COMPARATIVE MATING STRATEGIES OF MALE AND FEMALE *ECTROPIS OBLIQUE* (LEPIDOPTERA: GEOMETRIDAE)

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ABSTRACT

The mating strategies of male and female *Ectropis oblique* Prout were investigated with the aid of male antennae as an electroantennogram (EAG) detector and capillary-GC analysis. Each male was capable of mating with several females, but females that had received a spermatophore mated only once. Antennae dissected from males 0, 1, 2, 3, and 4 d post-mating and antennae from virgin males of corresponding ages displayed similar EAG responses to sex pheromone extracts from sexually active females. Pheromone extracts of mated females elicited significantly weaker male EAG responses than the pheromone extracts of virgin females. EAG responses of males to sex pheromone extracts taken from mated females at 0, 1, 2, 3, and 4 d post-mating were consistently weak. Pheromone production in the pheromone glands of mated females was strongly suppressed and declined during each of 4 successive nights after they had mated.

Key Words: EAG, GC, mating, sex pheromone emission, spermatophore

RESUMEN

Las estrategias de apareamiento de machos y hembras de *Ectropis oblique* Prout fueron investigadas con el uso de la antena del macho como un detector electro-antenogramatico (EAG) y de CG capilar análisis. Cada macho fue capaz de aparearse con varias hembras, pero las hembras que han recibido un espermatóforo se aparearon solamente una vez. Las antenas diseccionadas de los machos 0, 1, 2, 3, y 4 dias después de aparearse y las antenas de machos virgenes de edades corespondientes mostraron respuestas de EAG similares a los extractos de feromonas sexuales de hembras sexualmente activas. Los extractos de feromonas de hembras apareadas provocaron una respuesta del EAG de los machos significativamente mas debíl que los extractos de feromonas de las hembras vírgenes. Las respuestas de EAG de machos hacia los extractos de feromonas sexuales tomados de hembras apareadas a los 0, 1, 2, 3 y 4 dias después de aparear fueron consistentemente mas debiles. La producción de feromonas en las glandulas de feromonas de hembras apareadas fue fuertemente suprimida y declinó durante cada una de las noches consecutivas después de que las hembras se aparearon.

Male insects typically mate many times during their lifetime, while females display diverse mating strategies (Arnqvist & Nilsson 2000). In some species, females need to mate once or only a few times to produce an optimal number of viable eggs; in many other species females mate frequently to maximize reproductive potential (Radwan & Rysińska 1999). No matter what strategies females use, they tend to discontinue sex pheromone production after mating, either temporarily or permanently; and this avoids problems associated with excessive male sexual harassment (Giebultowicz et al. 1991). This phenomenon was demonstrated by bioassay or chemical analysis in many studies (Webster & Cardé 1984; Coffelt & Vick 1987; Ahn et al. 2002). In contrast to the numerous studies on changes in female reproductive behavior after mating, few studies have focused on male response to diminished pheromone production of mated females. Recently, the electroantennogram (EAG) has been widely used in studies on semiochemical involvement in sex pheromones (Park et al. 2001; Gökçe et al. 2007). An EAG response profile is thought to represent the sensitivity and relative abundance of olfactory receptor neurons on the

antennae that are tuned to the compounds tested. The EAG response amplitudes are thought to represent the quantity of semiochemicals (Pouzat & Ibeas 1989). Thus, the EAG may be used as a tool to investigate the sensitivity of males to variable amounts of sex pheromone.

Ectropis oblique Prout (Lepidoptera: Geometridae) is an important tea bush pest in Southeast China. Population outbreaks can completely defoliate leaves on the bushes (Hu et al. 1994). The sex pheromone components of the female *E. oblique* were identified as (Z,Z,Z)-3,6,9-octadecatriene and 6,7-epoxy-(Z,Z)-3,9-octadecadiene (Yao et al. 1991). Most efforts to control *E. oblique* populations are focused on the development of methodologies to disrupt reproduction. Therefore, understanding the behavior of *E. oblique* is necessary. In this study, the mating frequency and longevity of *E. oblique* males and females was investigated. At the same time, the different mating strategies of the two genders were examined using male antennae as EAG detectors. To verify the results of the electrophysiological analysis, changes in sex pheromone titers produced by mated and virgin females were also investigated using capillary-GC analysis.

