Endogenous suppression of pheromone production in virgin female moths

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Summary. Sexual receptivity generally is reduced in moths after mating. We found that even in virgin *Heliothis zea* females the titer of pheromone declines after the third night of adult life, although the number of eggs laid increases. Reduction in pheromone titer is not due to reduced amounts of 'pheromone biosynthesis activating neuropeptide'. We have discovered that a substance present in the bursa, ovaries and hemolymph of senescing virgins suppresses pheromone production. A similar factor was found in 2-day-old mated females indicating that both virgin and mated females use this factor to suppress pheromone production.
Key words. Sex pheromone; pheromone biosynthesis activating neuropeptide; bursa copulatrix; *Heliothis zea.*

Insects are extraordinarily successful organisms not only because of their ability to adapt to diverse environmental conditions, but also because they have optimized their mating strategies. This efficiency of mating is usually accomplished by sexual signalling using pheromones only during peak reproductive periods. Sexual receptivity is reduced at other times, particularly after mating has occurred [1]. We have discovered a new endogenous factor in senescing virgin females of a moth, *Heliothis zea*, which suppresses pheromone production and thus reduces the likelihood of engaging in futile mating. This same factor, which may also suppress pheromone production in younger mated females, very probably contributes to the overall mating success of the species.

In flies, reduced sexual receptivity after mating is caused by transfer of a substance from the male to the female [2]. After fertile mating in the moth, *Hyalophora cecropia*, an elevated rate of oviposition and cessation of pheromone release behavior is triggered by a female produced factor [3]. We have observed that, unlike females of *H. cecropia*, virgin females of *Heliothis zea* begin to oviposit at a significant rate after the 3rd day of adult life, and produce smaller amounts of sex pheromone than young adults. We have discovered that a substance present in the bursa copulatrix, the ovaries and the hemolymph of older virgins of *H. zea* counteracts the effect of 'pheromone biosynthesis activating neuropeptide' (PBAN)[4], thus suppressing pheromone production. This is the first documentation of endogenous suppression of sex pheromone production by virgin females of a moth species.

Materials and methods

Insects used in all experiments were obtained from a laboratory colony [5, 6]. PBAN was prepared from homogenates of the brain subesophageal ganglion complex as described elsewhere [5, 6]. All experiments were conducted during the light period when pheromone titer is very low [5, 7] with incubations lasting 1 h after injection of PBAN. Insects were injected with test substances during the 3rd photophase unless stated otherwise. Excision and extraction of the pheromone gland, and analysis of the extracts by capillary gas chromatography on 30 m × 0.25 mm (i.d.) SPB1 and Supelcowax 10 (Supelco, Inc.) fused silica capillary columns as well as by chemical ionization mass spectroscopy was conducted as described elsewhere [5, 6] to determine the amount of pheromone produced.

The bursa copulatrix or other tissues were dissected out of 5-day-old females and, unless stated otherwise, washed for 3 min in each of 4 vials containing 5 ml of 0.1 M phosphate buffer (pH 6.8) containing 0.35 M sucrose. The tissue was then dried (ca 1 min) on filter paper and homogenized in sucrose phosphate buffer using a Wheaton 1-ml homogenizer. The homogenate was centrifuged at 1000 × g for 10 min and the supernatant was removed and lyophilized using a Savant Speed Vac concentrator. The material was reconstituted of 15 µl/female equivalent (FE) in water and injected, along with 10 µl (1 FE) of PBAN solution. In some experiments tissue was homogenized in 15 µl of buffer/FE without washing. In other experiments the aqueous tissue homogenate was extracted 2 times with equal volumes of either pentane, hexane, or iso-octane to remove lipids like juvenile hormone that might be present. Then the aqueous phase of these samples was concentrated as above and the organic phase was concentrated to dryness under a stream of N_2 and dissolved in sufficient acetone to yield a 0.5% acetone/buffer solution after addition of 15 µl of buffer/FE.

Hemolymph was obtained by first inserting the needle of a 50-µl syringe through the thorax into the abdomen of 5-day-old females and injecting 40 µl of cold 0.1 M phosphate buffer (pH 6.8). After 10 min the buffer-hemolymph solution (ca 95% recovery of the buffer injected) was drawn off using a 50-µl syringe [8]. Hemolymph was frozen in a vial surrounded by solid CO_2. Frozen material was lyophilized and reconstituted to 1 FE of hemolymph per 5 µl of buffer.

