

Age Influence on Sexual Behavior of the Lesser Cornstalk Borer, *Elasmopalpus lignosellus* (Zeller) (Lepidoptera: Pyralidae)

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Abstract

The objective of this work was to evaluate the reproductive behavior and response of *Elasmopalpus lignosellus* (Zeller) males to calling females. Frequency of mating was recorded in couples during the first 7 days of the adult stage. Calling behavior of females was observed during the first 4 days of the adult stage and responses of males, in the same age intervals, to calling females were recorded in wind tunnel bioassays. The maximum number of matings occurred when the couple was between 24 and 48 h old. The scotophase period significantly influenced mating behavior, which peaked between 6 and 8 h of darkness and the mean mating duration was 93.9 ± 4.2 min. Calling females, when evaluated in a wind tunnel, attracted significantly more males than in bioassays with clean air (control). The number of individuals in calling behavior was significantly lower for females that were between 0 to 24 h old compared to the other females evaluated, but this did not influence male response. A lower proportion of males between 48 to 72 h old responded to calling females and these responses were delayed in comparison with males of other ages (0 to 24, 24 to 48, and 72 to 96 h old). These results indicate that the age of *E. lignosellus* males influences the response to conspecific calling females.

Introduction

The lesser cornstalk borer (LCB), *Elasmopalpus lignosellus* (Zeller) (Lepidoptera: Pyralidae), is an important insect pest widely distributed in the tropical and temperate regions of the western hemisphere from the south of the USA to Argentina and Chile. More than 60 species of host crops have been described for the LCB (Stone 1968, Viana 2004). This insect is a serious pest in Brazil, especially in maize, soybean, cotton, rice, sorghum, wheat, peanut, and sugarcane (Viana 2004). The larvae of *E. lignosellus* feed on plants up to 30 cm in height (Pinto *et al* 2004), boring a tunnel into the plant stem that can kill the host when it reaches the apical meristem, often known as dead heart (Pinto *et al* 2004).

The sex pheromone of LCB from a North American population (USA) was identified as a mixture of (Z)-7-tetradecenyl acetate, (Z)-9-tetradecenyl acetate, (Z)-9-tetradecenol, and (Z)-11-hexadecenyl acetate (proportion 37:20:4:39), and activity of this mixture was confirmed in a field test (Lynch *et al* 1984). However, this mixture failed to attract males of LCB in Brazil (Pires *et al* 1992). The chemical analysis of the sex pheromone of Brazilian populations from Goiânia (Goias State), Sete Lagoas (Minas Gerais State) (Lynch *et al* 1984, Jham *et al* 2005, 2007), and Paraná State (de Viana 2015) identified a specific blend of *E. lignosellus* for each population, which was different from that of the North American population.

The effective population monitoring of *E. lignosellus* with the use of sex pheromone could help develop IPM strategies

for this pest in Brazil. To do this, it is necessary to identify the specific sex pheromone blend for the Brazilian populations of this pest. In moths, variability of the sex pheromone blend between populations, the circadian dynamics of female signaling and male response to pheromones are factors that should be considered to understand the sexual communication and selection process (Ma *et al* 2015, Groot 2014, Groot *et al* 2016).

Pires *et al* (1994) observed that LCB female calling behavior starts on the first night of becoming an adult, and 70% of females called at least once during the second night. These authors also found that scotophase influences calling behavior that is limited to the second half of the dark phase and is influenced by age; older females start their calling earlier. However, there is no information about the influence of male age on the behavioral response to the female sex pheromone, which is important because traps with the sex pheromone can be employed to monitor the male population. In this study, an evaluation was carried out on LCB reproductive behavior and the male response to calling females in a wind tunnel. The aim of this work is to answer two questions: (1) what are the periodicity, the duration and the age at which mating occurs? (2) Is the frequency of male responses to calling females related to their age?

Material and Methods

Insects

A laboratory colony of *E. lignosellus* started at Embrapa Genetic Resources and Biotechnology (Brasília, Brazil), from insects of a mass rearing at Embrapa Milho e Sorgo (Sete Lagoas, Minas Gerais State) that was originally collected in sugarcane fields in Viçosa (Minas Gerais State, Brazil, 20°45' S, 42°52' W). The larvae were reared on an artificial diet (Chalfant 1975), modified by Viana (1993), maintained at 28.0 ± 0.5°C, and 70 ± 10% r.h., under a reversed photoperiod of L12:D12 (scotophase starting at 6:00 am). All the experiments with *E. lignosellus* in this work were carried with individuals from ten laboratory generations.

