Female *Diaphania glauculalis* (Guenée) Calling Behavior Diel Rhythm and Temporal Change in Pheromone Production[1]

Tao Ma[2,3], Na Lin[2,3], Xianhui Shi[2], Lili Zhou[2], Qiuhong Zhou[2], Cai Wang[2], Shengnan Zhang[2], Zhitao Liu[2], Zhaohui Sun[2], Xiaoyang Chen[2], Yurong He[4], and Xiujun Wen[2,5]

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Abstract *Diaphania glauculalis* (Guenée) is a defoliating pest of the Laran tree, *Anthocephalus chinensis* (Lamk.) Rich. ex Walp., which is an important tree species for reforestation efforts in tropical and subtropical regions. Currently, little information is available about the calling behavior and pheromone production of *D. glauculalis* females, but such information is important in developing pheromone-based monitoring systems for the pest. Thus, female calling behavior and its correlation with pheromone production was studied using electroantennogram (EAG) technology, qualitative analysis of gas chromatography-mass spectrometry (GC-MS), and Y-tube olfactometer assays. Calling females release a sex pheromone from a gland located on the dorsal membrane between the eighth and ninth abdominal segments, as observed with a stereomicroscope. Peak calling behavior occurred with females 3 d after emergence and 6 h into the scotophase. Pheromone release typically increased with the female age and scotophase and then decreased gradually. GC-MS analysis showed that the amount of pheromone in extracts from the pheromone glands were greatest at the onset of the calling behavior and decreased thereafter. Y-tube olfactometer assays confirmed that pheromone gland extracts at 6 h into the scotophase elicited stronger attractiveness in comparison to 5, 7, and 8 h after emergence.

Key Words *Diaphania glauculalis*, pheromone gland extracts, calling periodicity, electroantennogram, Y-tube olfactometer

Diaphania glauculalis (Guenée) (Lepidoptera: Crambidae) is the most damaging and important insect pest of the Laran tree, *Anthocephalus chinensis* (Lamk.) Rich. ex Walp. This fast-growing evergreen is distributed in southern China in Guangdong, Guangxi, Fujian, and Hunan and also occurs outside of China in Burma, Vietnam, Malaysia, and Sri Lanka (Fox 1971, Ma et al. 2015a). *Diaphania glauculalis* larvae skeletonize and defoliate *A. chinensis* (Guo 1982). Improved knowledge of the chemical communication and associated behavior of *D. glauculalis* could assist in the management of this pest. Currently, sex pheromone components of calling females of *D. glauculalis* have been only preliminarily studied (Ma et al. 2015b), and there are no reports on the diel periodicity of female calling

[1]Received 01 June 2017; accepted for publication 10 August 2017.
[2]Guangdong Key Laboratory for Innovative Development and Utilization of Forest Plant Germplasm, College of Forestry and Landscape Architecture, South China Agricultural University, 510642, Guangzhou, China.
[3]These authors contributed equally to this work.
[4]College of Agriculture, South China Agricultural University, Guangzhou 510642, China.
[5]Corresponding author (e-mail: wenxiujun@scau.edu.cn).

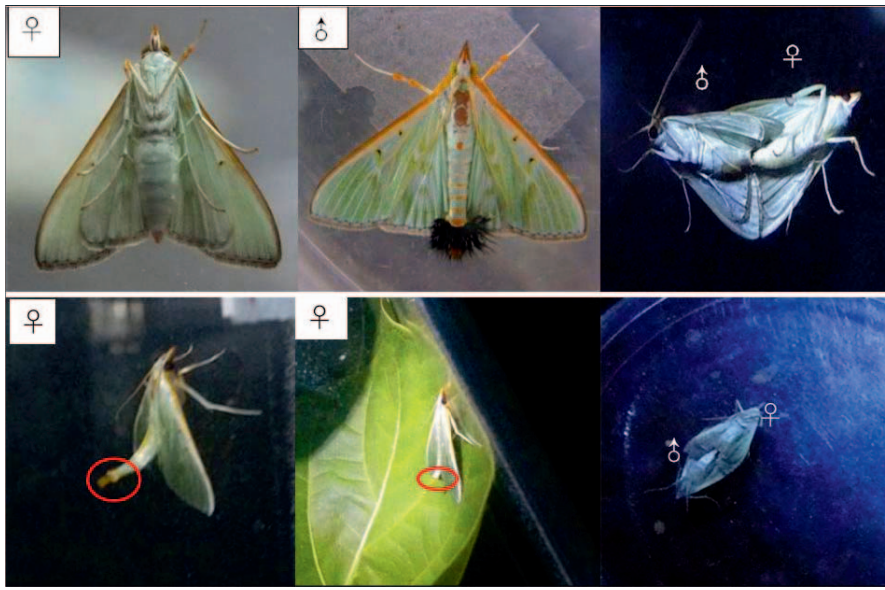

Fig. 1. Female calling and mating behavior of *Diaphania glauculalis*.

behavior, the amounts of pheromone produced by females, or the morphology of pheromone-producing structures. To this end, we herein report the calling behavior, the periodicity of female sex pheromone production, and the effect of female age and scotophase on sex pheromone production.