Preliminary studies indicated that the suppression factor is not retained on a C-18 solid phase extraction cartridge preconditioned with 5 ml of acetonitrile followed by 5 ml of water containing 0.1% trifluoroacetic acid (TFA). PBAN is retained on the preconditioned C-18 cartridge

and is eluted with 80% acetonitrile in 0.1% TFA. PBAN (10 FE) was mixed with 20 FE of the extract of the bursa and incubated for 30 min at 25 °C. The mixture was applied to a preconditioned C-18 cartridge and washed with 3 ml of 0.1% TFA then eluted with 80% acetonitrile in 0.1% TFA. The void volume plus 0.1% TFA eluants were combined and concentrated. The 80% acetonitrile fraction was concentrated. Samples were reconstituted to 1 FE/5 µl in water. The fraction containing the suppression factor (2 FE) was injected into 3-day-old females immediately before injection of 1 FE of fresh PBAN. The fraction containing PBAN was tested alone.

Results and discussion

The production of sex pheromone by females of *H. zea* is regulated by a circadian rhythm with the peak period of production being between the 3rd and 5th h of the scotophase [7]. Little or no pheromone is present during the photophase [5, 7]. This circadian rhythmicity of pheromone production is regulated by the release and action of PBAN on the terminal abdominal ganglion, which then induces the cells of the pheromone gland to synthesize pheromone [6]. However, females of *H. zea* can be induced to produce pheromone at any time by injection of PBAN. In fact, injection of PBAN into females during the third photophase, when the pheromone titer is normally less than 1.5 ng, resulted in production of the same amount of pheromone after 1 h as was present in the glands of females sampled at the peak of pheromone production during the 3rd scotophase [5]. Under natural conditions age also has an effect on the titer of pheromone produced by virgins who produce the greatest amount of pheromone during the 3rd scotophase [7]. On the 4th and subsequent nights virgins produced significantly less pheromone [7]. Given that the amount of pheromone produced after injection of PBAN increases with increasing doses of the neuropeptide [4], we hypothesized that the reduction in pheromone titer associated with increasing age was due to the production of smaller amounts of PBAN by older virgins.

We tested our hypothesis by injecting 3-day-old females during the photophase when little or no pheromone was present with PBAN obtained from donor insects which were 5 days old. These insects produced as much (Z)-11-hexadecenal (Z11-16:AL), the major component of the pheromone [55.3 ng (± 13.0 SD, n = 5)] as was produced [53.6 ng (± 16.4 SD, n = 5)] when females were injected with PBAN from 3-day-old moths. Extracts obtained from glands of females injected with only buffer (control insects) contained an average of 1.1 ng (± 0.6 ng, SD) of Z11-16:AL. When we injected PBAN obtained from 3-day-old adult females into 5-day-old females they produced only 17.0 ng of Z11-16:AL (± 7.8, SD, n = 5). Therefore, although PBAN obtained from both groups of insects was equally active, 5-day-old females were unable to produce as much pheromone as females that were at their reproductive peak. From this we concluded that PBAN was not the limiting factor in pheromone production by older virgin females.

The reduction in titers of pheromone associated with senescence could not be associated with changes in female physiology related to maturation of eggs because fully mature, chorionated eggs are present on the second day of adult life [9]. This is 24 h before the peak age of pheromone production. However, knowing that the bursa copulatrix of *H. cecropia* releases a substance which causes a shift from 'virgin' to 'mated' behavior [3] and that secretory cells are present in the bursa copulatrix of *H. zea* [9] we hypothesized that some factor, responsible for suppressing pheromone production, might be present in the bursa. We demonstrated that this was the case, because when we injected 2 FE of the homogenate of the bursa obtained from 5-day-old females along with PBAN into 3-day-old females, the amount of pheromone present in the gland 1 h later was significantly lower than that present in glands of females from the same cohort injected with PBAN alone (table). Injection of 2 FE of the homogenate of either the cervix bursae or corpus bursae [9] along with PBAN also resulted in significant reductions in the titer of pheromone (table). No decrease in pheromone titer was noted when extracts of the bursa obtained from females during the 1st photophase after adult emergence were tested (data not shown). But, insects injected with 1 FE of PBAN plus 2 FE of the bursa extract obtained from females during the 3rd photophase produced less than 50% of the amount of pheromone produced by females which had been injected with PBAN alone. Therefore, the titer of the inhibitory factor present in the bursa increases with increasing age.

