Effects of male age and mating status on response to the female sex pheromone of Copitarsia decolora (Lepidoptera: Noctuidae)

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Effects of male age and mating status on response to the female sex pheromone of *Copitarsia decolora* (Lepidoptera: Noctuidae)

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Abstract

The effects of male age and mating status on the behavioral response of *Copitarsia decolora* (Guenée) males to the glandular extract of the female sex pheromone were studied by conducting wind tunnel bioassays and electroantennography (EAG). To study the effect of age, 2-3, 4-5, 6-7, 8-9, 10-11 day old males were used to measure attraction in the wind tunnel. To study the effect of age on virgin male antenna depolarization, individual males 3-, 6- and 9-days old were used. The effect of male mating status was studied with 4-6 day old males and subsequent pheromone response was measured after a period of 24 and 48 h. Both male age and mating status significantly affected male response to the female sex pheromone. In the wind tunnel, male attraction to the extract reached its maximum in males 4-7 days old and then decreased as moth age increased. The glandular extract provoked a significantly greater depolarization of the antennae of 6 day old males than in 3 and 9 day old males. In the wind tunnel, virgin males were more attracted to female extracts than mated males. Significantly greater depolarization was observed in the antennae of virgin than mated males.

Key Words: *Copitarsia decolora*; sex pheromone; age; mating, wind tunnel; electroantennography

Resumen

Se estudió el efecto de la edad del macho y el estado de apareamiento sobre la respuesta de comportamiento de machos de *Copitarsia decolora* (Guenée) al extracto glandular de la feromona sexual de la hembra, mediante bioensayos en túnel de viento y electroantennografía (EAG). Para medir la atracción en túnel de viento se utilizaron machos de 2-3, 4-5, 6-7, 8-9, 10-11 días de edad y para estudiar el efecto de la edad sobre la despolarización de sus antenas, se utilizaron individuos de 3, 6 y 9 días de edad. Para estudiar el efecto del apareamiento se utilizaron machos de 4-6 días de edad y su respuesta a la feromona se midió 24 y 48 horas después. Ambos, la edad del macho y el estado de apareamiento, afectan de manera significativa la respuesta de los machos a la feromona sexual de las hembras. En el túnel de viento, se observó que la atracción de los machos al extracto fue mayor a los 4-7 días y después disminuyó conforme incrementó la edad. El extracto glandular provocó una despolarización significativamente mayor en las antenas de los machos de 6 días de edad que en los machos de 3 y 9 días de edad. En túnel de viento los machos no apareados fueron más atraidos al extracto glandular de las hembras que los machos apareados. Se observó una despolarización significativamente mayor en las antenas de los machos no apareados que en los machos apareados.

Palabras Clave: *Copitarsia decolora*; feromona sexual; edad, apareamiento; túnel de viento; electroantennografía

Males moths use sex pheromones emitted by females to locate a mate. However, male response can be affected by numerous factors, including the physiological state of the insect (Anton et al. 2007; Evenden & Gries 2008) and experience (Anton et al. 2007). Pheromones are detected by olfactory receptor neurons (ORN) housed within cuticular sensilla mainly on the antennae (Keil 1999). The axons of ORNs project via the antennal nerve into the antennal lobe (AL). There they make synaptic contact with intrinsic AL neurons, the local interneurons, and with AL projection neurons, which transfer information to higher brain centers (de Belle & Kanzaki 1999). The AL consists of a species-specific number of glomeruli (Hansson & Anton 2000). Male moths have large numbers of olfactory receptor neurons tuned to single compounds of the sex pheromone produced by females, and large glomeruli, called the macroglomerular complex, are entirely dedicated to the processing signals pertaining to these sex pheromones (Hansson & Anton 2000).

For some moth species such as *Plutella xylostella* (L.) (Zhang et al. 2009), *Agrotis ipsilon* (Hufnagel) (Anton & Gadenne 1999; Gadenne & Anton 2000; Gadenne et al. 2001; Barrozo et al. 2010a, 2010b, 2011), *Heliothis virescens* (F.) and *Heliothis subflexa* (Guenée) (Soques et al. 2010) it has been demonstrated that age and mating status can modify olfactory reception and sexual pheromone processing in males. In these species, the male response to the female sex pheromone is regulated by hemolymph titers of juvenile hormone (JH) (Gadenne et al. 1993; Duportets et al. 1998; Anton & Gadenne 1999; Gadenne & Anton 2000), and by neuromodulators that act on ORNs and AL neurons (Anton et al. 2007).
Materials and Methods

INSECTS

Insects used in the bioassays were obtained from a C. decolora colony at the Centro de Desarrollo de Productos Bioticos, National Polytechnic Institute (IPN) in Yautepec, Morelos, Mexico. The insects were maintained at 25 ± 3 °C, 60 ± 5% RH and 12:12 light:dark cycle. The prepupae and scotophase reversed with respect to the natural light cycle in order to allow pheromone extraction and bioassays during the day. Larvae were fed with a special artificial diet for lepidopterans (Cibrián-Tovar & Sugimoto 1992). Adults were fed with a 50% sucrose solution placed on cotton wool, which was replaced every 3rd day.