Materials and Methods

Insects. *Diaphania glauculalis* larvae were collected from *A. chinensis* trees growing on the Zengcheng (N 23.5°, E 113.3°) Experiment Station of South China Agricultural University and a forest at the ZhaiwuTown (N 22.8°, E 112.9°) of Jiangmen, Guangdong province. Larvae were transported to the laboratory and reared in plastic containers (60 × 30 × 30 cm), fed ad libitum on fresh *A. chinensis* leaves, and maintained at densities of approximately 200 larvae per container at 25–28°C, 12:12 h light:dark, and 75–80% relative humidity (RH). Containers were checked daily until pupation. Pupae were separated by sex to avoid any exposure of males to female sex pheromone (Liu et al. 2014), and adults were fed with a 10% (v/v) honey solution on a piece of cotton.

Adult emergence and female calling behavior. Emergence of adults was observed and recorded. Adults emerging within the same 24-h period were placed together to insure homogeneous age groups, with 1 d after emergence being 24 h after the event. Females were considered as in calling behavior when the abdominal tip was extruded. Calling behavior initiation and duration was observed and recorded in days after emergence. All dark phase observations were made using a dimmed red light.

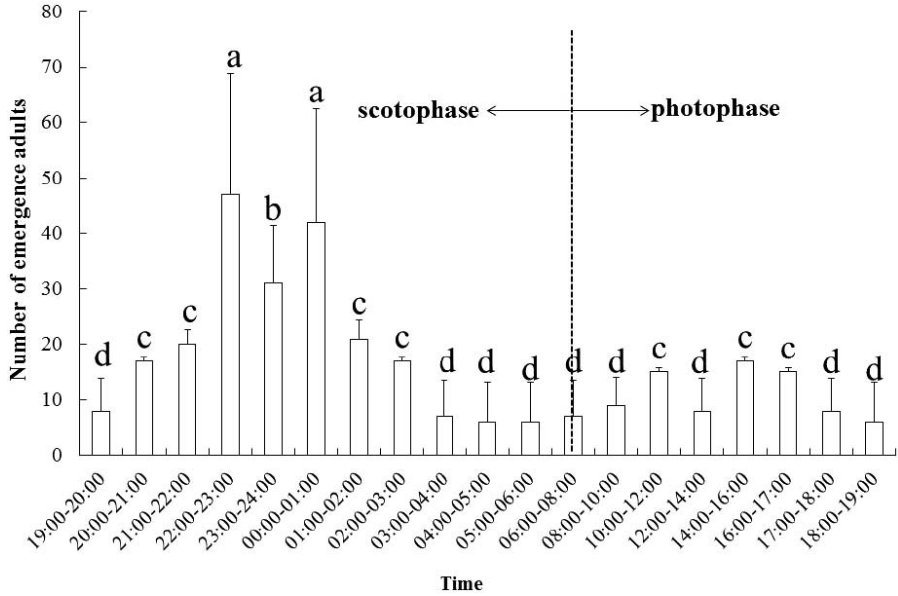

Fig. 2. The diurnal emergence rhythm of *Diaphania glauculalis* adults. Means followed by the different lowercase letters in every bar are significantly different ($P < 0.05$).

Pheromone glands. The fully extruded terminal abdominal segments of calling female moths were gently pressed with fingers and excised with a transverse cut rostral to the seventh abdominal segment. These samples were fixed in 10% sodium hydroxide (w/v) solution, rinsed in distilled water, air dried, and observed with a stereomicroscope (Olympus CX31, Japan) equipped with a computer (Lenovo, China). To determine whether there were active sex pheromone components, 10 ovipositors from scotophase females were cut and immersed in hexane (100 μL). The resulting extracts were further analyzed by gas chromatography-mass spectrometry (GC–MS).

Extraction of pheromone gland chemicals. Polar (dichloromethane, ethanol) or nonpolar (hexane, heptane) solvents also were compared for male reactivity to pheromone gland extracts. Females were randomly selected and placed into a freezer at −20°C for approximately 1 min. Terminal abdominal segments were then excised and placed together in organic solvent (HPLC grade; 10 μL solvent per abdominal tip), extracted at room temperature for 60–70 min, and then transferred to a clean glass vial (Agilent Technologies, USA). Gland extracts were stored at −20°C until they were used. Assessment of the gland extracts was performed with electroantennograms (EAG) to identify reaction to the chemicals in the various solvents.

Electrophysiological analyses. Male EAG response was recorded using a Syntech EAG equipped with a data acquisition interface box (IDAC-2), a probe/micromanipulator (MP-15), and an air stimulus controller (model CS-55). An