To determine if the effect was a specific response to an endogenous factor, rather than just a nonspecific response to the injection of tissue extracts, we injected homogenates of washed thoracic muscle, abdominal fat body, or ovaries. Although neither homogenates of the washed fat body nor washed thoracic muscle suppressed the pheromonotropic effect of PBAN, injection of homogenates of the washed ovary resulted in a significant reduction in pheromone (table). Thus, the suppressive

Effect of injection of PBAN plus 2 FE of tissue extracts obtained from 5-day-old virgin females on pheromone production in 3-day-old virgin females of *H. zea* *

Treatment	Pheromone	
1 FE PBAN + 20 µl buffer (n = 10)	100.0%	A
1 FE PBAN + 2 FE unwashed bursa copulatrix (n = 10)	2.9%	E
1 FE PBAN + 2 FE washed bursa copulatrix (n = 5)	5.1%	E
1 FE PBAN + 2 FE corpus bursae (n = 5)	32.9%	BC
1 FE PBAN + 2 FE cervix bursae (n = 5)	57.0%	B
1 FE PBAN + 2 FE washed ovary (n = 5)	12.4%	D
1 FE PBAN + 2 FE washed fat body (n = 5)	91.3%	A
1 FE PBAN + 2 FE washed thoracic muscle (n = 5)	104.7%	A
1 FE PBAN + 2 FE unwashed thoracic muscle (n = 5)	32.5%	BC
1 FE PBAN + 2 FE hemolymph (n = 5)	25.3%	C

* Pheromone production as indicated by the titer of Z11-16:AL in gland extracts. Percentages followed by the same letter are not significantly different as indicated by a Duncan's multiple range test at p = 0.05 using the amount (ng) of Z11-16:AL present in extracts.

effect is tissue-specific, being associated with the bursa and ovaries, two closely associated and interconnected organs. Although homogenates of the washed thoracic muscle did not contain the suppression factor, injection of PBAN plus homogenates of the thoracic tissue prepared without prewashing resulted in the production of significantly less pheromone than that produced by insects treated with PBAN alone (table). This suggested that the suppression factor was present in the hemolymph of older females. When hemolymph obtained from older females was injected along with PBAN a decrease in pheromone production was noted but the reduction was not as great as that resulting from injection of extracts of the bursa. This may be accounted for by the relatively small volume of hemolymph obtained from these insects relative to the total blood-volume. Nonetheless, hemolymph does contain the suppression factor.

Knowing that juvenile hormone (JH) affects pheromone production in several species of insects [1, 10] and that topical application of a juvenile hormone analog caused females of the omnivorous leafroller moth to oviposit while inhibiting both calling behavior and pheromone production [11], we hypothesized that juvenile hormone might be the factor responsible for suppressing pheromone biosynthesis. However, extraction of the homogenates of the bursa with pentane, hexane, or iso-octane, solvents known to extract juvenile hormones from aqueous homogenates [12, 13], did not alter the suppressive activity of the aqueous phase nor was any activity associated with the lipids extracted into the organic solvents. Gas chromatographic analysis of the lipids extracted by the organic solvents did not reveal the presence of JH I, II, or III. Therefore, the substance present in the bursa copulatrix and ovaries is not one of these juvenile hormones. We also tested the effect of injection of ecdysteroids on pheromone biosynthesis because ecdysone is known to regulate pheromone biosynthesis in houseflies [14]. Experiments in which dosages from 10 to 400 ng of either ecdysone or 20-hydroxy ecdysone mixed with 1 FE of PBAN were injected into 3-day-old females did not result in the production of less pheromone than that produced by control insects injected with PBAN alone. Thus, the suppression factor is not one of the ecdysteroid hormones tested.

