remained for 24h. Eggs of the same age were processed as above for development and pupation. Emerging adults were recorded and sexed daily, with the results being analyzed by Chi-square.

Results and Discussion

Our rearing protocol, with over 85% viability, led to the development of robust insects which were essential for pheromone identification (Leal *et al.* 2005). Unlike our laboratory-raised males, field collected males did not give strong and consistent responses by gas chromatography with electroantennographic detection (GC-EAD) and were useless for behavioral studies. In addition, we were able to generate virgin females for detailed behavioral studies.

Time, age, and duration of first copulation and number of copulations. Preliminary observations indicated that copulation took place for 1h 30 min, starting in the last hour of the scotophase and ending at the first 30 min during the photophase, i.e., between 7 AM and 8:30 AM PST. Most of the copulation (56%) occurred between 7:31 AM and 8 AM, with 22% of them taking place before and 22% after that time period. More detailed observations confirmed that most of the mating indeed took place during the last hour of the scotophase, particularly in the last 30 min of the scotophase (57.4%), with limited (11.6%) mating occurring after lights went on (Table 1).

Table 1. Percent of mating in *A. transitella* kept at 25 ± 1°C, 65,0 ± 10.0% relative humidity and 16L:8D photoregime, with photophase starting at 8 AM US Pacific Time. Data were analyzed by Tukey's test at $P < 0.05$. Statistically significant values are indicated by different letters.

Time (h)	% mating
7:00-7:30	31.0 ± 8.17 c
7:31-8:00	57.4 ± 8.82 a
8:01-8:30	11.6 ± 4.13 b

The majority of mating took place in the first two days after adult emergence and decreased thereafter (Table 2). Among 80 couples (10 males and 10 females with 8 replicates) observed, 66.3% mated until day 7, with females mating only once, whereas 54.9% of males mated only once, 40.2% mated twice and 4.9% mated three times. Landolt & Curtis (1991) reported multiple mating of the navel orangeworm in the field as they found multiple spermatophores in 24% of female moths collected by light traps in almond orchards. In our experimental design, in which a mated female had a chance to encounter other unmated males (but not the same male) in subsequent nights, we did not find multiple mating.

Periodicity of pheromone release/calling behavior in the navel orangeworm has been previously investigated by Proshold (1967), Srinivasan (1970) and Coffelt *et al.* (1979), but the literature was inconsistent. Srinivasan (1970) reported that pheromone release, male response to female pheromone and mating follow a circadian rhythm and that there was

Table 2. Percentage of the age of adults *A. transitella* at the time of first mating. Insects were kept at 25 ± 1°C, 65,0 ± 10.0% relative humidity and 16L:8D photoregime, with photophase starting at 8 AM US Pacific Time. Data followed by the same letter are not significantly different by the Tukey's test at $P < 0.05$.

Age (d)	% mating
1	42.7 ± 3.86 a
2	36.9 ± 3.67 a
3	10.6 ± 4.61 b
4	1.6 ± 1.56 b
5	3.3 ± 2.20 b
6	3.3 ± 2.20 b
7	1.6 ± 1.56 b

a peak of mating activity 8-12h after the scotophase on a 12L:12D photoregine and at 26ºC. Coffelt *et al*. (1979) reported that female calling and pheromone release at 25ºC and 14L:10D occurred in the last 30 min of the scotophase thus coinciding with the last part of the major mating activity observed by us. On the other hand, Srinivasan (1970) found no precise diurnal pattern of pheromone production and male response in field observations, but noted a peak of mating activity from 1 AM to 3 AM when temperature was 9ºC to 12ºC. In addition, Proshold (1967) reported that copulation, corresponding with male trapping, occurred from 11 PM to 6 AM in the field. Some of these discrepancies might be explained by Landolt & Curtis (1982) who observed in the field an apparent linear relationship between temperature and the time most females began calling and males began to be captured. They noticed that on cooler nights mating activity started progressively earlier. However, female calling and male capture never started more than 9h before sunrise. In an environmental chamber at 13ºC, females began calling at 5.5h and 5h before the beginning of the photophase, whereas at 18ºC and 27ºC female calling started 1.5h and 1h before the photophase, respectively (Landolt & Curtis 1982).