Materials and Methods

Insect Culture

The *E. oblique* insects were obtained from Qianshan County (31.5N°, 116.3E°), Anhui Province, China, and maintained for many generations in the laboratory. Larvae were reared on tea leaves. Adults and larvae were maintained in controlled conditions at 22 ± 3°C, 60-70% relative humidity, and a photoperiod of 14L:10D, with scotophase and photophase reversed from a natural light cycle to permit scotophase observations during normal working hours. Pupae were sexed based on the morphology of the 8th-10t^{h} abdominal segments, and maintained in moist sand for eclosion. Adults were kept individually in 240-mL plastic jars and fed a 10% honey solution soaked in cotton.

Effect of Mating Frequency on Longevity of Females and Males

In preliminary observations of behavior, both females and males copulated during the first scotophase after emergence, and were sexually active at the second scotophase. Therefore, a 1-d-old female (0 d after emergence) and a 2-d-old male were paired. Copulation occurred in the scotophase lasted approximately 6 h. After mating, the female insects did not resume calling during the same scotophase (Yang et al. 2008). Thus, each female in this experiment was replaced daily with another 1-d- old virgin female. When the female died, the number of times it had mated was ascertained by counting the spermatophores present in her bursa copulatrix. The experiment was repeated 40 times. The number of times a male *E. oblique* mated was determined by keeping a record of the number of females mated during successive scotophases. The experiment was also repeated 40 times. The longevity of each male and each female was recorded in order to determine whether mating affected longevity of either gender.

Extraction of Sex Pheromones

Active sex pheromones were extracted from the glands of the virgin females. The terminal section of the abdomen, which included the pheromone gland, was excised from the virgin female moth 6 h after the onset of the second scotophase, when the virgin female had been calling for 1 h. Experimental procedures were performed under a red light to facilitate observation without disturbing the insects. Each excised abdominal tip was immersed in 10 µL of redistilled hexane for 4-6 h at room temperature. Then, the tip was removed and the extract without any purification was submitted for EAG or GC analysis. The procedure for extracting sex pheromones from either mated or virgin females was the same.

Electroantennographic Analysis of the Effect of Mating on Pheromone Production and on Male Responsiveness at Various Days after Mating

Electroantennograms (EAG) were obtained with an EAG apparatus (Syntech Co., 79199 Kirchzarten, Germany). The antennae of either mated or virgin males were excised at the bases and a few distal segments were cut off to facilitate conductivity. The antennae were then attached to the electrodes of the EAG probe with Spectra 360 Electrode Gel (Parker Laboratories Inc., Orange, New Jersey). Antennal preparations were exposed to a stream of humidified and charcoal-filtered air emitted at 4 mL s^{-1}after having flowed through a 35-cm long glass tube (inner diameter, 8 mm; outer diameter, 10 mm). To facilitate insertion of the Pasteur pipette used to administer the pheromone test stimulus, a 3-mm hole was bored 5 cm from the outlet of the glass tube. Ten µL of the extract (test stimulus) was applied to a piece of filter paper (1 × 5 cm1 × 5 cm). The filter paper was placed in a Pasteur pipette after the solvent (hexane) had been allowed to evaporate for 5 min. Each test stimulus was delivered within a 0.5 s pulse of 4 mL s^{-1} of air with a stimulus controller (type CS-55) to transport the volatiles to the antenna. The EAG signal was amplified 10× through an intelligent data acquisition controller (type IDAC-2) and viewed on an oscilloscope. A period of at least 30 s was allowed between 2 successive stimuli for the recovery of antennal responsiveness. Redistilled hexane (10 µL) was used as a

control stimulus in every test. The absolute EAG amplitude (mV) minus the solvent response was used for data analysis.