We found that the effect of the suppression factor increases in a linear fashion with increasing doses of extract of the bursa (fig. 1). Complete suppression of pheromone production resulted when 2 FE of the extract were administered along with PBAN. Also, we have found that injection of this extract both 30 min and 60 min prior to injection of PBAN completely suppressed pheromone production (fig. 2). The glands of females injected with the extract of the bursa 30 min after injection of PBAN but extracted after an additional 30 min contained an average of 25.1 ± 6.8 ng (n = 5) of Z11-16:AL. Similarly, the glands of females injected with PBAN and extracted after 30 min contained an average of 21.6 ± 5.6 ng

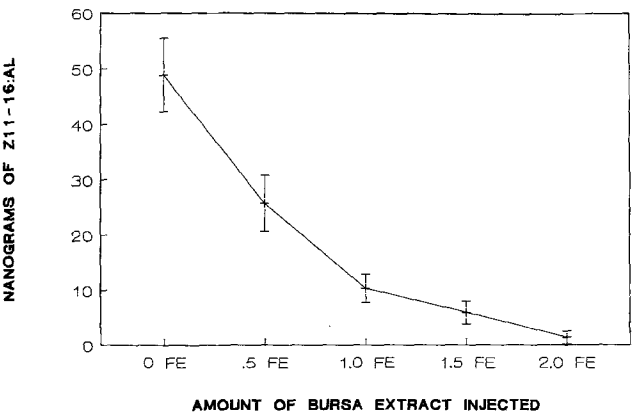

Figure 1. Amount of Z11-16:AL produced 1 h after injection of different concentrations of the extract of the bursa copulatrix obtained from 5-day-old females together with 1 FE of PBAN obtained from 3-day-old females (n = 10, each concentration, ± SD).

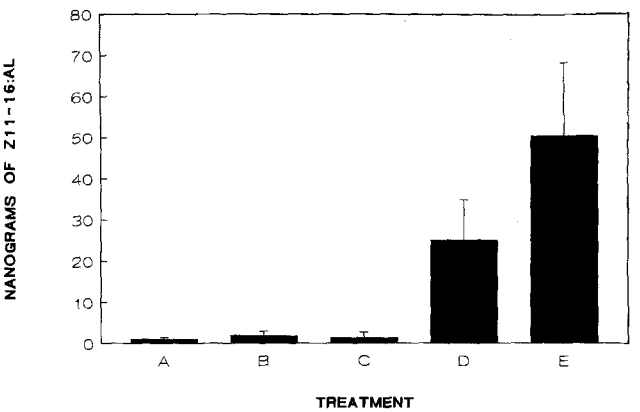

Figure 2. Effect on production of Z11-16:AL of injection of 2 FE of the extract of the bursa copulatrix obtained from 5-day-old females before, at the same time as, or after injection of 1 FE of PBAN obtained from 3-day-old females compared to females injected with PBAN alone. Glands were extracted 60 min after injection of PBAN (n = 5, each treatment, ± SD). *A* Injection of bursa extract 60 min prior to PBAN, *B* injection of bursa extract 30 min prior to PBAN, *C* injection of bursa extract at the same time as PBAN (mixed together), *D* injection of bursa extract 30 min after PBAN, *E* injection of PBAN alone.

(n = 5). This indicated that the suppression factor does not cause increased metabolism of pheromone already present in the gland.

When the extract of the bursa was incubated for 30 min with PBAN and subsequently the PBAN was separated from the suppression factor using a C-18 solid phase extraction column, females injected with treated PBAN produced an average of 31.0 ng (± 7.56, n = 5) of Z11-16:AL. Control insects treated with buffer produced 0.7 ng (± 0.5) and insects treated with PBAN incubated with only buffer prior to elution from the C-18 column produced 32.9 ng (± 5.83, n = 5) of Z11-16:AL. When the suppression factor from the C18 column was injected along with fresh PBAN 2.1 ng (± 0.6, n = 5) of Z11-16:AL was produced. Controls treated with fresh PBAN plus the unaltered bursa extract produced 2.9 ng (± 0.7,

n = 5). Consequently, it appears that the factor present in the bursa copulatrix acts to suppress the action of PBAN very rapidly but does not alter the activity of PBAN.

