mM potassium phosphate, pH 7.5, containing 2 mM EDTA, 1 mM DTE, and 0.1 mM PMSF. The precipitate was removed by centrifugation, and the supernatant containing the PRAI activity was applied to a DEAE-Sepharose fast-flow column, equilibrated with the same buffer, and eluted with a linear phosphate gradient. After addition of 10% glycerol, the pure proteins were stored at −70°C. Typically, 5 to 10 mg of protein was obtained from 1 g of wet cells.
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Involvement of Juvenile Hormone in the Regulation of Pheromone Release Activities in a Moth

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Juvenile hormone has been implicated in the mediation of several reproduction-related events in adult insects, but had previously been found to play no role in the regulation of sex pheromone production and release behavior ("calling") in moths. In females of the true armyworm moth, *Pseudaletia unipuncta*, juvenile hormone is shown to be essential to the initiation of both calling behavior and pheromone production. Females without corpora allata, the source of juvenile hormone, do not call and do not produce pheromone, but injection of juvenile hormone into allatectomized females restored these activities. The armyworm's control system has likely evolved in response to the adults' migratory behavior which may necessitate that mating be restricted to the period following migration.

FEMALES OF MANY INSECT SPECIES attract mates by means of a sex pheromone, the release of which is frequently associated with a characteristic behavior termed "calling" (*1*). Early studies of the physiological regulation of pheromone emission indicated that the presence of corpora allata (CA), the source of juvenile hormone (JH), was essential for mate attraction in some cockroaches, but that pheromone release was unaffected by CA removal in the females of two species of moth (*2*). Subsequent investigations on other moths yielded similar results (*3, 4*). We now report that, unlike the moth species previously studied, females of the true armyworm, *Pseudaletia unipuncta* (Noctuidae), require CA in order to synthesize pheromone and express calling behavior.

After comparing the reproductive cycle of cockroaches with that of moths, Barth (*2*) proposed the hypothesis that neuroendocrine control of pheromone release activities would be expected to evolve only in insects that are long-lived as adults and in which there are periods when mating is suppressed. Unlike cockroaches, the moths that he and others examined have short adult lives and are physiologically ready to mate soon after eclosion, making the involvement of a complex endocrine control system unnecessary. In contrast with these moths, *P. unipuncta* females do not mate immediately after emergence. Instead, they delay all mating-related activities for varying lengths of time in response to environmental conditions. When they are subjected to temperatures and photoperiods that mimic those encountered in the spring or in the fall [which are thought to be associated with long-distance flights (*5*)], females initiate calling at a much older age than when they are exposed to summer-like conditions (*6*). Because migratory behavior, and the often-associated arrest in ovarian development, appear to be under the control of JH in some species of insects (*7*), we tested the hypothesis that the same hormone might play a key role in either suppressing or stimulating pheromone production and calling behavior in armyworm moths.

We have demonstrated that the brain-suboesophageal ganglion (SOG) complex of *P. unipuncta* females contains a substance with effects similar to the pheromone biosynthesis-activating neuropeptide (PBAN) discovered in *Heliothis zea* (*8*). Armyworm females that were neck-ligated during the photophase did not produce pheromone during the following scotophase (dark phase) (*9*). However, ligated females injected with a brain-SOG homogenate contained relatively large amounts of pheromone 3 hours following injection, whether the injection was performed during the scotophase or the photophase (Table 1). This indicates that the true armyworm's pheromone gland, unlike that of *H. zea* (*10*), can produce pheromone during the photophase.

We have also shown the existence of a clear relation between pheromone release activities and ovarian development in *P. unipuncta*, and we proposed two models to explain the possible involvement of JH as the factor governing the initiation of pheromone production and calling (*11*). In one model (Fig. 1A), JH acts directly on the central nervous system (CNS) to allow the release of PBAN [possibly by the corpora cardiaca (*10*)] for pheromone biosynthesis and the sending of neural messages (*4*) that initiate the expression of calling behavior. The second model (Fig. 1B) involves an intermediate hormone released by the ovary, in response to JH stimulation. We present data that support the former model.

The CA were removed (allatectomy) from newly emerged females, anesthetized under CO_2. These, along with untreated controls and sham-operated females, were observed daily during the last 1.5 hours of the scotophase to determine the age of first calling (*12*). At the end of the fifth scotophase, all females were sacrificed for pheromone titer analysis and ovarian development assessment.