First, the influence of the male's mating status on the male's EAG responses to active sex pheromones was studied. To obtain mated males and females, the insects were paired in the first scotophase and allowed to mate. Mating pairs were then removed. After mating, the mated females were used for the next experiment. With the same procedure as described above, the antennae of the mated male were dissected at 0, 24, 48, 72, and 96 h after mating for use as EAG test detectors. Sex pheromone extracts from sexually active females were used as stimuli. The EAG responses of antennae dissected from virgin males at each of these times post-mating were compared to the EAG responses of antennae obtained from mated males at corresponding times post-mating. Each treatment was repeated 6-8 times.

Secondly, the influence of the female's mating status on the EAG responses of a male was studied with sex pheromone extract of a mated female as the stimulus to elicit an EAG response from a 2-d-old virgin male. As described above, the sex pheromone of the mated females was extracted at 0, 24, 48, 72, and 96 h after mating. The EAG responses of antennae of 2-d-old virgin males exposed to pheromone extract obtained from virgin females at each of the above times post-mating were compared to the EAG responses to pheromone extract obtained from mated females at corresponding times post mating. Each treatment was also repeated 6-8 times.

Pheromone Titer Analysis

To assess the effect of mating on pheromone production, the sex pheromone titers of the mated and virgin females were analyzed by the procedure described above. Thus sex pheromone extract was analyzed in a gas chromatograph (Agilent 6890) equipped with a capillary column (DB-5, 60m × 0.5mm i.d × 0.25 μm film). The oven temperature was programmed at 50°C for 2 min, then 15°C min^{-1} to 250°C and held for 5 min. The temperatures of the injector and detector were 200°C and 250°C, respectively. Nitrogen with a flow velocity of 40 mL min^{-1} was used as the carrier gas. To quantify the pheromones in the female gland, only the amount of epo3,Z6,Z9-19:H, the major sex pheromone component of *E. oblique,* was determined. Each treatment was repeated 6-8 times.

Statistical Analysis

The data were analyzed by one-way ANOVA, followed by a LSD multiple comparison test at $P < 0.05$ (SPSS 11.0 for Windows, 2002; SPSS Inc., Chicago, IL).

Results

Mating Frequencies of Females and Males

The results of mating frequency are shown in Table 1. When 40 females were paired individually with 2-d-old virgin males on successive days until they died, only 1 spermatophore was detected in the abdomens of 31 females (77.5%), while no spermatophore was detected in the abdomens of the rest of the females (9, 22.5%). Thus any female that had received a spermatophore in 1 mating did not copulate again. When males were repeatedly offered 2-d-old virgin females, the numbers of males that mated various times during their lifespan and the corresponding percentages were as follows: 0 matings (10; 25.0%); 1 mating (9;22.5%); 2 matings (8; 20.0%), 3 matings (7; 17.5%), 4 matings (5; 12.5%); 5 matings (0; 0%) and 6 matings (1; 2.5%).

The Effect of Mating on the Longevity of Adults

Males lived significantly longer than females (Table 1), whether mated or unmated ($P < 0.05$).

Table 1. Mating frequency of *Ectropis oblique* and its effect on female and male longevity.

Mating time	No. of females observed	Female mating rates (%)	Female Longevity (days)	No. of males observed	Male mating rates (%)	Male longevity (days)
0	9	22.5	*11.36 ± 2.67 a	10	25.0	14.2 ± 0.85 b
1st	31	77.5	10.07 ± 2.87 a	9	22.5	19.6 ± 1.82 c
2nd	0	0	**	8	20.0	16.0 ± 1.47 b
3rd	0	0	—	7	17.5	15.5 ± 2.50 b
4th	0	0	—	5	12.5	15.5 ± 1.50 b
5th	0	0	—	0	0	—
6th	0	0	—	1	2.5	15

*Values are mean ± SE. Different letters indicate significant difference ($P < 0.05$) by LSD test.
** not tested.

In addition, the males that mated only once lived significantly longer than unmated males or males that had mated more than once ($P < 0.05$). However males that had mated 2 times did not live significantly longer than males that had mated either 3 or 4 times. The life spans of mated and unmated females did not differ significantly.