PREPARATION OF SEX PHEROMONE EXTRACT

The gland that produces the sex pheromone, situated between the seventh and ninth abdominal segment (Rojas et al. 1995), was dissected from virgin 3-5 day old females that presented sexual calling behavior during the final third of the scotophase (Rojas et al. 2006). Sixty glands were placed in 2 mL glass vials containing 1 mL of dichloromethane as a solvent for 10 min. The supernatant was concentrated to 100 µL under a nitrogen flow. Each extract had a concentration of 3 female equivalents (FE) for each 5 µL. The gland extracts were stored at -4 °C until chemical analysis or use in the bioassays.

ANALYSIS AND IDENTIFICATION OF VOLATILE COMPOUNDS

Two µL of extract were injected into a gas chromatograph (GC) (HP 6890) coupled with a mass spectrometer (MS) (HP 5972) (Agilent, USA). The samples were analysed using a non-polar HP 5MS column (30 m long, 250 µm internal diameter and 0.25 µm film thickness; Agilent Technologies, Santa Clara, California). The initial oven temperature was 60 °C, increasing 15 °C/min until it reached 280 °C. The carrier gas was helium at a constant flow of 1 mL/min. The injector temperature was 225 °C and the auxiliary was 280 °C; the injector functioned in splitless mode for 0.30 min. The MS functioned by electron ionization (70 EV), in SCAN mode and at a mass interval of 35 to 550 AMU. Compound identification was carried out by considering retention time (RT) and by comparing mass spectra to spectral libraries (NIST/HPA/NIM 2002) and synthetic standards. The standards (Z)-9-tetradecenylacetate (Z9-14:Ac) and (Z)-9-tetradecenol (Z9-14:OH), with 98% purity were obtained from Sigma Aldrich (Toluca, Mexico). In all of the extracts used in the bioassays, the presence of Z9-14:Ac and Z9-14:OH in similar proportions to those reported by Rojas et al. (2006) were verified.

WIND TUNNEL BIOASSAYS

In the wind tunnel bioassays, volatile chemicals from the glandular extract were carried to the insects by a current of air (0.4 m/s) produced by a fan (Siemens 1RA3055-4YK31, México). The experiments were conducted at 25 ± 3 °C, 60 ± 5% RH. In preliminary studies the following concentrations were tested: 3, 1.2, 0.12, 0.012 and 0.0012 FE. The 3FE concentration caused the greatest attraction response and landing, so this was used in both experiments (wind tunnel and EAG). 5 µL (3 FE) of glandular extract was individually placed on filter paper (2.0 × 2.0 cm, Whatman no. 1), then left for 20 s at the same height as the air flow entrance to allow the solvent to evaporate. A single male was released at this height but at the opposite end of the tunnel. Each moth was tested for response within 300 s and with one test per moth.

The following types of behavior were measured in the wind tunnel: no response, short flight (flight less than 100 cm), long flight (flight more than 100 cm but less than 150 cm), and landing on the source. The number of males that displayed their claspers was also recorded. Furthermore, latency to activation (antenna and wing movement and displaying claspers) and latency to landing on the scent source were recorded.

ELECTROANTENNOGRAPHY

The measurement and analysis of the EAG responses were conducted using Syntech EAG equipment (Kirchzarteng, Germany). Once the antenna of the insect was dissected, it was mounted between 2 silver (Ag) electrodes and conductor gel was added. The signal generated by the antenna was transmitted to an IDAC-2 amplifier and was observed on a monitor with software for EAG recording and analysis. Antenna stimulation continued for 1 s with an air flow of 0.5 L/min transported to the antenna by a pump (stimulus controller SC-55) with a constant flow of humidified purified air (0.7 L/min). For stimulus application, 5 µL (3 FE) of glandular extract of the sex pheromone were placed on filter paper (1.0 × 0.5 cm, Whatman No. 1). In each experiment the solvent was allowed to evaporate for 20 s and the interval between each stimulus was 120 s. In these bioassays, the antenna polarization response to solvent was subtracted from response to glandular extract before analysis. Each antenna was used only once.