More than half of the sham-operated and control females called by day 5, whereas none of the allatectomized ones did (Fig. 2A). Furthermore, allatectomized females

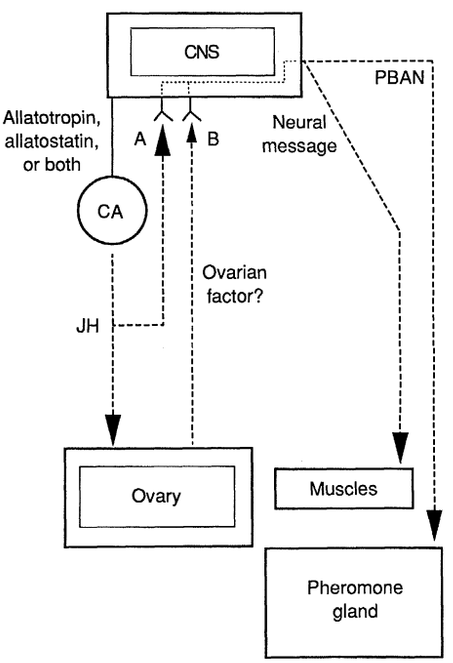

Fig. 1. Diagram illustrating the two pathways (A and B) proposed by Cusson and McNeil (*11*) to explain the role of JH in the regulation of pheromone production and calling behavior in *Pseudaletia unipuncta*.

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had little or no pheromone in their glands, as well as undeveloped ovaries, whereas those with intact CA had substantial amounts of pheromone and mature oocytes. When allatectomized females were injected with JH II one day after the operation (*13*), calling behavior, pheromone titers, and ovarian development were similar to those of controls (in Fig. 2A) by the end of the remaining four days of the experiment (Fig. 2B). These results demonstrate the important role of JH in the regulation of oocyte growth, pheromone production, and the expression of calling behavior in *P. unipuncta*.

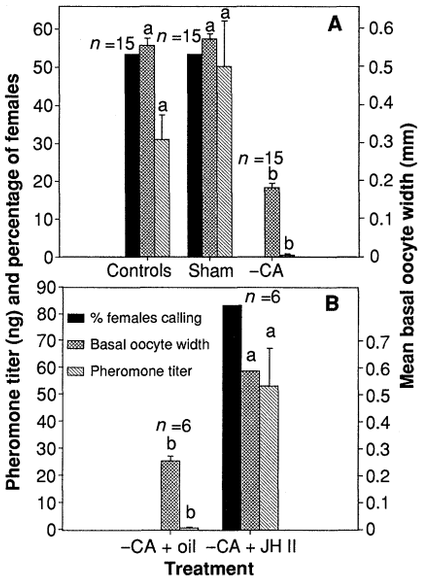

Fig. 2. (**A**) Effect of allatectomy (−CA) and sham-operation (Sham) on pheromone titer, basal oocyte width, and percentage of *Pseudaletia unipuncta* females initiating calling. (**B**) Effect of 10 μg of JH II in mineral oil (−CA + JH II), or just mineral oil (−CA + oil) on pheromone titer, basal oocyte width, and percentage of allatectomized *Pseudaletia unipuncta* females initiating calling. Error bars indicate standard errors of the mean; means followed by the same letter are not statistically different ($P = 0.05$, Mann-Whitney U test).

As pheromone release activities do not begin until basal oocytes have reached a critical size (*11*), we tested the possibility that JH might exert its effect through an intermediate ovarian hormone. Females were ovariectomized as ultimate or penultimate instar larvae (*14*) and the resulting adults were followed for a period of 5 days, under the same protocol described above. Ovariectomized females did not differ from sham-operated or control individuals in either the amount of pheromone present in their gland at the end of day 5 (Kruskal-Wallis test statistic = 0.81, $P = 0.668$) or in the proportion that had initiated calling ($\chi^2 = 0.06$, df = 2, $P = 0.972$; Table 2), indicating that in *P. unipuncta*, the ovaries are not directly involved in the regulation of pheromone production or calling behavior. Similar results have been obtained with the Gypsy moth (*15*).

During the scotophase which follows the day of emergence, *P. unipuncta* females never produce pheromone (compare 1-day-old and 3-day-old females in Table 1). However, (i) their brain-SOG complex contains PBAN activity, as evidenced by the production of pheromone by ligated females injected with brain-SOG homogenates from 1-day-old donors, and (ii) they are able to synthesize pheromone when injected with a brain-SOG extract (Table 1). In addition, the response of allatectomized females to brain-SOG extracts (*16*) is similar to that of 1-day-old females (Table 1). These results, along with the observation that allatectomy suppresses both calling behavior and pheromone biosynthesis while injection of the putative PBAN activates pheromone production without inducing calling behavior (*17*), suggest that JH acts to allow the release of both PBAN and the neural signals which presumably mediate calling (*4*). However, JH may also increase, directly or indirectly, the capacity of the pheromone gland to synthesize pheromone, possibly by stimulating the production of key enzymes involved in pheromone biosynthesis, as suggested for the spruce budworm (*18*). This might explain why the injection of brain-SOG extracts in 1-day-old and allatectomized females resulted in lower levels of pheromone synthesis than in females ligated after their first night of calling (Table 1).