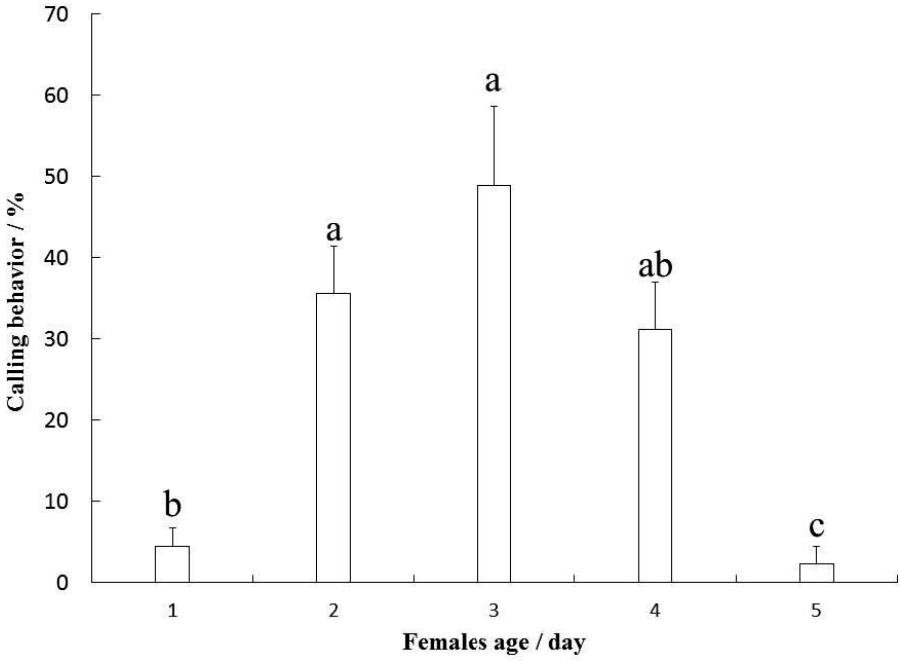

Fig. 3. Percentage of calling *Diaphania glauculalis* females under laboratory conditions at different ages. Each treatment, consisting of 25 females, was replicated three times. Means followed by the different lowercase letters in every bar are significantly different ($P < 0.05$).

isolated male antenna (1 per male adult), cut on both ends, was mounted onto an antenna holder for the EAG probe (PRG-2) with electrically conductive gel (Spectra 360, Parker Lab. Inc., Orange, NJ, USA). The stimuli (extracts) were applied to individual strips of filter paper (0.5 × 3.0 cm) which were inserted into a 15-cm Pasteur pipette. A puff of air containing no extract or chemical was pushed through the apparatus after each test of a stimulus. The tip of the pipette was inserted into the main airflow tube (8 mm diameter) in which a charcoal-filtered and moistened air stream (100 ml/min) was constantly flowing toward the antenna. The stimuli were provided to the antenna by puffing the humidified air for 0.3 s from the Pasteur pipette. Each antenna was exposed to each of the extracts, presented in random order, with at least 40 s between stimulations. This process was repeated three times for each of the five antennae for all stimuli. EAG values were averaged and the data were analyzed with the EAG software. When pheromone gland extracts were not in use, they were stored at −20°C.

Timing of pheromone production. Two experiments were conducted for monitoring the hourly and daily rhythm of female sex pheromone production. The first experiment used females that had emerged 2 or 3 d earlier to measure the male antennal EAG at 1-h intervals. Pheromone glands were dissected from 30 unmated females at hourly intervals starting at 2 h and ending at 8 h into the

