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Neural regulation of sex pheromone biosynthesis in *Heliothis* moths

(pheromone biosynthesis activating neuropeptide/neurohormone/terminal abdominal ganglion/abdominal nerve cord/pheromone gland)

P. E. A. TEAL*, J. H. TUMLINSON, AND H. OBERLANDER

Insect Attractants, Behavior and Basic Biology Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Gainesville, FL 32604

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ABSTRACT **Pheromone biosynthesis in females of *Heliothis zea* is regulated endogenously by a neuropeptide produced in the subesophageal ganglion. We have found that the ventral nerve cord must be intact for normal induction of pheromone biosynthesis and that pheromonotropic activity is associated with extracts of the abdominal nerve cord, but only during the period when pheromone is produced. We did not find evidence of pheromonotropic activity in hemolymph obtained from females that were producing pheromone. Extracts of the brain–subesophageal ganglion complex, which contain pheromone biosynthesis activating neuropeptide (PBAN), induced pheromone biosynthesis when applied to the terminal abdominal ganglion only if nerves from this ganglion to the pheromone gland were intact. Brain–subesophageal ganglion extracts did not induce biosynthesis when applied directly to the pheromone glands *in vitro*. From our results, we conclude that the target site of PBAN is not the pheromone gland but the terminal abdominal ganglion, and we hypothesize that the abdominal nerve cord transports PBAN to the terminal abdominal ganglion.**

Endogenous regulation of reproductive physiology and behavior associated with pheromone production and release in many insects results from the action of nervous, neuroendocrine, and/or endocrine stimulation (1–4). However, while the chemistry and biological effects of sex pheromones are understood more fully for moth species than for other groups of insects (5), the endogenous mechanisms that regulate the initiation and inhibition of pheromone biosynthesis by moths remain largely unexplored. Neural, neurohormonal, and hormonal regulators effectively control the periodicity of sexual signaling and are thus of prime importance for the reproductive success of moth species. Although these processes are tightly coordinated, no single neural, neuroendocrine, or endocrine factor regulates the complete system. In fact, there are conflicting reports (6–11) regarding the endogenous mechanisms that control even a single aspect of the pheromone communication system in moths: induction of pheromone biosynthesis.

Studies on the corn earworm moth *Heliothis zea* (6–8) have firmly established that injection of extracts of the subesophageal ganglion induces pheromone biosynthesis. It was hypothesized that the neuropeptide responsible, pheromone biosynthesis activating neuropeptide (PBAN), was released from the corpora cardiaca, carried in the hemolymph, and acted directly on the cells of the pheromone gland (4, 6). However, gypsy moths, which also produce PBAN (6), do not require either the corpora allata or corpora cardiaca for production and release of the sex pheromone but do require that the ventral nerve cord be intact for production and release of normal amounts of pheromone (9–11). Thus, in this case neural rather than hormonal factors appear to be the main components regulating biosynthesis.

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These apparent discrepancies in the regulation of pheromone production caused us to investigate the mechanism by which PBAN induces biosynthesis. We have found that females of the corn earworm, like the gypsy moth, require the intact ventral nerve cord for pheromone biosynthesis. Furthermore, we have discovered that pheromonotropic activity is associated with the entire abdominal nerve cord (ANC), but only during the period when pheromone is produced. We have also found that PBAN induces pheromone production when applied to the terminal abdominal ganglion only if nerves from this ganglion to the gland are intact. PBAN does not induce biosynthesis when incubated with pheromone glands *in vitro*. These results show that the target site of PBAN is not the pheromone gland but the terminal abdominal ganglion that innervates the glandular area. Our experiments are most consistent with the hypothesis that the ANC is the conduit by which PBAN reaches the terminal abdominal ganglion.

MATERIALS AND METHODS

Insect Cultures. Virgin females of *H. zea* were obtained as pupae from a laboratory colony and were maintained under both natural and reversed 14:10 light/dark cycles at 25°C and 70% relative humidity. All experiments were conducted with insects during the third or fourth photophase after adult emergence unless stated otherwise.

