The loss of female sex pheromone after mating in the corn earworm moth *Helicoverpa zea*: Identification of a male pheromonostatic peptide

(insect/mating behavior/male seminal fluid)

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ABSTRACT   Female moths often become depleted of sex pheromone after mating as the various components of virgin behavior are switched off. In examining a potential male contribution to these events in the corn earworm moth *Helicoverpa zea*, we have characterized a basic polypeptide from the tissues producing (accessory glands) and storing (duplex) the seminal fluids. The peptide evokes the depletion of sex pheromone when injected into virgin females. This pheromonostatic peptide (PSP) is 57 amino acids long and contains a single disulfide bridge. It is blocked at the N terminus with pyroglutamate and at the C terminus by amidation. As little as 23 ng of peptide evokes the near-complete depletion of pheromone in decapitated (neck-ligated) females that had been injected with pheromone biosynthesis-activating neuropeptide. Activity is ≈15-fold less in intact virgins, showing that the head limits the expression of activity in these injected females. Females mated to surgically impaired males, capable of producing a spermatophore but not transferring spermatozoa or seminal fluids, are depleted of pheromone by injected peptide. Females whose abdominal nerve cords have been severed are not depleted of pheromone after mating. Thus, neural signals either descending or ascending via the nerve cord are required for the depletion of pheromone after mating. PSP, from the seminal fluids, may participate in this process by direct or indirect action on the glandular tissue; if so, it represents an unusual mechanism in insects for the regulation by seminal fluids of postmating reproductive behavior.

Mating in most species of insects causes a transient or permanent switch in the reproductive physiology and behavior of females. In moths, for instance, females often begin ovipositing within hours of mating, and the total number of mature eggs produced in the female's lifetime may be increased. In addition, behaviors associated with the attraction of males may be turned off, or deterrent behaviors may be activated, thus ensuring that she is not distracted by new suitors when oviposition begins. While these changes associated with mating have been described in a number of groups of insects, including the moths, few studies have sought to determine the mechanisms by which they are evoked. In principle, the individual components of the mated state could be evoked by a single aspect of mating; however, the accumulated body of evidence shows that control of these components is independent but parallel. In the moths, as in other groups of insects, the presence of viable spermatozoa or testicular secretions in the spermatheca have been implicated in the oogenic response to mating (1, 2); depletion of sex pheromone in gypsy moths after

mating may be similarly mediated (3). In other moths or butterflies, inflation of the bursa evoked by a normal spermatophore is required for oogenesis (4) and the mate refusal posture (MRP) typical after mating (5). Because the spermatheca (3) and bursa (4–6) of female moths receive sensory innervation, it seems likely that neural mechanisms participate in some aspects of the switch to the mated state, as has recently been shown for the pheromonostatic response to mating in the lightbrown apple moth (7) and the redbanded leaf roller (8).

The possibility that humoral mechanisms also participate in evoking the mated state has been advanced by the demonstration that the hemolymph of the mated female is able to evoke MRP in the cabbage white butterfly (9) and caused the depletion of pheromone in the corn earworm moth (10). If humoral mechanisms operate, they could be indirect as well as direct; the former was shown by the observation that empty bursa copulatrices from mated cecropia moths, when implanted into virgins, elicited the mated pattern of oviposition (11). If direct mechanisms operate, then seminal fluid transferred with spermatozoa may serve as a source of chemical messengers.

As in many species of moths, the mated state in the corn earworm moth *Helicoverpa zea* (Boddie) is characterized by a depletion of pheromone, cessation of "calling" or pheromone-releasing behavior, and loss of sexual receptivity (10, 12). The transient (24 h) depletion of pheromone does not depend on transfer of spermatozoa or testicular secretions (10), and a spermatophore *per se* is not sufficient (12); rather, the seminal fluids normally contained in the spermatophore must also be transferred (12). Thus, we wanted to know whether pheromonostasis could be associated with a specific component of the seminal fluid; furthermore, we sought to determine whether neural mechanisms could participate in the requirement for the seminal fluids.