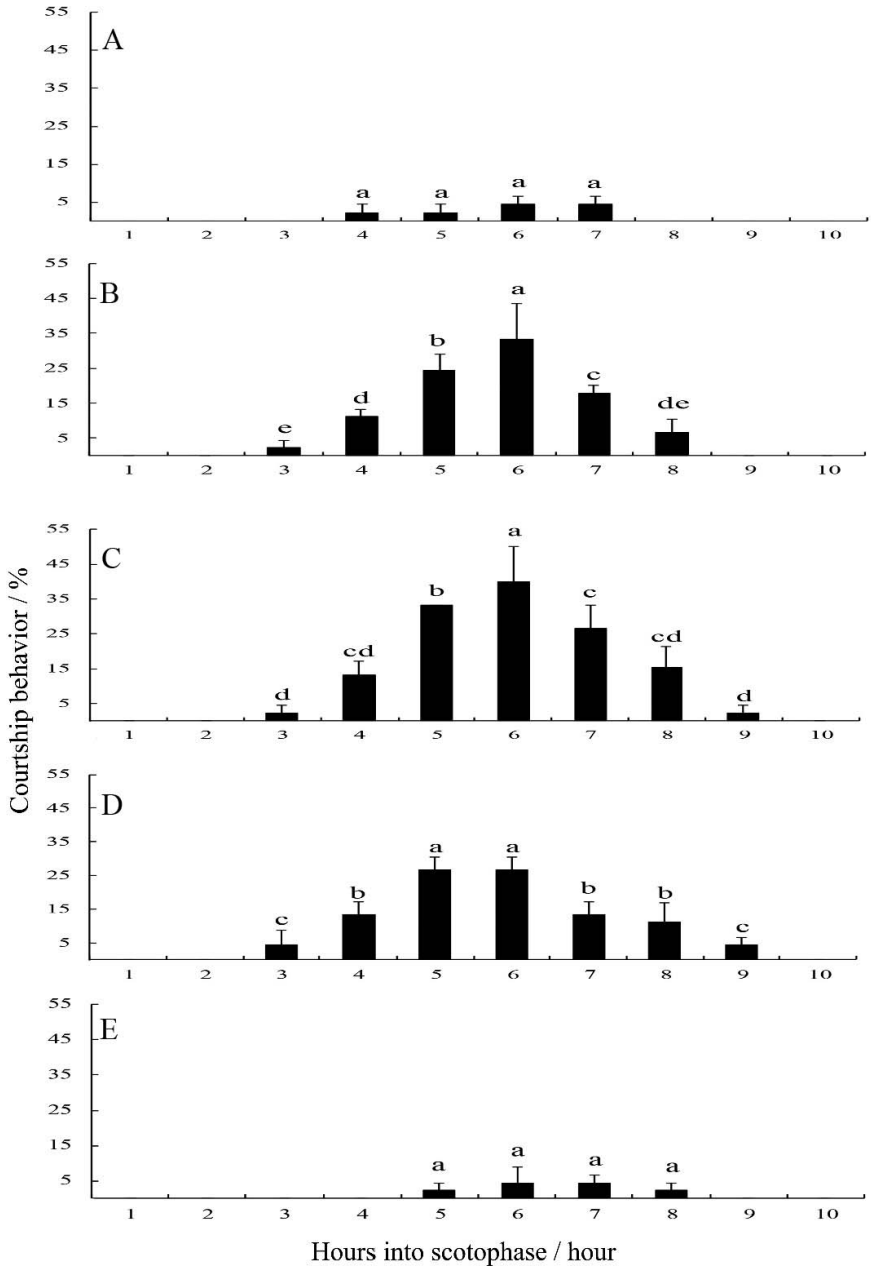

Fig. 4. Calling rhythm of female *Diaphania glauculalis* with different scotophase. Each treatment, consisting of 25 females, was replicated three times. Means followed by the different lowercase letters in every bar are significantly different ($P < 0.05$). (A) 1 d old; (B) 2 d old; (C) 3 d old; (D) 4 d old; (E) 5 d old.

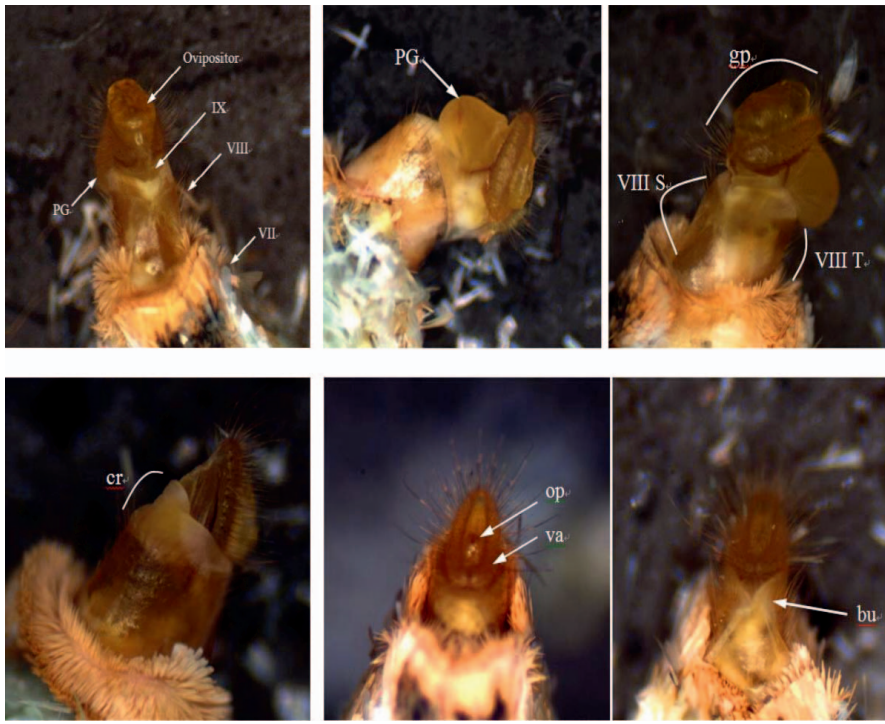

Fig. 5. Morphology of pheromone gland in female *Diaphania glauculalis*. VII: the seventh abdominal segment; VIII: the eighth abdominal segment; IX: the ninth abdominal segment; PG: Pheromone gland; gp: genital papilla; VIII T: eighth abdominal tergite; VIII S: eighth abdominal sternite; bu: bursa; cr: crescent; op: oviporus; va: valvula.

scotophase. Chemicals extracted using a solvent concentration of 1 female equivalent (FE) per 10 μl. The second experiment used females of different ages following emergence (1–5 d) to measure the sex pheromone production by age. Again, extracts of 30 unmated females were obtained daily by dissecting the glands and immersing in the solvent at 1 FE per 10 μl. Both experiments used males that emerged 2 d earlier to measure the male EAG response with hexane used as a chemical control. All the EAG experiments were conducted between 6:00 p.m. and 9:00 p.m.

Qualitative analysis of pheromone. Pheromone components and titer were detected with GC-MS (7890A-5975C, Agilent Technologies, USA). Hourly changes in pheromone titer were examined in glands of females that emerged 2 d earlier, and daily changes were examined at 6 h into the scotophase. Extracts were analyzed on DB-5 capillary columns (30 m × 0.25 mm id, 0.25-μm film thickness; Agilent Technologies, USA). The column oven was maintained at 50°C for 2 min, then the temperature was increased to 270°C at 10°C/min, at which point it was held constant for 10 min. Helium was used as the carrier gas at a constant flow rate of 1