Tissue Extracts. Extracts of the brain–subesophageal ganglion complex, known to contain PBAN (6–8), were prepared with tissue obtained from females during the 3rd day after adult emergence. The tissue was removed from the heads of decapitated insects with dissecting forceps and was stored (until 50 brain subesophageal ganglias were obtained) in a microhomogenizer (Erway Glass Blowing, Oregon, WI) surrounded by solid CO_2. Prior to homogenization, the tissue was warmed to 25°C and 250 μl of 0.35 M sucrose in 100 mM phosphate buffer (pH 6.8) was added. After homogenizing, the sample was centrifuged for 10 min at 2000 × *g*, the supernatant containing PBAN was removed, and the pellet was rehomogenized and centrifuged with 250 μl of buffer as described above. The combined supernatant [final concentration, 1 female equivalent (FE) per 10 μl] was tested for activity as described (8) and stored at −60°C until use. No loss in activity was found after 2 weeks of storage at −60°C.

Extracts of the ANC were prepared by removing the entire ANC or portions of it from insects during the 3rd hr of the third scotophase or the 5th hr of the third photophase after emergence. The tissue was homogenized in cold sucrose phosphate buffer (20 μl per FE) and injected into females during the light period as described (8).

Hemolymph was collected from females during the 4th hr of the third scotophase after emergence. The technique used

Abbreviations: PBAN, pheromone biosynthesis activating neuropeptide; ANC, abdominal nerve cord; FE, female equivalent; TAG, terminal abdominal ganglion; Z11-16:AL, (*Z*)-11-hexadecenal.

*To whom reprint requests should be addressed at: P.O. Box 14565, Gainesville, FL 32604.

for collection of hemolymph was the same as that described by Raina and Klun (6), who reported using 10 μl of hemolymph. We inserted the needle of a 50-μl syringe through the dorsal intersegmental membrane between abdominal segments 4 and 5 and drew up all available hemolymph. However, the volume of hemolymph obtained in this manner was quite variable (between 5 and 12 μl). After collection, hemolymph was placed in a microcentrifuge tube held in an ice bath, and a volume of cold (4°C) sucrose phosphate buffer equal to that of the hemolymph sample was added. Each day hemolymph from six to eight individuals was pooled as described above. The final concentration of hemolymph was adjusted with buffer to 1 FE per 20 μl. Females were injected with either 2 FE of hemolymph solution or 40 μl of buffer as indicated above. In some experiments, 4 FE of hemolymph was used.

***In Vitro* Incubation.** *In vitro* studies were conducted using the excised terminal abdominal segments where the pheromone gland is located (12, 13). Terminal segments were opened by cutting down the dorsal midline to ensure penetration of PBAN and placed in a microvial with 1 FE of PBAN. Glands were also incubated in 1 FE of PBAN in 20 μl of buffer containing the terminal abdominal ganglia (TAGs) or TAG homogenates of two insects that had been injected with 1 FE of PBAN 30 min prior to removal of the TAGs. All preparations were incubated for 1 hr at 25°C prior to extraction with two aliquots (20 μl each) of a solution (0.5 ng/μl) of myristyl acetate (internal standard) in hexane. The organic phase was removed and concentrated under N_2 to 5 μl prior to analysis.

***In Vivo* Studies.** Experimental controls for all *in vivo* studies included insects that were injected with 1 FE of PBAN or insects injected with a volume of buffer equal to the volume of the test sample as described (8). All preparations were incubated for 1 hr after injection of PBAN or buffer prior to excision and extraction of the pheromone glands (8).

In initial studies, the abdominal nerve cord was severed either anterior or posterior to the TAG by cutting through the appropriate sternite (14). In sham-operated insects, the dorsal aorta was cut at the seventh abdominal tergite. Wounds were sealed with New Skin (Medtech Laboratories, Cody, WY). After 20 min, 1 FE of PBAN was injected. All insects survived the 1-hr incubation period after injection of PBAN, and autopsies performed after excision of the pheromone glands confirmed that either the nerve cord or aorta had been severed.

For insects in which the TAG was bathed in PBAN, the ANC was exposed by making posterior incisions on both sides of the dorsal aorta. The cuticle and aorta were peeled back and cut at the seventh intersegmental membrane. The preparation was pinned in a wax-bottom dish and the ovaries and fat body were lifted out exposing the nerve cord. The gland was elevated by placing a dissecting pin under it so that PBAN did not contact the gland. Then 1 FE of PBAN was applied to the TAG. Nerves extending to the gland from the TAG were cut prior to bathing the TAG in PBAN in other preparations. After 1 hr, the pheromone glands were excised, extracted, and analyzed. Studies in which the TAG was bathed in 1% methylene blue in buffer indicated that elevation of the gland effectively eliminated movement of the stain into the gland.