It has been reported that the titer of pheromone drops significantly during mating due to the transfer of a male-produced factor but that the action of this 'male factor' is short-lived and can be overcome by restimulation with PBAN[15]. Nonetheless, mated females produce less pheromone than their virgin counterparts for several days after mating[7]. We have found that injection of PBAN plus the extract of the bursa of young females (3rd photophase) sampled on the day after mating resulted in the production of only 3.1 ng (± 0.9, n = 5) of Z11–16:AL whereas 34.0 ng (± 3.8, n = 5) of Z11–16:AL were produced when extracts of the bursa obtained from virgin females of the same age plus PBAN were injected. Therefore, we believe that under natural field conditions, production and release of this factor we have termed 'pheromone biosynthesis suppression factor' (PBSF), are stimulated by mating. Such release of PBSF may result in the maintenance of the much reduced pheromone titer present in the glands of mated females for several days, thereby rendering them unattractive to males. Endogenous regulation of this type is advantageous to moth species because it reduces competition between mated and virgin females, thus increasing the probability that virgins will mate and thereby increasing genetic diversity. The refractory period after mating, when pheromone titer is very low, would allow females to lay fertilized eggs without threat of being accosted by sexually aroused males. Furthermore, the inhibition of pheromone production among senescing females, be they virgin or mated, would be advantageous to males because males would not be attracted to and would not waste reproductive effort and energy on females likely to die before laying fertilized eggs.

Although data reported here are for *H. zea*, we have also found that this factor suppresses pheromone production in several other noctuid moth species including *H. subflexa*, *H. virescens*, and *Spodoptera frugiperda*. Additionally, we have evidence that a similar substance is produced by females of a sphyngid moth, the tobacco hornworm, thus suggesting that the factor is produced by a large number of moths from several families.

1 Barth, R. H., and Lester, L. J., A. Rev. Entomol. *18* (1973) 445.
2 Leopold, R. A., Terranova, A. C., and Swilley, E. M., J. exp. Zool. *176* (1971) 353.
3 Riddiford, L. M., and Ashenhurst, J. B., Biol. Bull. *144* (1973) 162.
4 Raina, A. K., Jaffe, H., Klun, J. A., Ridgeway, R. L., and Hayes, D. K., J. Insect Physiol. *33* (1987) 809.
5 Teal, P. E. A., and Tumlinson, J. H., Can. Ent. *121* (1989) 43.
6 Teal, P. E. A., Tumlinson, J. H., and Oberlander, H. O., Proc. natl Acad. Sci. *86* (1989) 2488.
7 Raina, A. K., Klun, J. A., and Stadelbacher, E. A., Ann ent. Soc. Am. *79* (1986) 128.
8 Raina, A. K., and Klun, J. A., Science *225* (1984) 531.
9 Callahan, P. S., Ann. ent. Soc. Am. *51* (1958) 413.
10 Cusson, M., and McNeil, J. N., Science *243* (1989) 210.
11 Webster, R. P., and Cardé, R. T., J. Insect Physiol. *30* (1984) 113.
12 Schooley, D., in: Analytical Biochemistry of Insects, p. 241. Ed. R. B. Turner. Elsevier, New York.
13 Feyereisen, R., and Tobe, S., Analyt. Biochem. *111* (1981) 372.
14 Adams, T. S., Dillwith, J. W., and Blomquist, G. J., J. Insect Physiol. *30* (1984) 287.
15 Raina, A. K. J. chem. Ecol. *14* (1988) 2063.

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Effect of recombinant human granulocyte colony-stimulating factor on human neutrophil adherence in vitro

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Summary. We measured the effects of recombinant human granulocyte-colony stimulating factor (rhG-CSF) on the adherence of human neutrophils by using a dacron fiber system to assay the adhesive ability of neutrophils. rhG-CSF enhanced neutrophil adherence to dacron fibers. N-formyl-methionyl-leucyl-phenylalanine (fMLP) induced neutrophil-neutrophil interaction (neutrophil aggregation) in addition to neutrophil-dacron interaction, whereas rhG-CSF did not cause neutrophil aggregation. These results indicated that rhG-CSF increases the adhesive ability of neutrophils without neutrophil-neutrophil interaction, and the action of rhG-CSF in neutrophil activation is different from the neutrophil activation caused by fMLP.
Key words. Recombinant human G-CSF; neutrophil; adherence; dacron.

G-CSF stimulates the growth of progenitor cells of neutrophils[1,2]. Human G-CSF was isolated from the human bladder carcinoma cell line 5637[3], and was cloned and expressed in *E. coli*[4]. Recombinant human G-CSF (rhG-CSF) was shown to increase the peripheral blood neutrophil count in humans[5,6]. G-CSF binds to specific