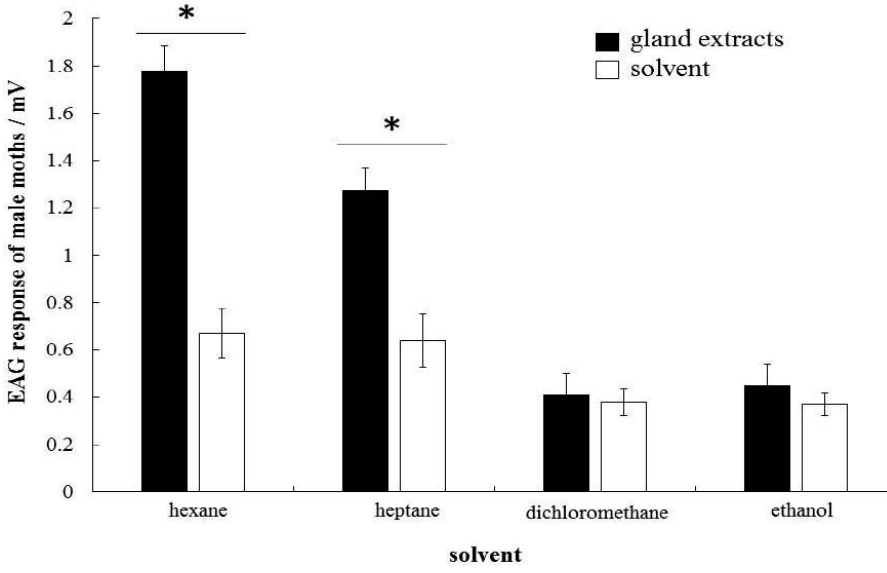

Fig. 6. EAG response of male moths to pheromone gland extracts with different solvent. Bars with asterisks represent significant differences between different pairs tested ($P = 0.05$).

ml/min, and the GC inlet temperature was 260°C. Electron ionization mass spectra were recorded from m/z 30 to 330 at 70 eV with the ion source temperature of 230°C.

Y-tube olfactometer assay. Male responses to the pheromone gland extracts at different scotophase intervals (5, 6, 7, 8 h) were measured in a glass Y-tube olfactometer, with a main column (90 cm long, 5.5 cm diameter) and two arms (50 cm long, 5 cm diameter) at 45° angles. The airflow speed was maintained at 500 ml/min by connecting the olfactometer arms to a vacuum pump. Air was charcoal-filtered and humidified before entering the system. Each treatment was replicated four times with 15 males per replicate. Response of the males was checked 5–10 min under a red lamp in the darkened room at 25 ± 2°C and 80% RH. Y-tubes were cleaned with ethanol before each trial. The response rate, rate of selective response, and selection coefficient were calculated with formulae provided by Zhou et al. (2009) as: Response Rate = $[(A + B) / C] \times 100\%$; Rate of Selective Response = $[A / (A + B)] \times 100\%$, and; Selection Coefficient = $[(A - B) / (A + B)] \times 100\%$, with A = number of males in tube with pheromone gland extracts, B = number of males in the control tube without pheromone gland extracts, and C = total number of males.

Statistical analyses. Data were analyzed using one-way analysis of variance (ANOVA) for means comparison. Both EAG responses to pheromone gland extracts (mV) and antennal responses were normalized. The mean response was compared to that of hexane. Multiple comparisons were performed using Tukey's