The effect of severing the ANC on production of pheromone was studied by severing the nerve cord either immediately anterior to the TAG or anterior to the first abdominal ganglion of females during the 5th hr of the third photophase after emergence. Sham operations were performed by severing the aorta at the sixth abdominal intersegment. All wounds were sealed with molten dental wax. Some operated insects were injected with 1 FE of PBAN during the 3rd hr of the subsequent dark period and glands of all surviving females (87% survival) were extracted during the 4th hr of the scotophase after operation. This is the time when pheromone titer is greatest (15). Glands of untreated females that were producing pheromone were extracted at the same time as treated individuals. All insects were autopsied to ensure that operations had been successful.

Chemical Analysis. Previous work has demonstrated that the ratio of all of the compounds present in the naturally produced pheromone (16) is the same when females are artificially induced to produce pheromone by injection of PBAN (8). Therefore, we monitored pheromone production in our studies by measuring the titer of (*Z*)-11-hexadecenal (Z11-16:AL), the compound present in greatest amount in the pheromone blend (16). Analysis of the amount of Z11-16:AL present in extracts obtained from glands of individual females was conducted with a Hewlett–Packard 5890 gas chromatograph (GC) equipped with split/splitless capillary injectors and flame ionization detectors. Data were acquired and processed with a Nelson Analytical 3000 data system. Capillary columns used included an apolar 30 m × 0.25 mm ID SPB1 and a polar Supelcowax 10 of the same dimensions (Supelco). Conditions of GC were as follows: initial temperature, 60°C; injector operated in the splitless mode for 0.5 min; oven temperature increased at 30°/min after 1 min; final temperature, 200°C; helium carrier gas linear flow velocity of 18 cm/sec. GC mass spectroscopy (MS) was conducted with a Nermag R10-10 MS interfaced to a Hewlett–Packard 5790 GC having a split/splitless injector and equipped with the SPB1 column used in GC studies. Chemical ionization spectra were obtained by using methane as the reagent gas.

RESULTS AND DISCUSSION

Adult females of *H. zea* do not produce pheromone during the light period (15). However, when we injected PBAN into the abdomens of females during the day, significant amounts of pheromone were produced (Fig. 1). It has been suggested that PBAN is released from the corpora cardiaca into the hemolymph, as are other insect hormones (17), and acts on cells of the pheromone gland directly (3, 6). However, when we incubated glands with PBAN *in vitro*, pheromone was not produced (Fig. 1). Thus, either PBAN did not act on the pheromone gland or it acted in concert with another factor(s) to stimulate biosynthesis.

We suspected that the ANC might be involved in inducing pheromone production because severing the ANC of larval or pupal gypsy moths reduced or prevented pheromone production and altered pheromone release behavior in adults (9–11). Severing the ANC of female *H. zea* anterior to the TAG prior to PBAN injection did not reduce the amount of pheromone produced (Fig. 1). In a control experiment, no reduction in pheromone titer was observed when the dorsal aorta was severed prior to injection of PBAN. However, when nerves extending from the TAG into the area of the gland were severed prior to PBAN injection no significant level of pheromone was produced (Fig. 1). When the TAG was bathed in PBAN while still attached to the gland, so that no PBAN contacted the gland, pheromone was produced. Severing the nerves extending to the gland inhibited pheromone production in these preparations (Fig. 2). Therefore, PBAN exerts direct action not on the cells of the pheromone gland but on the TAG. Neurons arising in the TAG then send another signal to the gland to induce pheromone production. These findings are of considerable interest because insect neurohormones usually act directly on the effector organ (17), in this case the pheromone gland.