Periodicity and length of mating

To assess the periodicity and length of mating, 60 couples of *E. lignosellus* were placed individually into plastic pots within 2 h after adult emergence, fed with an alcohol-free beer, and maintained in a separate room in the same environmental conditions as described above for larval rearing. The couples were monitored by direct observation during the scotophase of the reversed photoperiod previously described, during 7 days (0 to 168 h old) after adult emergence. Scotophase observations were conducted using fluorescent red light (40 W) in intervals of 5 min. The observations were grouped

in periods of 2 hours. Therefore, for a scotophase of 12 h, the observations were grouped into six periods. The start of mating and mating duration were registered.

Wind tunnel bioassays

Behavioral tests were carried out in a wind tunnel to evaluate orientation of virgin males towards virgin calling females. The wind tunnel was constructed with a glass cylinder with a flight section of 120 cm × 10 cm internal diameter. Airflow was produced by two computer fans (15 cm diameter), one at the up-wind extremity of the glass cylinder to create the airflow, and the other at the opposite side, working as an exhaust. Prior to the up-wind fan, the incoming air was driven by a cylinder (15 cm diameter × 5 cm long) with activated charcoal. To create a laminar flow, a cardboard "honey comb" circle (3 cm × 15 cm diameter) was adapted to the up-wind fan. The wind speed was calibrated at 0.5 m/s.

All bioassays were performed under the scotophase of the reversed photoperiod (12L:12D), between 4 to 12 h after the initiation of the scotophase, and in the same environmental conditions described above for larval rearing. The wind tunnel was illuminated from above with two 40 W fluorescent red lights. The males were grouped within age classes of 0 to 24, 24 to 48, 48 to 72, and 72 to 96 h old (hours after adult emergence). Their orientation to virgin calling females of the same age classes in all combinations of male and female ages was evaluated. Fifteen females of the same age class were placed inside a plastic cylinder cage (8 × 20 cm diameter to length) with the open ends closed with a voile cloth, which was placed inside the wind tunnel in front of the air entrance. The plastic cage was arranged with its open ends in the same direction of the airflow. At least 40 bioassays were performed for each combination of male and female age. Additionally, control bioassays were performed using the plastic cages without females ($N = 10$ for each male age). The males were released individually at the downwind extremity of the wind tunnel, 1 m away from caged females. The males were placed in a plastic cylinder (6 cm × 3 cm diameter) with its ends closed with cotton wads and maintained in the same environmental conditions for 10 min before its use in the bioassays. After this time, the cylinder was introduced into the wind tunnel and the cotton wads were removed gently.

The males inside the wind tunnel were observed for 10 min recording the vibration of wings or antennae and direct flight or walk to the odor source. Since this small moth naturally performs short and erratic flights (Xavier, LMS and Laumann, RA, field observations), a response in the wind tunnel was considered positive when the males left the liberation point and flew up-wind, reaching at least half distance to the caged females. For males that showed a positive response, the activation time (time since the males were introduced in the wind tunnel to when they left the

liberation area flying or walking) was also registered. In addition, during this set of bioassays, we recorded the number of females, in each group and age class, with their typical calling behavior (female at rest with open wings and abdomen pointing upwards).

Statistical analysis

Chi-square (χ^2) tests were performed to analyze the differences in the number of couples mating, considering different periods of the scotophase and age class. The response of males in the wind tunnel was analyzed using generalized linear models (GLM) and deviance analyses (ANODEV) with binominal distribution (positive responses or no response) of errors and log as link function. The model included two factors, male age class and female age class. For factors with significant effects, means were compared using contrast analyses. The activation times of males in each age class were compared using GLM and ANODEV with Gaussian errors and identity as link function followed by comparison of means using Tukey's test. To compare the number of females in calling behavior, GLM and ANODEV were used, with Poisson errors and log as link function followed by mean comparison using contrast analyses. All the analyses were carried out in software R (R statistical Development Core Team 2010) at 5% significance level.