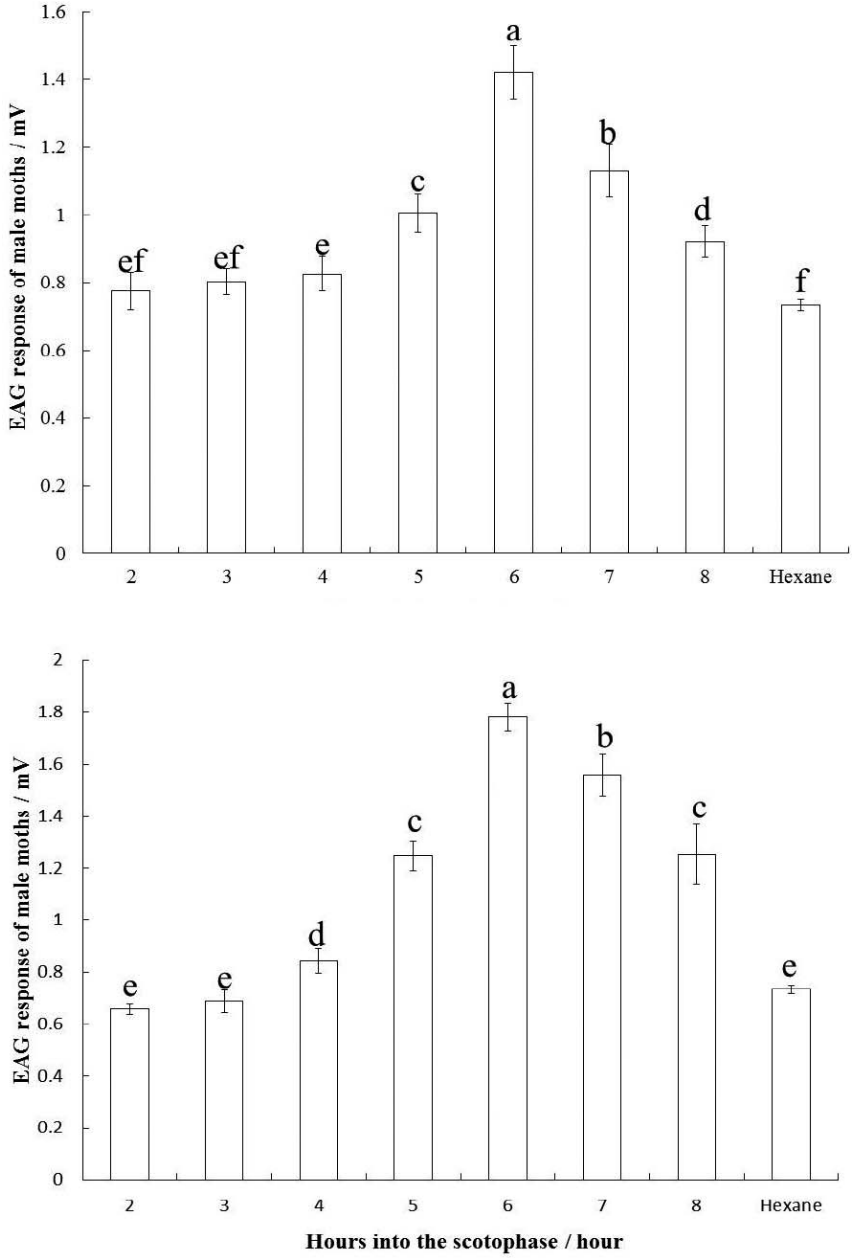

Fig. 7. The EAG response of male moths to pheromone crude extract from 2-d-old (upper) and 3-d-old (lower) virgin females at different times during scotophase. Each treatment, consisting of 30 females, was replicated five times ($n = 5$, or 5 male antenna). Means followed by the different lowercase letters in every bar are significantly different ($P < 0.05$).

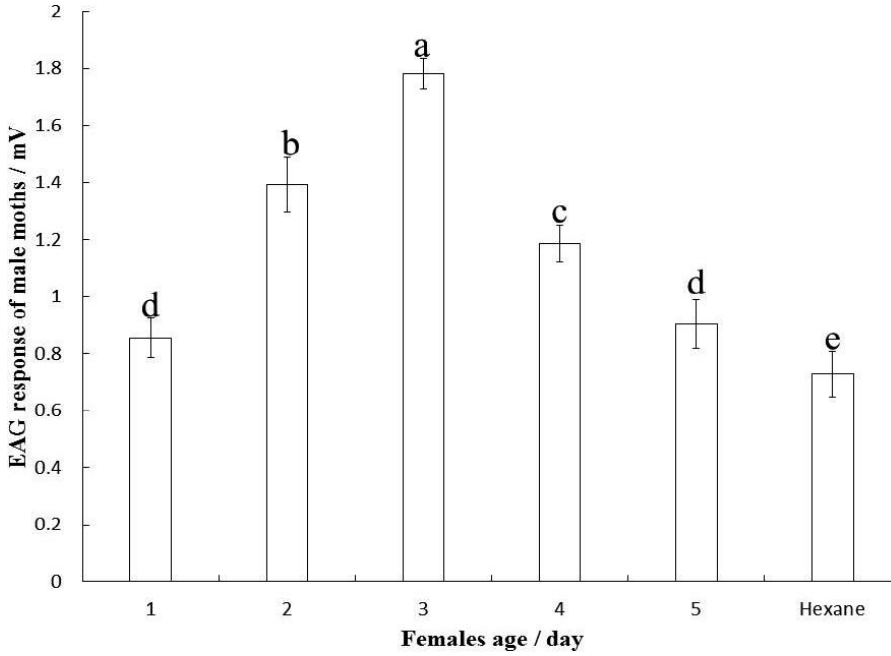

Fig. 8. The EAG response of male moths to pheromone crude extract from 1 to 5-d-old virgin females, 6 h into scotophase. Each treatment, consisting of 30 females, was replicated five times ($n = 5$, or 5 male antenna). Means followed by the different lowercase letters in every bar are significantly different ($P < 0.05$).

honestly significant difference (HSD) test (SPSS 17.0). The level of significance in all tests was set at $\alpha = 0.05$.

Results

Female and male adults of *D. glauculalis* were very easily distinguished, with noticeable sexual dimorphism (Fig. 1). Males possessed hair-like spines at the tip of the abdomen when at rest while females lacked these structures and characteristic. This was not observed on the closely related *D. indica* Saunders (Ganehiarachchi 1997; Kinjo and Arakaki 1997).

Adults were more active in darkness than in light (Fig. 2). Of the total number of adults observed, 72.3% emerged between the hours of 7:00 p.m. to 7:00 a.m., with peak emergence between 10:00 p.m. and 1:00 a.m. Some females initiated calling behavior (e.g., releasing pheromone) as soon as 1 d after emergence from pupation. Calling females elevated their abdomen and curved the abdominal tips upward, with the pheromone glands continuously exposed to air (Fig. 1). The proportion of females exhibiting calling behavior increased from 1 to 3 d after emergence and then gradually decreased from 4 to 5 d after emergence (Fig. 3).