In vitro incubation of glands with PBAN plus either intact isolated TAGS or homogenates of TAGs from females previously stimulated by PBAN did not increase pheromone production (Fig. 2). Similarly, no increase in production was

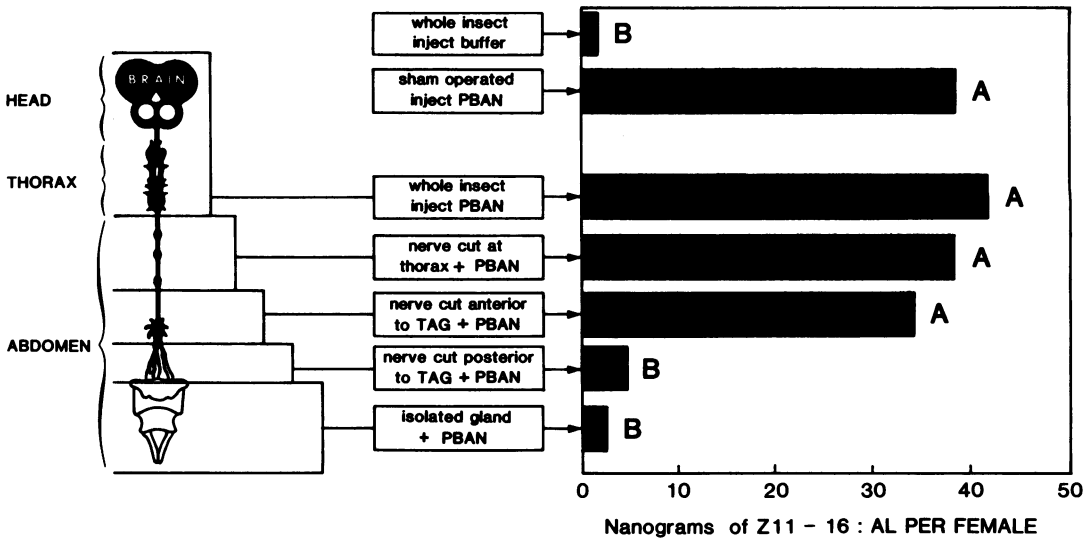

FIG. 1. Amount of Z11-16:AL produced by the pheromone gland in the 1-hr period after injection of 1 FE of PBAN into intact females, females in which the abdominal nerve cord was cut anterior or posterior to the TAG, or sham-operated females, and on *in vitro* incubations of glands plus PBAN. All experiments were conducted during the third or fourth photophase after adult emergence and were replicated 10 times. Means were analyzed by a Duncan's multiple range test. Bars capped with the same letter are not significantly different ($P = 0.05$).

found when isolated TAGs or TAG homogenates from non-PBAN-stimulated females were incubated with PBAN plus isolated glands. The lack of activity in these treatments suggests that continuous stimulation of the pheromone gland by the TAG and nerves extending from it is required for pheromone biosynthesis. This hypothesis is supported by the fact that severing or clamping nerves extending from the TAG to the gland at various times after injection of PBAN stops the continued production of pheromone (P.E.A.T. and J.H.T., unpublished results). Although histological studies (12, 13) have not revealed the presence of synaptic connections between pheromone gland cells and nerves extending from the TAG, there are fine neuronal branches that appear to be efferent, associated with the pheromone gland in both *Heliothis virescens* and *H. zea*. (H. Itagaki, T. A. Christensen, and J. G. Hildebrand, personal communication). Therefore, we suspect that the signal from the TAG to the gland involves the release of a neurosecretion.

The knowledge that the nerve cord must be intact for normal production of pheromone by gypsy moths (11) and that PBAN acts on the TAG, the most posterior ganglion of the nerve cord, suggested that the entire ANC might play a role in regulation of pheromone biosynthesis. We tested this hypothesis by severing the ANC immediately anterior to the TAG during the light period and then analyzing for the presence of pheromone 12 hr later during the peak period of pheromone production. These insects produced only a small amount of pheromone (Fig. 3). Insects in which the dorsal aorta was severed produced pheromone. Furthermore, when insects in which the ANC had been severed during the light period were injected with PBAN during the peak of pheromone production, they produced pheromone (Fig. 3).