Results

Forty-one couples of the 60 observed mated (68.3%). The mating distribution in relation to couple age was significantly different ($\chi^2_6 = 59.29$, $P < 0.05$). Higher frequency (31.9%) of mating was observed when the insects were 24–48 h old (Table 1). Differences in mating related to scotophase periods were significant ($\chi^2_5 = 69.07$, $P < 0.05$). The mating occurred preferentially from 6 to 8 h after the onset of scotophase; 68.3% of mating was observed in this period (Table 2). Courtship behavior in males is characterized by wing fanning when a female approaches. The mean mating duration was 93.9 ± 4.2 min.

The age of males influence their responses to calling females (ANODEV, $\chi^2_3 = 9.06$, $P = 0.03$, $df = 3$), while female age did not show a significant effect on male response (ANODEV, $\chi^2_3 = 1.92$, $P = 0.59$, $df = 3$). In addition, there were no significant interactions between male and female age (ANODEV, $\chi^2_9 = 14.46$, $P = 0.11$, $df = 9$). Considering this result, the responses of males from different age classes were analyzed independently of the female age class. Males within 48–72 h old show a lower response level (Fig 1). Only 7.5% of males (3 of 40), considering all age classes, introduced in the wind tunnel without females (control) left the plastic tube and moved up in the wind tunnel.

Table 1 Percentage of *Elasmopalpus lignosellus* couples that mated at different age intervals (0–168 h after emergence) during scotophase of a photoperiod of 12L:12D ($N = 60$).

Age of couples in hours	Couples mating (%)
0–24	7.3
24–48	31.9
48–72	17.0
72–96	7.7
96–120	6.5
120–144	4.2
144–168	0.0
Total mating	68.3

The mating distribution in relation to couple age was significantly different ($\chi^2_6 = 59.29$, $P < 0.05$).

Similarly, the mean for activation time showed significant variation only according to the age of males ($F_{3,177} = 7.78$, $P < 0.05$). Males that were 48–72 h old showed a higher activation time than the other ages evaluated (Fig 2).

The mean number of calling females in wind tunnel tests, for the four ages analyzed, differed significantly (ANODEV, $\chi^2_3 = 124.25$, $P < 0.01$, $df = 3$). The females between 0 to 24 h old showed significantly lower frequency in calling behavior compared to the other females evaluated (Fig 3). On the other hand, females that were 72–96 h old had higher number of females in calling behavior (Fig 3).

Of the 110 males observed, 100% of them vibrated their antennae after being introduced in the wind tunnel, 7.3% fanned their wings, 56.4% flew directly to the odor source, 26.4% walked linearly towards the females, 3.6% walked in circles, and 38.2% walked in zigzag movements to the odor source.

Discussion

The lesser cornstalk borer showed mating peaks when the adults were between 24 and 48 h old; the highest frequency of mating was observed between 6 to 8 h after the beginning

Table 2 Percentage of *Elasmopalpus lignosellus* couples that mated at different periods in the scotophase (photoperiod 12L:12D) ($N = 41$).

Hours scotophase	Couples mating (%)
0–2	0.0
2–4	2.4
4–6	21.9
6–8	68.3
8–10	7.3
10–12	0.0

The mating distribution in relation to scotophase periods was significantly different ($\chi^2_5 = 69.07$, $P < 0.05$).

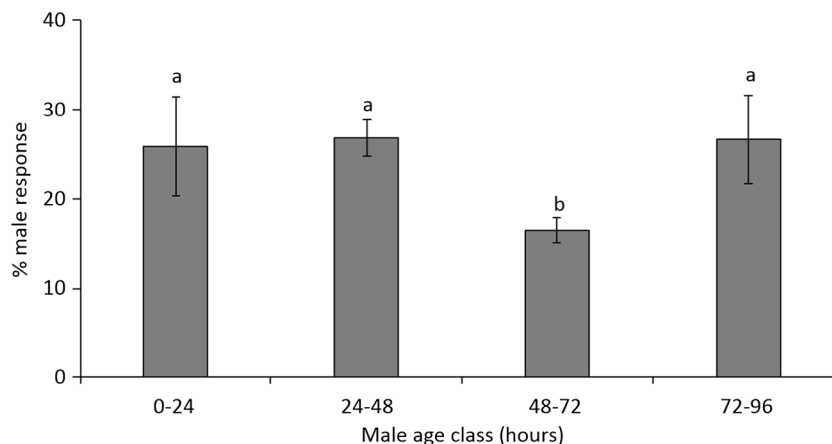


Fig 1 Percentage (mean \pm SE) of *Elasmopalpus lignosellus* males of four different ages attracted to calling females in wind tunnel bioassays. The bars represent the mean percentage of males of each age class responding to females of the four age classes (0–24, 24–48, 48–72, and 72–96 h, $N = 4$ for each male age class). Bars followed by different letters are significantly different (ANODEV, $\chi^2_3 = 9.06$ $P = 0.03$, $df = 3$ and mean comparisons by contrast analysis ($P < 0.05$)).