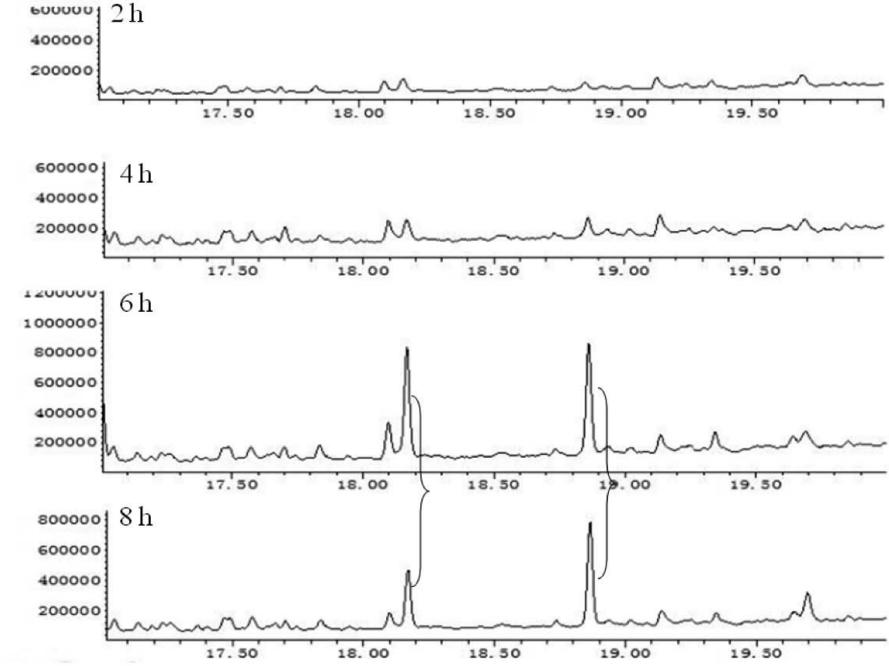

Fig. 9. Total ion chromatogram (TIC) of pheromone crude extracts from 2-d-old virgin females ($n = 10$) at different times during scotophase. The scale of TIC is the same and the peak area is relative content of sex pheromone.

Calling during the scotophase varied over time with most participating females being 2 to 4 d after emergence (Fig. 4).

The ovipositor, oviporus (op), and valvula (va) seen in Figure 5 were easily observed, with the sex pheromone being synthesized in and released from the pheromone gland (PG), which was associated with the intersegmental membrane between the eighth and ninth abdominal segments of the female. We found that the PG was fully exposed and that two sacs were everted from the dorsal intersegmental membrane with the characteristic calling behavior during scotophase.

Male EAG responses to gland extracts varied with polarity of the solvents tested (Fig. 6). The nonpolar solvents elicited stronger EAG responses than did the polar solvents. However, male response to hexane extracts reached 1.78 $\pm$ 0.11 mV, which was significantly higher than the other solvents tested. Therefore, hexane was used as the solvent for extraction of pheromone gland chemicals for the tests in our study.

Male EAG responses to PG extracts from unmated females 2 and 3 d after emergence revealed that release of pheromone began 4–5 h into the scotophase (Fig. 7). Production peaked at 6 h into the scotophase and then decreased gradually. Extracts from females at 6 and 7 h elicited significantly higher male EAG

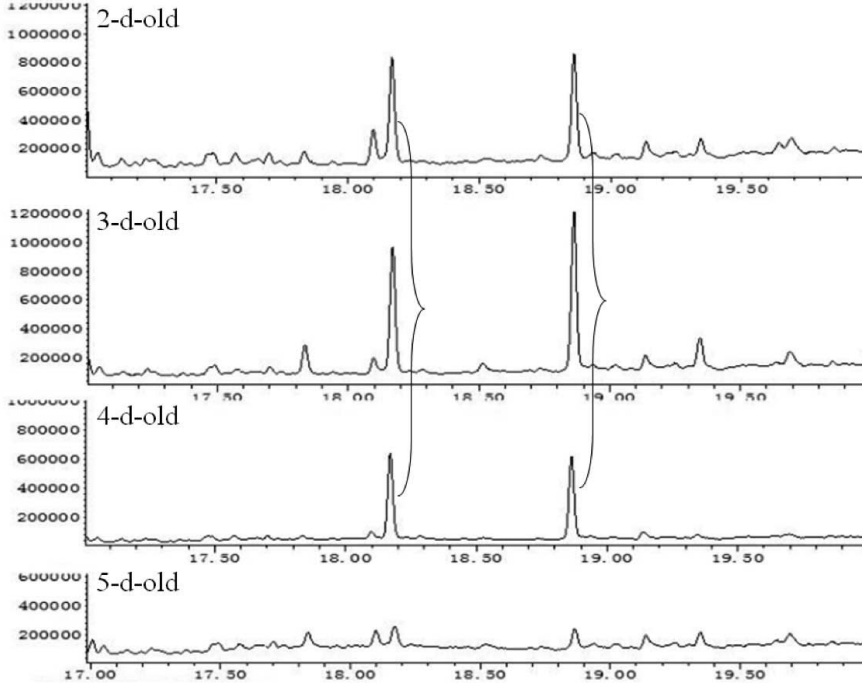

Fig. 10. Total ion chromatogram (TIC) of pheromone crude extracts from 2- to 5-d-old virgin females ($n = 10$), 6 h into scotophase. The scale of TIC is the same and the peak area is relative content of sex pheromone.

responses compared to the other hours tested (2 d after emergence: $F = 85.14$; df = 7, 32; $P < 0.05$; 3 d after emergence: $F = 232.40$; df = 7, 32; $P < 0.05$).

Females began to produce significant amounts of pheromones as early as 1 d after emergence (Fig. 8). Pheromone production increased significantly over the next 2 d, peaking at 3 d, and then gradually decreasing. Extracts from females 3 d after emergence elicited significantly higher male EAG responses than did those extracts from females 1, 2, 4, and 5 d after emergence ($F = 136.13$; df = 5, 24; $P < 0.05$). Extracts from females 5 d after emergence produced significant EAG responses, but no significant EAG responses were observed with extracts from females >5 d after emergence.