To test whether connection of the nerve cord between the head and abdomen was required, we severed the ANC anterior to the first abdominal ganglion during the day. Analysis during the peak of pheromone production on the next night showed that insects did not produce pheromone (Fig. 3). However, these insects could be stimulated to produce pheromone by injection of PBAN. These data showed that the nerve cord between the thorax and abdomen

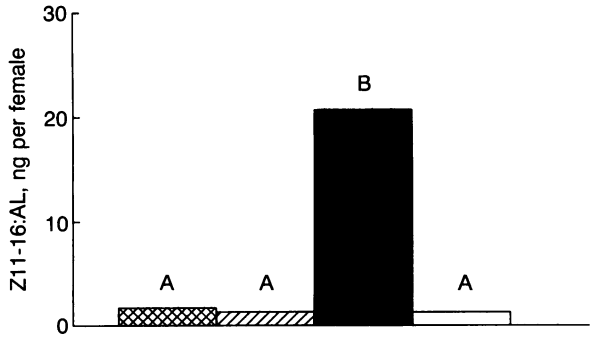

FIG. 2. Effect of *in vitro* incubation of glands in PBAN plus intact TAGs obtained from females stimulated with PBAN (cross-hatched bar) or homogenates of TAGs obtained from PBAN-stimulated females plus PBAN (hatched bar). The effects on pheromone production of bathing the exposed TAG in PBAN while it was still connected to the pheromone gland (solid bar) or after severing the nerves extending from the TAG to the gland (open bar) are also shown. Bars capped with the same letter are not significantly different in a Duncan's multiple range test ($P = 0.05$) ($n = 10$ for each treatment).

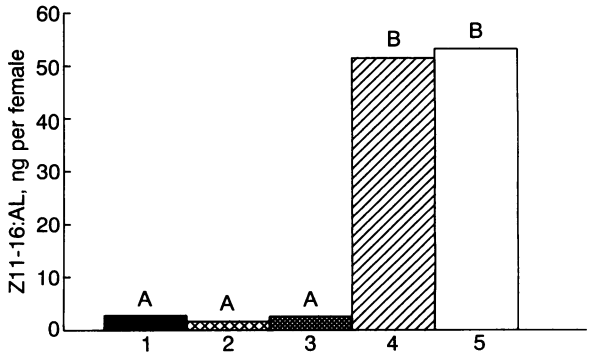

FIG. 3. Amount of Z11-16:AL present in glands of females under the following conditions: *1*, during the light period (solid bar); *2*, ANC severed anterior to the TAG during the day but sampled at the peak of pheromone production of the subsequent night (cross-hatched bar); *3*, ANC severed anterior to the first abdominal ganglion and treated as in *2* (small cross-hatched bar); *4*, ANC severed as in *2* but injected with 1 FE of PBAN 1 hr prior to extraction during the subsequent night (hatched bar); *5*, during the peak of pheromone production in untreated females (open bar). Bars capped with the same letter are not significantly different in a Duncan's multiple range test ($P = 0.05$) ($n = 10$ for each treatment).

must be intact to induce pheromone biosynthesis under normal conditions.

Next, we tested the hypothesis that PBAN was carried in the hemolymph (6) by injecting 2 FE of hemolymph obtained from females during the peak of pheromone production into non-pheromone-producing females. In more than 30 trials, females injected with hemolymph did not produce significantly more pheromone ($\bar{x}$ = 4.14 ± 0.60 ng; n = 32) than control females injected with buffer ($\bar{x}$ = 3.30 ± 0.51 ng; n = 32). We tested the activity of hemolymph further by injecting 4 FE of hemolymph, obtained in the same fashion but without addition of buffer, and found that glands of those females contained an average of 3.92 ng of Z11-16:AL (±0.78; n = 5), which was not statistically different from the 3.09 ng (±0.86 ng; n = 5) of Z11-16:AL present in control insects injected with 10 μl of buffer. This indicated too little PBAN was present in the hemolymph of our samples to have an effect.

Knowing that an intact ANC was required for induction of biosynthesis in normal insects we hypothesized that either PBAN or another pheromonotropic substance might be present in extracts of the ANC but only when females were producing pheromone. When we injected homogenates of the ANC obtained from females that were producing pheromone into nonproducing females, the treated insects produced pheromone. Homogenates of ANC obtained from females that were not synthesizing pheromone had only limited activity (Fig. 4).