MATERIALS AND METHODS

Insects. *H. zea* were reared on an artificial diet in the laboratory under a reversed 16 h/8 h (light/dark) photoperiod as described (12). Tissues producing and storing the seminal fluids in males, the accessory glands and duplexes, were removed and stored as described (12). Insects are d1 (day 1) on the day of emergence. Females for mating were d2; males and females were operated on during the photophase of d1.

Bioassays. The ability of fractionated extracts and purified pheromonostatic peptides (PSPs) to cause the depletion of sex

Abbreviations: CAD MS, collision-activated dissociation mass spectrometry; ESMS, electrospray mass spectrometry; RPLC, reverse-phase high-performance liquid chromatography; PBAN, pheromone biosynthesis-activating neuropeptide; PSP, pheromonostatic peptide; RG, radical gonadectomy; VNC, ventral nerve cord.
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pheromone was assessed in neck-ligated females (12). Females received injections of (*i*) 1–2 pmol of pheromone biosynthesis-activating neuropeptide (PBAN) and, 1 h later, (*ii*) fractionated extracts or peptides. The activity of purified peptides was also assessed in intact scotophase females. In both assays, the tip of the ovipositor, containing the glandular tissue that produces pheromone, was extracted with heptane 2 h after injection of PSPs. The principal component of the pheromone blend, (*Z*)-11-hexadecenal, was quantified by gas chromatography (13).

Surgical Procedures. Radically gonadectomized (RG) male moths were produced by the removal of the fused testes, accessory glands, and duplex early in the photophase as described (12). RG males were mated in the following scotophase. For ventral nerve cord (VNC) transection, females were anesthetized with CO_2 and then immersed under cold saline (14). The VNC was severed anterior to the 5th abdominal ganglion through a transverse ventral incision, and the wound was then sealed with beeswax. Sham-operated females were similarly prepared, and the VNC was touched with forceps but not cut.

Purifications. Combined accessory glands and duplexes were extracted in batches from 400–800 insects and fractionated by batchwise ion-exchange chromatography and by one reverse-phase HPLC (RPLC) step as described (12). Four adjacent active fractions were pooled and fractionated by cation-exchange HPLC (SP-5-PW; Bio-Rad) in 20 mM sodium phosphate/20% CH_3CN, pH 3.0, with a gradient of NaCl (increasing from 0 at the rate of 10 mM/min) and by RPLC (RP-300, a 4.6-mm-diameter C_8 column; Applied Biosystems) with a gradient of CH_3CN (increasing from 10% at 0.67%/min) in 50 mM sodium phosphate (pH 6.0) (15). Two active components were separated in the latter procedure, and these

were then fractionated separately by RPLC (RP-300, a 2.1-mm-diameter column otherwise identical to that described above) with a gradient of CH_3CN in 0.1% trifluoroacetic acid. Protein in purified peptides was quantified with BCA protein assay reagent (Pierce).

Sequence Determination. The early-eluting peak of activity (Fig. 1) was fragmented with trypsin in either 50 mM NH_4-HCO_3 (pH 8.0) or 100 mM Tris·HCl (pH 8.3) at 37°C for 4–12 h. The mixture was treated with dithiothreitol and cysteines were carboxymethylated; the mixture was then analyzed directly by electrospray ionization tandem mass spectrometry (ESMS; Finnigan TSQ-70, Finnigan-MAT, San Jose, CA), with samples introduced to the ionization source by microcapillary RPLC (16). Some fragments produced by trypsin digestion were analyzed directly by collision-activated dissociation mass spectrometry (CAD MS), while others were first isolated by RPLC and then subdigested with endoproteinase Asp-N in 50 mM NH_4HCO_3 (pH 8.0) at 37°C for 0.25 to 2 h. These Asp-N fragments were then analyzed by CAD MS, automated Edman degradation (Applied Biosystems, model 473A pulsed liquid protein sequencer), or amino acid analysis (16).