of the scotophase. This is directly in relation to LCB female calling behavior, which shows maximum frequency in this period of the scotophase (Pires et al 1994, de Viana 2015) and after females reach 24 h in adult age (Fig 3).

Pires et al (1994) and data on Fig 3 show that calling behavior of LCB females start on the first day after emergence, suggesting that they are sexually mature at this time. In this work, the maximum number of matings were observed when the insects were 24–48 h old, which suggests that male at this age are in optimum physiological state for reproduction, a fact also observed in other species of Pyralidae (Witethom 1992, Parra-Pedrazzoli & Leal 2006).

In general, lepidopteran males are highly attracted to the sex pheromone of females after they become sexually mature (Baker 1989, Roelofs 1995, Jarriault et al 2009), and in some moth species, the male response to female pheromones increases with adult age (Anton & Gadenne 1999, Gadenne & Anton 2000, Evenden & Gries 2008). For

example, Baker & Cardé (1979) reported the highest response to sex pheromone in *Grapholita molesta* Busk (Tortricidae) when males were 72 to 96 h old. In our wind tunnel bioassays, *E. lignosellus* males showed a lower level of response to calling females at 48–72 h old. However, males at 72–96 h old responded in a similar proportion as younger males, suggesting that complex physiological or behavioral mechanisms could be responsible for the alterations in the response to volatiles from calling females.

One of the explanations for these observations could be endocrinological changes, such as JH levels and the status of accessory glands, which modify the level of response to sex pheromones, as was demonstrated in other Lepidoptera (Happ 1984, 1992, Anton & Gadenne 1999, Gadenne et al 2001, Jin & Gong 2001, Carlsson & Hansson 2003, Gillott 2003, Wedell 2005, Jarriault et al 2009, Soques et al 2010). Another reason could be a differential expression of odorant binding proteins related to male age, a phenomenon

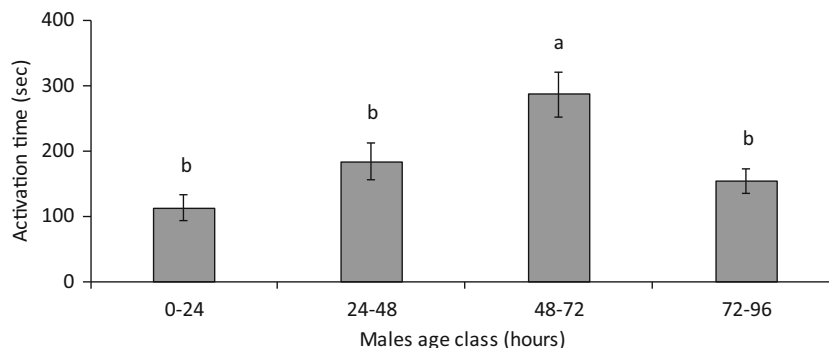


Fig 2 Activation time (sec) (mean \pm SE) of *Elasmopalpus lignosellus* males of four different ages when were stimulated with calling females in wind tunnel bioassays. The activation time was characterized as the time from the introduction of the males in the wind tunnel until they left the liberation area by flying or walking. The bars represent mean values of activation time for males of each age class responding to females of the four age classes ($N = 42, 48, 39$ and 55 for males of 0–24, 24–48, 48–72 and 72–96 h). Bars followed by different letters are significantly different (ANODEV $F_{3,177} = 7.78$, $P < 0.05$ and mean comparisons by Tukey's test ($P < 0.05$)).