Thus, the true armyworm appears to conform to Barth's hypothesis in that it has a relatively complex reproductive biology and in that both calling behavior and pheromone production are under hormonal control. The age of first calling is likely to be determined by the brain through the release of neurohormones (such as allatotropin or allatostatin), which will either stimulate or inhibit JH production by the CA (Fig. 1). In the context of seasonal migration, environmental cues (spring and fall versus summer conditions) are expected to affect the release of these neurohormones which, in turn, will influence JH hemolymph titers. Under spring or fall conditions, JH titers probably remain low for a longer period of time after emergence than in the summer, thereby preventing the initiation of pheromone release activities during migration. This possibility deserves further attention. Once JH titers reach the necessary levels to initiate pheromone biosynthesis and calling behavior, the diel rhythms of these tightly linked activities are apparently governed by PBAN and neural signals, respectively. The mechanisms responsible for the synchrony between pheromone production and the expression of calling behavior (*19*) remain to be determined.

Table 1. Effect of ligation, allatectomy, and injection of brain-SOG complex homogenates on pheromone titer (mean ± SE) in *Pseudaletia unipuncta* recipient females. Unless otherwise stated, donors and recipients were sexually mature, that is, calling had begun (*9, 16*).

Treatment	*n*	Titer (ng)
Ligated	10	0.2 ± 0.2 a†
Ligated + saline	6	0.0 a
Ligated + brain-SOG (scotophase)	15	48.5 ± 5.3 c
Ligated + brain-SOG (photophase)	10	33.4 ± 6.7 bc
Untreated 3-day-old females (first calling night)*	45	44.4 ± 4.6 bc
Untreated 1-day-old females*	10	0.0 a
One-day-old females + brain-SOG	8	14.8 ± 3.3 b
Allatectomized + saline	3	0.0 a
Allatectomized + brain-SOG	10	17.8 ± 5.8 b
Ligated + brain-SOG of 1-day-old female	5	27.5 ± 8.3 bc

*Pheromone extracted at lights-on. †Means followed by the same letter are not significantly different ($P = 0.05$, Newman-Keuls multiple range test following ANOVA on ranks).

Table 2. Effect of ovariectomy on pheromone titer (mean ± SEM) and proportion of virgin *Pseudaletia unipuncta* females initiating calling. There were no statistically significant differences among mean pheromone titers (Kruskal-Wallis test) and among proportions (χ^2).

Treatment	Pheromone titer (ng)	Females calling
Controls	55.9 ± 8.1	11/14
Ovariectomized	58.8 ± 11.5	10/12
Sham-operated	46.2 ± 8.6	10/11

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9. Females were reared at 25° ± 1°C, 16L:8D, 65 ± 5% relative humidity. At the time of injection, donor and recipient females were in the second to third hour of the scotophase that followed their first night of calling. Females anesthetized with CO_2 were neck-ligated in the middle of the preceding photophase with the use of cotton thread. Brain-SOG homogenates were prepared as described in (*8*). Recipient females for the photophase treatment were in the second hour of the photophase at the time of injection. The titer of the main pheromone component (Z11-16:Ac) was determined by gas-liquid chromatography of a gland extract in hexane (performed 3 hours after injection of brain-SOG homogenate), with methyl heptadecanoate used as an internal standard [see (*19*) for details on the temperature program and column used].
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12. Rearing conditions were the same as in (*9*). Only females emerging during the photophase were used. Allatectomies were performed 1 hour after eclosion. The scales covering the head capsule and the cervical area were brushed off and the insect immobilized in plasticine at the bottom of a dish supplied with a continuous flow of CO_2. With sharp forceps and micro-scissors, a small opening was made in the head capsule near the neck membrane, and the CA was gently excised under a microscope. A few crystals of streptomycin were applied to the wound before replacing the cuticule. Sham-operated females underwent the same treatment, but the CA were left untouched. A lamp covered with Kodak Wratten filter (No. 29) was used for observations during the scotophase at 10-min intervals. To assess ovarian development, we measured the width of the penultimate basal oocyte in each ovariole of the right ovary with an ocular micrometer. Pheromone titer analysis was performed as described in (*9*).
13. One-day-old allatectomized females were injected between the fourth and fifth abdominal segment with either 5 μl of light mineral oil (Atlas), or 5 μl of light mineral oil containing 10 μg of JH II (Sigma). This dosage may appear too high (non-physiological), but the kinetics of JH release from oil carriers are such that it should result in a relatively low JH titer [L. M. Riddiford and A. M. Ajami, *J. Insect Physiol.* **19**, 749 (1973)]. We chose the C_{17}JH (JH II) since HPLC analysis indicated that it is the main JH produced by *P. unipuncta* females.
14. Larvae were immobilized with CO_2 and anchored in plasticine on the bottom of a dish, with metal restrainers. With micro-scissors, a small slit was made on the dorsum of the animal to expose the yellowish ovaries which were excised with fine forceps (or left intact in sham-operated controls). Streptomycin was applied to the wound which was then sealed with Super Glue.
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16. Allatectomy was performed as described in (*12*) and allatectomized females were injected with brain-SOG homogenates from donor females similar to those described in (*9*), or saline 3 to 4 days later.
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A Novel Vasodilatory Peptide from the Salivary Glands of the Sand Fly *Lutzomyia longipalpis*