Electroantennographic Analysis of the Effect of Mating on Pheromone Production and on Male Responsiveness at Various Days after Mating

The effects of mating and lapsed time after mating of males on their EAG responses to sex pheromone extracts from sexually active females are shown in Fig. 1. No significant ($P > 0.05$) differences in EAG responses to sex pheromone extracts from sexually active females were observed between the mated and virgin males. The antennae of males that had been amputated 0, 1, 2, 3, and 4 d post-mating exhibited the same magnitude of the EAG responses to sex pheromone extracts from sexually active females as those of antennae of the corresponding virgin males.

The male EAG responses to female sex pheromone extracts from virgin females and mated females (Fig. 2) differed profoundly ($P < 0.05$) with the former evoking much stronger responses than the latter. Moreover the male EAG responses to sex pheromones extracted from the females at 0, 1, 2, 3, and 4 d post mating remained at very low levels (data not shown).

Pheromone Titer Analysis

The virgin females began to produce sex pheromones during the first scotophase after emergence (Fig. 3). Maximal pheromone titers were present in the glands during the second and third scotophase after emergence. Thereafter the pheromone titer decreased gradually. When the females mated on the first night after eclosion, the sex pheromone titers decreased strongly and significantly compared with the titers of virgin females. Pheromone production in mated females remained suppressed during each of 4 successive nights after they had mated. These results are consistent with the weak male antennographic responses coinciding with the male response to mated female sex pheromones.

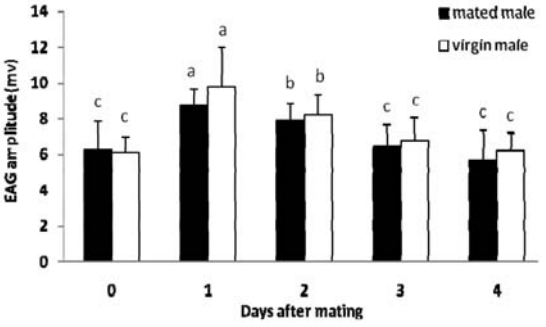

Fig. 1. Influence of the mating status of males on their EAG response to female sex pheromone extracts from sexually active females. Antennae of unmated males (solid bar) and virgin males (open bar) amputated 0, 1, 2, 3, and 4 d post-mating were used as EAG detectors. Data were presented as mean values ± SE (n = 6-8) and analyzed by one-way ANOVA, followed by an LSD multiple range test ($P < 0.05$). Significant differences among various dosages of the same stimulant are indicated with different letters.

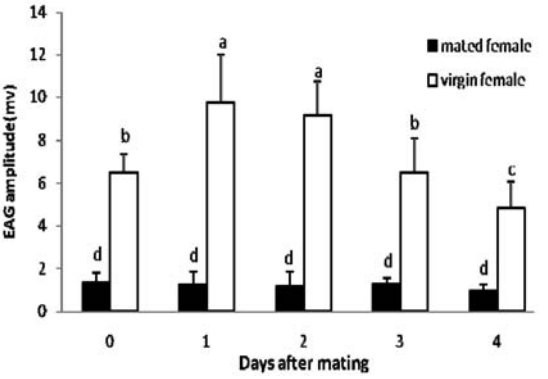

Fig. 2. Influence of differential mating status of females on male EAG responses to their pheromones. Sex pheromone extracts of mated females (solid bar) and virgin females (open bar) obtained 0, 1, 2, 3, and 4 d post-mating were used as stimuli. Data were presented as mean values ± SE (n = 6-8) and analyzed by one-way ANOVA, followed by an LSD multiple range test ($P < 0.05$). Significant differences among various dosages of the same stimulant are indicated with different letters.