TREATMENTS

To study the effect of age, the pupae were separated by sex. After emergence, adults were placed in plastic containers (20 x 20 x 20 cm) to obtain single sex groups of known age. In the wind tunnel bioassays, virgin males that were 2-3, 4-5, 6-7, 8-9 and 10-11 days old were used, with 10 moths tested per group. For EAG response, virgin males that were 3, 6 and 9 days old were tested, with 6 moths tested for each age. Selection of the age groups for EAG tests was based on results of wind tunnel tests.

For tests of the effect of mating, virgin males and females, 4-5 days after emergence, were individually paired and held in plastic containers (200 mL). The pairs were observed for mating and time when mating ended was recorded. Male responses in wind tunnel bioassays and EAG to the extracts were measured 24 and 48 h after mating. All of the evaluated mated males in both wind tunnel (n = 10) and EAG (n = 6) were 5-7 days old. The depolarization values obtained from the groups of mated males were compared against a group of virgin 4-6 day old males. The females from each mated pair were dissected and
the presence of the spermatophore used to confirm that matings were successful.

**STATISTICAL ANALYSIS**

The behaviors of the males in the wind tunnel were analysed by the Chi-square test with Yates correction. Regarding the latency of the responses observed in the wind tunnel, the median and interquartile range (Q1 to Q3) were determined for each group. The interquartile range is little affected by extreme scores, so it is a good measure of spread for skewed distributions. The latency data were analysed by a Kruskal-Wallis test and Tukey’s test ($P = 0.05$) was used for comparison of means.

The data obtained from the depolarization in the male antennae were analysed by ANOVA. Comparisons of means were carried out using a Tukey’s test. Sigma Plot 11 (Systat Software Inc., Chicago, Illinois) was used for all statistical analyses.

**Results**

**EFFECT OF AGE**

Male attraction to the extract was greatest for 4-5 and 6-7 day old males and then decreased with age in wind tunnel tests (Table 1). These groups were the most responsive, although there was no difference between these 2 groups. There were also significant differences between 4-5, 6-7 and 2-3 (Chi square = 52.769; df = 1; $P < 0.001$), 8-9 (Chi square = 52.769; df = 1; $P < 0.001$) and 10-11 day old males (Chi square = 72.521; df = 1; $P < 0.001$) regarding flight behavior patterns when landing on the scent source. In addition, there were significant differences between 4-7 and 2-3 day old (Chi square = 20.056; df = 1; $P < 0.001$), 8-9 and 10-11 day old males (Chi square = 8.526; df = 1; $P = 0.004$) for displaying claspers (Table 1).

The 4-5 and 6-7 day old males presented activation and landing latencies significantly less than those displayed by 2-3, 8-9 and 10-11 day old males ($H = 28.557$, df = 4, $P < 0.001$ and $H = 24.167$, df = 4, $P < 0.001$, respectively) (Fig. 1).

The EAG responses by age group are shown in Fig. 2. The extract elicited a significantly greater depolarization in antennae of 6 day old males than in 3 and 9 day old males ($F = 9.965$; df = 2, 17; $P = 0.002$).

**EFFECT OF MATING**

In the wind tunnel bioassays, the virgin males were more attracted (both long flight and landing) than males evaluated 24 and 48 h after mating (AM). Differences were found in source landing behavior for 24 AM males (Chi square = 18.484; df = 1; $P < 0.001$) and 48 AM males (Chi square = 48.505; df = 1; $P < 0.001$); as well as in long flight behavior for 24 AM males (Chi square = 11.281; df = 1; $P < 0.001$) and 48 AM males (Chi square = 36.214; df = 1; $P < 0.001$) (Table 2).

Mating had an effect on both activation and landing latencies (activation latency $H = 17.867$, df = 2, $P < 0.001$; landing latency $H = 14.885$, df = 2, $P < 0.001$). The males from the 24 AM and 48 AM groups spent more time in activation and landing on the scent source than the virgin males group (Fig. 3). Similarly, a significantly greater depolarization was observed EAG responses of virgin males than mated males (24 AM and 48 AM groups) ($F = 9.814$; df = 2, 17; $P = 0.002$) (Fig. 4).

**Discussion**

In this study, we found that antennal sensitivity to the sex pheromone by male *C. decolora* moths varies in relation to age, reaching a maximum at 6 days. Similar changes in antennal sensitivity due to age have been reported in other species of moths (Baker et al., 1992; Koskimies et al., 1994).