Description of calling, courtship, and copulation behavior. When inactive, both males and females kept their antennae in a resting position beneath the wings, but antennal movements were very characteristic of male and female sexual activity. During calling, females separated and lowered the wings, protruded the abdomen between the wings placing the distal segments perpendicular to the body, extruded an abdominal (pheromone) gland, and kept the antennae extended and in continuous up-down movements (antennation) (Figs. 2a, 3a). This is similar to the calling behavior observed by Landolt & Curtis (1982) for the navel orangeworm and by Mozuraitis *et al.* (2002) for *Phyllonorycter emberizaepenella* (Bouche) (Lepidoptera: Gracillariidae).

When exposed to calling females from a short distance, males started walking toward females while displaying wing fanning and anntenation (Fig. 3b). When a male reached a calling female and touched her abdomen with the antennae, the female lowered the abdomen (Fig. 3c, d). Calling females did not lower the abdomen when touched with an artificial substrate (e.g.: tip of a SPME syringe) thus suggesting that male-to-female signaling is involved in the acceptance process. Also, females that did not accept a male walked rapidly away from the approaching male. When a female lowered the abdomen, a male approached her laterally while performing strong wing funning and started antennating on her body from the distal part of the wing to the head (Fig. 3e). The male curved his abdomen towards the female (Fig. 3e,f) and attempted coupling the genital apparatus until the female accepted him and allowed copulation (Fig. 3g). Soon after copulation began, the male stopped wing fanning (Fig. 3g) and rotated his body 180º to rest in a linear, abdomen-to-abdomen position with the female (Figs. 3h and 2b). During copulation, both male and female kept their antennae beneath the wings with the male's wings resting on the female's wings. Copulation lasted on average of 3h30min ± 15min and after that male and female separated from each other and remained side by side in a resting position. To the best of our knowledge, this is the first complete description of the courtship behavior of the navel orangeworm.

Protandry in the navel orangeworm. Five hundred thirty-five larvae hatched from the eggs deposited by a total 100 females that were kept in a cage with 100 males. A majority (334 individuals) made it to the adult stage, with a an overall female ratio, F/(M+F), of ≈0.54 (179 females and 155 males), but predominantly more males in the first three days of emergence. The male ratio was clearly higher in the first days of the emergence period (Fig. 4). Chi-square tests indicated a significantly ($P < 0.05$) greater proportion of males in the first two days of emergence, thus supporting protandry in the navel orangeworm. This early emergence of males and the early and single mating in females suggest that protandry in the navel orangeworm is a strategy to maximize the chance of mating. Similar findings were reported by Pivnick & McNeil (1985) with field populations of the European skipper, *Thymelicus lineola* (Ochsenheimer) (Lepidoptera: Hesperiidae). On the other hand Baughman (1991) suggested that protandry alone

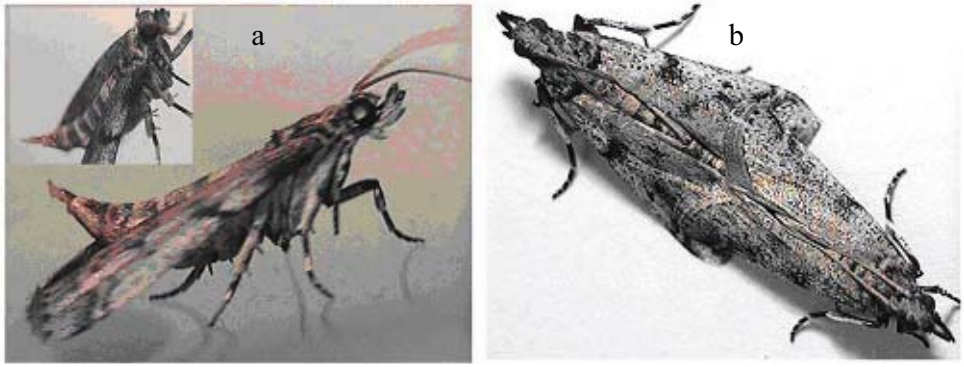

Fig. 2. (a) Typical position of navel orangeworm calling females. Note the extended wings, the exposed pheromone gland at the tip of the curved abdomen, and the blurring antennae (due to extensive antennation). (b) During mating, the male's wings rest on the female's wings.