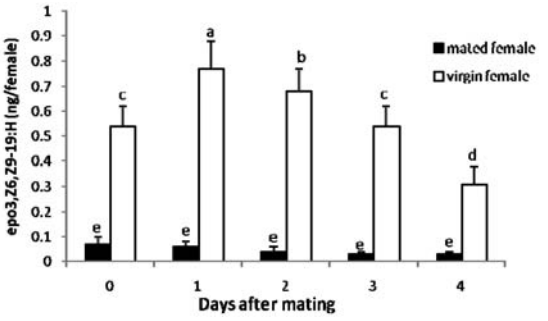

Fig. 3. Influence of mating on the production of 6,7-epoxy-(Z,Z)-3,9-octadecadiene, the major female sex pheromone. The female sex pheromone was extracted with hexane from pheromone glands of mated females (solid bar) and virgin females (open bar). Pheromone glands of mated females were extracted 0, 1, 2, 3, and 4 d post-mating.

Discussion

Our data suggest that the females in our laboratory colony of *E. oblique* are monandrous. Two different views exist concerning female mating strategies. Polyandry presents a variety of benefits to females, including full fertilization of their egg complement, increased genetic diversity of offspring, receipt of non-sperm nutrients, and reduced chances of fertilization by sperm that are genetically defective due to age. Conversely, polyandry may decrease female fitness due to the ecological cost of mating, including energy costs, and risks of physical injury and sexually transmitted pathogens and parasites (Arnqvist & Nilsson 2000). We observed that *E. oblique* females after having mated fended off males and did not accept a second mating partner. Mated females began laying eggs during the first scotophase and laid nearly all of their eggs before the fourth day (data not shown). The life spans of females ranged from about ½ to ⅔ of the lengths of the life spans of males. The lifespan of the female in the wild is likely to be even shorter than in the laboratory. Thus, a single mating is sufficient to fertilize nearly all eggs and minimize the above mentioned risks associated with multiple matings.

Males, on the other hand, are potentially polygynous. Male polygyny is to be expected because most evolutionary theories contend that the contributions and consequences of mating are much greater for females than for males (Thornhill & Alcock 1983).

This study revealed that multiple matings reduced the longevity of males but not of females. This result is in agreement with the results of studies on several other species (Proshold et al. 1982; Svensson et al. 1998). It is thought that allocation of nutritional reserves for egg development and maturation after mating may be responsible for causing the lifespan of mated females to be shorter than that of virgin females.

In most species, there is a causal relationship between male calling behavior and female pheromone emission. Only when the female pheromone gland becomes exposed to emit pheromone, may the male display calling behavior. Permanent or even temporary reductions in the emissions of sex pheromones caused loss of attraction and sexual receptivity in males (Kingan et al. 1995). The present study showed that *E. oblique* male EAG responses to mated female pheromone gland extracts were significantly diminished, which could be the result of reduced pheromone release. The results support the hypothesis that mating considerably suppressed pheromone production in females. Indeed according to our capillary-GC analysis, pheromone titers in pheromone gland extracts did not increase at all up to 4 d after mating.

Because the male EAG response to pheromone gland extract of already mated females did not show any increase up to 4 d after mating, it may be deduced that females may mate only once. This deduction is in accordance with the observation that each female actually mates only 1 time. Even though a few females were observed to copulate twice, but no more than 1 spermatophore was ever detected in a bursa copulatrix, possibly because the first copulation was an unsuccessful mating.

Mating-induced termination of sex pheromone production has been investigated in several moth species (Raina et al. 1994; Ando et al. 1996). The inactivation of pheromone production after copulation, which reduces the ability of females to elicit a sexual response in males, may be due to the secretion of pheromonostatic peptide (Kingan et al. 1995), or the presence of viable sperm in the spermatheca (Giebultowicz et al. 1991). The mechanisms involved in pheromone suppression after copulation in *E. oblique* are currently unknown. Thus, it is suggested that further studies must be conducted on this area.

The present results show that mating status did not appear to have a significant effect on the responses of male antennae to sex pheromone extracts of sexually active females. This suggests that a past mating does not cause the male to be unresponsive to the sex pheromone, and such a male can be expected to continue to seek females for additional matings. This deduction is also in accordance with direct observations of multiple matings by males. The same mating system described for *E. oblique* has been observed in some other species (Royer & McNeil 1993; Foster& Ayers 1996; Svensson et al. 1998).

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