Our data support the conclusion that while PBAN is present in the subesophageal ganglion (3, 6, 7), it is not carried directly to the pheromone gland by the hemolymph, nor does it act on the cells of the pheromone gland. Instead, it acts on the TAG, which sends a different message, not PBAN, to the pheromone gland. This second message induces pheromone biosynthesis. One hypothesis that would account for results reported by others (6), which indicate that small amounts of pheromonotropic activity are present in the corpora cardiaca and hemolymph, is that PBAN could be sequestered from the hemolymph and accumulated in the ANC before being transported to the TAG. Alternatively, hemolymph-borne PBAN could stimulate release of a second neurosecretory substance from cells in the ANC. We do not dispute the hypothesis that a small amount of PBAN may be present in the hemolymph, but it is insufficient to stimulate pheromone production. Our data show clearly that neural

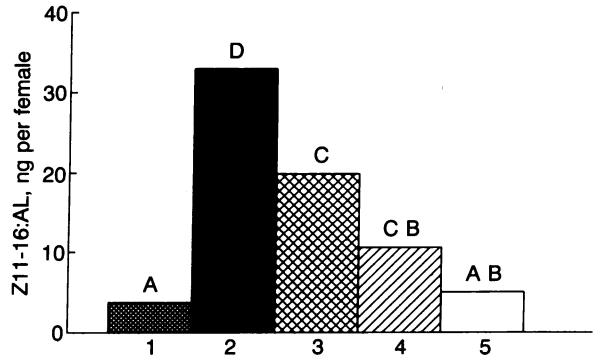

FIG. 4. Production of Z11-16:AL by intact females during the third photophase after emergence following injection of the following *1*, 10 μl of buffer (small cross-hatched bar) (n = 10); *2*, homogenates of the entire ANC obtained from calling females (solid bar) (n = 12); *3*, homogenates of the three ganglia and connectives anterior to the TAG from calling females (cross-hatched bar) (n = 10); *4*, homogenates of the TAGs obtained from females during the peak of pheromone production (hatched bar) (n = 10); *5*, effect of injection of 2 FE of homogenates of the entire abdominal ganglia obtained from females during the period in which the pheromone titer is lowest (n = 10). Bars capped with the same letter are not significantly different in a Duncan's multiple range test (P = 0.05).

connection between the subesophageal ganglion and ANC is required for induction of pheromone production under normal circumstances. Manifestly, if PBAN were released into the hemolymph and acted on the ANC to induce pheromone production, then connection between the ANC and thoracic nerve cord would not be required. We hypothesize that PBAN is transported from the subesophageal ganglion to the TAG via the ventral nerve cord because of the following results: (*i*) pheromonotropic activity is associated with the ANC only during the period when pheromone is produced, (*ii*) we found no activity associated with hemolymph, (*iii*) pheromone production can be blocked by eliminating the neural connection between the brain and TAG but can be stimulated by injection of PBAN, and (*iv*) one or two cells staining for neurosecretory material run the length of the *Heliothis* ventral nerve cord, connecting the subesophageal ganglion and TAG (H. Itagaki, T. A. Christensen, and J. G. Hildebrand, personal communication). If this hypothesis is correct, then axonal transport of PBAN must occur at a rapid rate, given that no pheromonotropic activity is associated with the ANC during the middle part of the photophase.

The fastest rates of axonal transport of neurosecretory material reported for insects are 11 and 12 mm/hr (18, 19). However, substantially faster rates have been reported in vertebrates (17). Thus, given the average length of the ventral nerve cord in dissected insects of 14.7 mm (±0.4 mm; mean ± SD; n = 10) and a rate of axonal transport of 12 mm/hr, a period of 1.25 hr would be required for PBAN to be transported from the subesophageal ganglion to the TAG down the ventral nerve cord before pheromone production could begin. We do not know what environmental signals initiate the release of PBAN. However, studies by Raina *et al.* (15) indicate that small amounts of pheromone are present 1 hr after the initiation of the dark period and that levels increase rapidly after that. This 1-hr lag between lights off and production of pheromone is similar to the calculated time of 1.25 hr for axonal transport of PBAN.

Although the data reported here are for *H. zea*, we have conducted similar experiments with *H. virescens* and *H. subflexa* as experimental organisms (data not shown) and have obtained results that parallel those found when using *H. zea*. Therefore, this intriguing mechanism of neuroregulation could provide the fine tuning required for explicit diel periodic control of pheromone production by *Heliothis* moths.

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