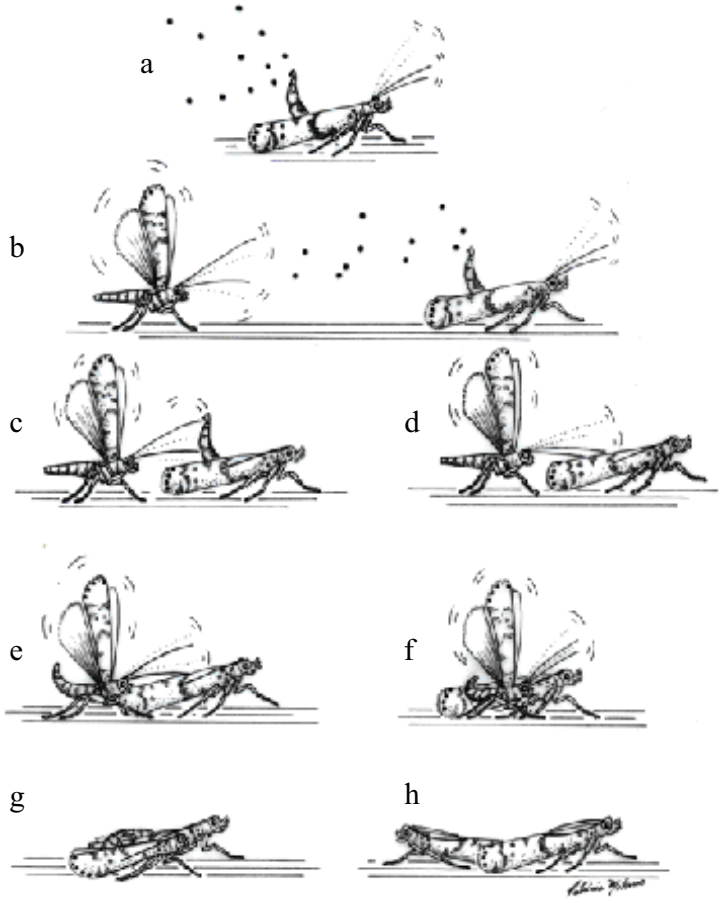

Fig. 3. Short-range mating behavior in the navel orangeworm. (a) Calling female; (b) Approaching male displaying wing fanning and antennation; (c) Approaching male touches female abdominal tip; (d) Female lowering the abdomen (stops calling); (e) Male keep antennating, wing fanning and bending of the abdomen when approaching the female laterally; (f) Male attempting to couple; (g) Wing fanning stops soon after succeeding; and (h) Male rotating to remain in a position opposite to the female.

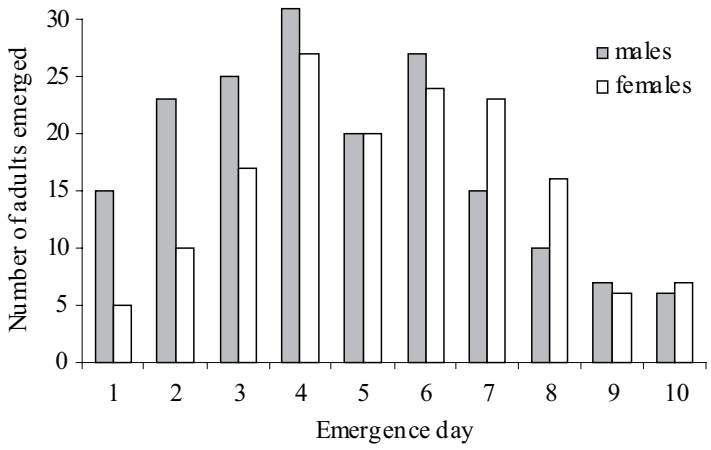

Fig. 4. Male-to-female ratio of emerged adults in a single generation in the lab at 28 ± 2°C indicating a clear higher ratio of emerging males, particularly in the first two days of emergence. Ratios in the other days (3-10) after emergence were not significantly different. Emergence of males was significantly different (x^2, $P < 0.05$) from female emergence in the first two days.

does not confer a fitness advantage relative to early-emerging males and that time of emergence does not seem to influence mating success in *Euphydryas editha bayensis* Sternitzky (Lepidoptera: Nymphalidae) populations.

In conclusion, we have developed an effective protocol for rearing navel orangeworm in the lab and gained a better understanding of the insect's courtship behavior. After a stereotypical calling behavior, mating occurred with a peak of activity prior to the scotophase and mainly in the first two days after adult emergence, which coincides with the period in which the sex ratio of emerged adults was skewed toward males. To succeed in controlling moth populations with pheromones, it is essential to understand what we want to disrupt. Here, we provided a hitherto unknown series of events in the short-range mating behavior for the navel orangeworm that may help improve mating disruption with the newly identified pheromone system (Leal *et al.* 2005).

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