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**Table 1.** Response (percentage) by age group of virgin *Copitarsia decolora* males to female sex pheromone extract (3fe) in wind tunnel bioassays.

| Behavior         | 2-3  | 4-5  | 6-7  | 8-9  | 10-11 |
|------------------|------|------|------|------|-------|
| No response      | 0 b  | 0 a  | 0 a  | 10 b | 50 b  |
| Short flight     | 30 b | 0 a  | 10 a | 30 b | 10 a  |
| Long flight      | 10 a | 10 a | 10 a | 20 a | 10 a  |
| Landed           | 40 a | 90 b | 80 b | 40 a | 30 a  |
| Claspers displayed | 80 a | 100 a| 100 a| 90 a | 90 a  |

Percentages followed by the same letter within a behavioral category are not significantly different (Chi-square, $n = 10$, $P < 0.05$).

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**Fig. 1.** Activation latencies (black bars) and landing latencies (gray bars) (Q1 < Median < Q3) by age group of virgin males in response to a glandular extract of female sex pheromone (3FE) in wind tunnel bioassays. Bars within a behavior headed by the same letter are not significantly different (Tukey’s mean separation test, $n = 10$, $P < 0.05$).

**Fig. 2.** Depolarization (mean ± SEM) of virgin male antennae in response to a glandular extract of female sex pheromone (3FE) by male age. Bars headed by the same letter are not significantly different (Tukey’s mean separation test, $n = 6$, $P < 0.05$).
observed in other species of moths such as *A. ipsilon* (Gadenne et al. 1993), *Spodoptera littoralis* (Boisdouval) (Martel et al. 2009) and *Mamestra brassica* (L.) (Pophof 2000). In these species, this has been attributed to the maturation of the ORNs (Martel et al. 2009), changes in hemolymph titers of the juvenile hormone (Gadenne et al. 1993; Gadenne & Anton 2000) and neuromodulation of biogenic amines that act on ORNs sensitivity (Pophof 2000; Grosmaître et al. 2001). These 3 mechanisms could explain the results observed in *C. decolora* as well; however studies are needed on these variables for this species.

In *C. decolora*, the observed changes in antennal sensitivity to the sex pheromone correspond to observed behavioral changes. During the initial days after emergence, male attraction response is low and it then begins to increase with age until reaching its maximum level at days 4-5 and 6-7, subsequently decreasing with age. In some species of moths, such as *A. ipsilon*, male response to the sex pheromone starts to increase in 3 day old individuals (Gadenne et al. 1993). In other moths such as *Spodoptera frugiperda* (Smith), the optimal age is 2 days (Rogers & Marti 1996), while for *Spodoptera exigua* (Hübner) it is reached at 1-2 days (Rogers & Marti 1996). It seems that young *C. decolora* males (2-3 days old) do not have sufficient sexual maturity to respond to the sex pheromone, and this also seems to be the case regarding recently emerged males (2-3 days old) do not have sufficient sexual maturity to respond to the sex pheromone. Mating triggers the release of neuromodulators that have the ability to modulate antennal lobe neurons in response to female sex pheromones and affect the development of the sex accessory glands, but antennal sensitivity is not affected (Duportets et al. 1998; Gadenne et al. 2001). In contrast, in *C. decolora* the antennal sensitivity is affected by mating. The decrease in pheromone response could be related to the fact that *C. decolora* males do not mate more than once during the night (Rojas & Cibrián-Tovar 1994) and can mate a total of 2 to 3 times during their lifetime (Castrejón-Gómez et al. 2000). This inhibition in the antennal and behavioral responses, combined with the effect of age, could represent a mechanism for temporally separated matings.

*Copitarsia decolora* males possibly need to recover from increased energy expenditure during mating and store sperm again (Gillott 2003). During this period of inhibition, the males can focus on searching for food to re-establish reserves in their accessory glands to allow sperm production and therefore mate again, as observed in *A. ipsilon* (Barroz et al. 2011).

The attraction response of male *C. decolora* to pheromone not only depends on the chemical composition (type of compounds and their relative proportions) (Rojas et al. 2006), but is also influenced by insect age and mating status, which affect the sensory input or processing that generates the necessary locomotion pattern to land on the stimulation source (Daly et al. 2007; Anton et al. 2007; Lemmen & Evenden 2009). In *C. decolora* males, neural plasticity induced by the physiological state allows males to modulate their response to the sex pheromone, which must be considered before that synthetic pheromone is used to manage this insect.

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