The two major components of the extracts are *E*11-16:Ald and *E*10*E*12-16:Ald as previously reported by Ma et al. (2015b). GC-MS analysis in this study showed similar variations in the production of these two components with scotophase and the age of the female after emergence. Based on the GC-MS analysis, pheromone production by unmated females 2 d after emergence reached a peak at 6 h into the scotophase, after which production decreased (Fig. 9). Titers of the pheromone components varied with age of the adult females after emergence, with peak levels observed from females 3 d after emergence; levels decreased with advancing age (4–5 d after emergence) (Fig. 10). Thus, based on these results, females release

Table 1. Olfactory response of *Diaphania glauculalis* to pheromone gland extracts from 3-d-old virgin females at different times during scotophase.*

Hours Into Scotophase/h	Response Rate (%)	Rate of Selective Response (%)	Selection Coefficient
5	61.67 ± 11.39 a	65.43 ± 4.84 ab	0.310 ± 0.090 b
6	70.00 ± 16.78 a	69.26 ± 4.010 a	0.390 ± 0.080 a
7	41.67 ± 6.380 b	59.64 ± 8.910 b	0.190 ± 0.180 c
8	30.00 ± 3.850 c	56.25 ± 14.93 b	0.130 ± 0.290 c

* Mean ± SE; values within each test followed by a different lowercase letter are significantly different at $P < 0.05$ (Tukey's HSD test).

the greatest amount of pheromone 3 d after emergence, 6 h into the scotophase. These observed variations were consistent with the EAG responses observed previously.

The Y-tube olfactometer assays showed the calculated Response Rates and Selective Response Rates of the male moths reached maximum values 6 h into the scotophase (Table 1). The Selection Coefficient revealed that all treatments could attract males, the maximum value obtained was 70**%**, and the hexane extracts 6 h into the scotophase was 0.39; these were significantly greater than with any other treatments.

Discussion

Diaphania glauculalis male adults frequently displayed the hair-like spines at the tip of their abdomens when at rest. This phenomenon differs from that of *Diaphania indica*, where females released sex pheromone by retracting a pair of hair-like spines. Under normal conditions, *D. glauculalis* males arch their abdomens dorsally and rotate a pair of hair-like spines. This same behavior also has been reported in congeneric species including *Diaphania nitidalis* Stoll (Elsey 1982), *Diaphania hyalinata* L. (Valles and Capinera 1992), and *D. indica* (Kinjo and Arakaki 1997). However, it is unknown whether such behavior is also related to the mating behavior of *D. glauculalis* males. Further studies are needed to elucidate the role, if any, that this behavior plays in mating of *D. glauculalis*.

The *D. glauculalis* female pheromone glands were found to be located on the dorsal membrane between the eighth and ninth abdominal segments. The GC-MS analysis further confirmed that the extracts from this anatomical location contained the major pheromone components (Figs. 9, 10) while analysis of extracts from other locations on the females did not detect pheromonal active components. Other lepidopterans have specialized pheromone glands at similar locations but with different morphologies. For example, the greatest pheromonal activity of *Manduca sexta* L. is found associated with the ventral membrane of the ninth abdominal segment (Itagaki and Conner 1988) while the pheromone gland of *Helicoverpa zea*

(Boddie) is a ring of specialized columnar cells almost encircling the abdomen between the eighth and ninth segments (Raina et al. 2000), and the pheromone gland of *Lymantria dispar* (L.) consists of a single layer of epithelial tissue on the dorsal and ventral membranes between the eighth and ninth abdominal segments (Solari et al. 2007).

Of the solvents compared in our study, hexane extracts evoked significantly greater EAG responses than did all others. We concluded that hexane was the better solvent for sex pheromone extraction from *D. glauculalis* females. Other solvents, however, have proven efficient in extraction of sex pheromone from other insect species (Ma et al. 2014; Ryall et al. 2010; Salerno et al. 2012; Witzgall et al. 2000).

We found that *D. glauculalis* adults emerged from pupation during a narrow range of time, preferring emergence during dark hours. Utilizing hexane extracts of the pheromone, we further determined that females possess sufficient amounts of sex pheromone to elicit male EAG response at 5–8 h into the scotophase, with releases beginning 5 h into the scotophase and peak production at 6 h.

Sex pheromone production in many lepidopteran species typically shows a marked reduction with increasing age (Howlander and Gerber 1986; Raina et al. 1986; Teal et al. 1990). We found the same trend with *D. glauculalis*. Females released sex pheromone 1 d after emergence with greater production at 2 d and maximum production at 3 d. Pheromone production decreased thereafter. This observed rhythm of pheromone production coincided with female calling behavior and mating and was consistent with reports with other *Diaphania* species (Elsey et al. 1984; Valles et al. 1991, 1992).

Female calling behavior and sex pheromone production is synchronous and usually depends on moth age as well as other endogenous and exogenous factors. It appears that *D. glauculalis* follows a calling and sex pheromone biosynthesis pattern that is common for *Heliothis subflexa* Guenée (Heath et al. 1991), *Sesamia nonagrioides* Lefèbvre (Babilis and Mazomenos 1992), *Helicoverpa assulta* Guenée (Kakimura and Tatsuki 1993), *Cydia pomonella* L. (Bäckman et al. 1997), and *Isoceras sibirica* Alpheraky (Liu et al. 2013). The sex pheromone production for these species occurs during the period in which females are calling and releasing pheromone.

Our findings of the periodicity and age effects on pheromone production and release by *D. glauculalis* females could be valuable in the development of pheromone-based monitoring systems for this pest. Studies are needed to further develop technology and methodology for deployment and to assess efficacy of the pheromone strategy.

Acknowledgments

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