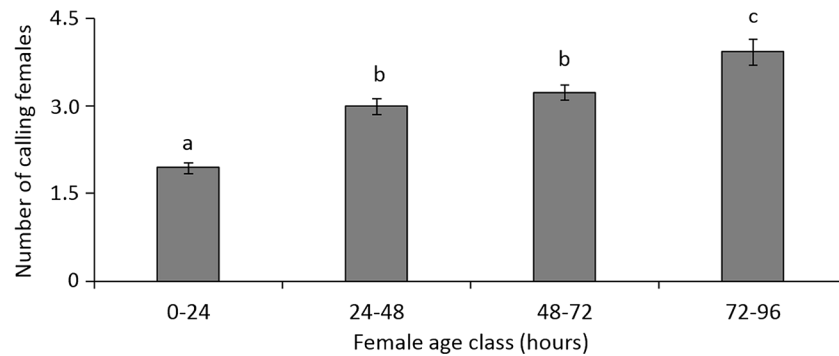


Fig 3 Number (mean \pm SE) of *Elasmopalpus lignosellus* calling females of different age classes. The number of calling females was counted from groups of 15 insects in wind tunnel bioassays. The number of groups observed by age class were 0–24 h = 163, 24–48 h = 211, 48–72 h = 213, and 72–96 h = 198. Bars with different letters are significantly different (ANOVA, $\chi^2_3 = 124.25$, $P < 0.01$, $df = 3$) and mean comparisons by contrast analyses ($P < 0.05$).

observed in two other pyralids, namely *Orthaga achatina* (Butler) (Liu *et al.* 2012) and *Cnaphalocrocis medinalis* (Guenée) (Liu *et al.* 2015). To better understand the degree of difference in response by *E. lignosellus* males at different age classes, further studies are needed including molecular, neural, and electrophysiological techniques. Pheromone production in moths generally decreases in older females, and as a result, they spend more time in calling behavior (Groot 2014, Umbers *et al.* 2015). In our study, the age of *E. lignosellus* females did not influence male attraction.

The results presented in this study under controlled conditions are in agreement with field observations in which *E. lignosellus* virgin females at 0 to 96 h old showed no significant differences in attracting males (Payne & Smith 1975). Notwithstanding, in wind tunnel bioassays, the number of females in calling behavior increased with their age, which could be the cause for similar male attraction to different female age classes. A similar pattern of calling behavior was reported by de Viana (2015) stating that 98% of females between 48 to 72 and 96 to 120 h old, displayed calling behavior during the scotophase, which is in contrast with 50% of the females between 0 to 24 h old. In this way, an increased number of *E. lignosellus* females in calling behavior could compensate the reduction in sex pheromone production. How this could be characterized as a group strategy needs to be addressed in future works.

In this research, we observed that insect age and scotophase period influences *E. lignosellus* mating behavior. Our results indicate that males reach maximal reproductive capacity after 24 h into the adult stage. These observations, associated with those previously reported by Pires *et al.* (1994) and de Viana (2015) in laboratory and field conditions, respectively, represent important information about the sexual behavior of this species.

The sex pheromone composition of *E. lignosellus* of different Brazilian populations from Goiânia (Goias State), Sete Lagoas (Minas Gerais State) (Lynch *et al.* 1984, Jham *et al.* 2005, 2007), and from Paraná State Viana (2015) showed a

specific blend of each population. These were also different from North American population, that was described as a mixture of (Z)-7-tetradecenyl acetate, (Z)-9-tetradecenyl acetate, (Z)-9-tetradecenol, and (Z)-11-hexadecenyl acetate (proportion 37:20:4:39) (Lynch *et al.* 1984). Studies of Brazilian populations identified the presence of (E)-8-hexadecenyl acetate, (Z)-9-hexadecenyl acetate, and (Z)-11-hexadecenyl acetate in the population from Goiânia. Only two acetates which are (Z)-9-hexadecenyl acetate and (Z)-11-hexadecenyl acetate were found in the population from Sete Lagoas, and a mix of two alcohols and three acetates non-identified in the population from Paraná (Jham *et al.* 2005, 2007, de Viana 2015). These differences could be a result of population isolation. However, host plants, climate conditions, rearing procedures, and experimental procedures in the laboratory could also contribute to originate differences in the pheromone blend.

Results presented here could be relevant in future works and may be useful in the identification of the sex pheromone blend of Brazilian populations of *E. lignosellus* and to determine biological activity of natural and synthetic sex pheromone in the laboratory.

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