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Salivary gland lysates of the sand fly *Lutzomyia longipalpis* contain a potent vasodilator that aids the fly to feed on the blood of its vertebrate hosts. Chromatographic analysis, antibody reactivity, and data obtained from bioassays of the salivary erythema-inducing factor indicate striking similarity with human calcitonin gene-related peptide. The erythema-inducing factor is, however, at least one order of magnitude more potent than calcitonin gene-related peptide.

IN THEIR QUEST FOR BLOOD, HEMATOphagous arthropods introduce into the skin of their hosts an array of salivary compounds that facilitate blood feeding by blocking the hemostatic reactions or by altering hemodynamics (*1*). Salivary gland homogenates of the sand fly *Lutzomyia longipalpis* contain trypsin-sensitive erythema-inducing factor (EIF) (*2*). As little as 10 ng of protein from a crude salivary lysate produced an erythema in rabbits (*2*). The EIF was differentiated from other known erythema-inducing substances such as histamine, serotonin, bradykinin, substance P, or prostaglandins on the basis of its sensitivity to trypsin, kinetics of erythema development, and absence of edema and itching. The neuropeptide CGRP (calcitonin gene-related peptide) has been characterized as the most potent and persistent vasodilator known. When injected into human skin it produces a delayed, long-lasting, erythema with a surrounding pallor (*3, 4*). In vitro, CGRP relaxes various isolated arterial preparations contracted by adrenergic agonists (*3, 5*). We investigated the extent to which the properties of EIF are similar to those described for CGRP.

We first investigated the nature of the response of human skin to the bite of a sand fly. As little as 30 s after a sand fly probed human skin, an erythema formed, reaching a maximum area at 60 min after the bite (Fig. 1). Between 30 min and 1 hour after the bite, the erythema develops pseudopod-type figures. A surrounding pallor, which contrasted with the sharp boundary of the erythema, was also observed (Fig. 1). In all four human beings tested, the erythema was still visible 24 hours after the bite, without any edema or itching.

To compare the potency of EIF with that of synthetic CGRP in inducing erythema, we injected serial dilutions of either salivary gland lysates or human α-CGRP intradermally into the back of a rabbit, starting with 100 ng of crude salivary gland protein (about 10% of one pair of glands) (*2*) or 100 ng of CGRP per injection site. The minimum dilution of the crude homogenate

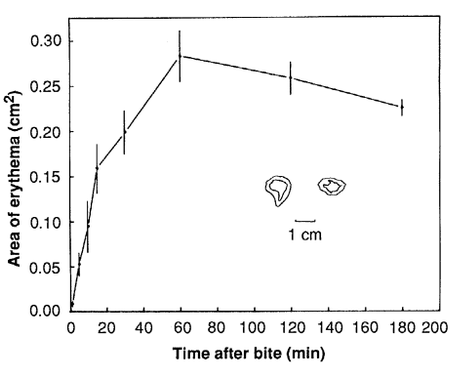

Fig. 1. Time course of the erythema induced by the bite of adult female *L. longipalpis* sand flies on humans. Flies were allowed to probe the forearm skin for 30 s. Symbols and bars represent the average and SE of four experiments. Inset: Typical contours of the erythema (inner contour) and associate pallor (outer contour) displayed by two humans 1 hour after being bitten for 30 s.

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