RESULTS

Purification of PSPs. Partial purification of extracts of accessory glands and duplexes of male *H. zea* yielded adjacent RPLC fractions capable of evoking 95–98% depletion of pheromone at 0.05 equivalent (12). Two PSPs were purified from this material to apparent homogeneity by ion-exchange chromatography and two additional RPLC protocols (Fig. 1; see legend for explanation of activity in the 1 min trailing the UV-absorbing peak in Fig. 1 *Lower*).
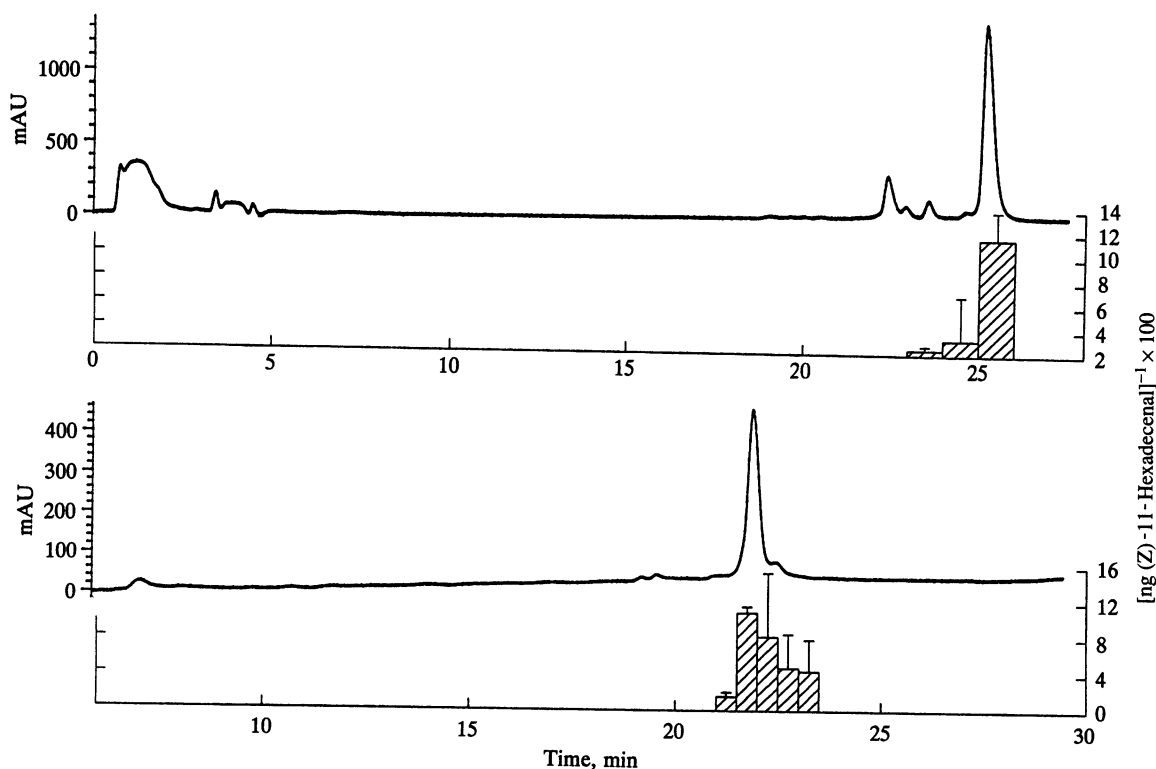

FIG. 1. Final RPLC step in the purification of PSPs. Active material was chromatographed in a reverse-phase column with a gradient of acetonitrile in 0.1% trifluoroacetic acid; either 1.0-min (*Upper*) or 0.5-min (*Lower*) fractions were collected. Neck-ligated and PBAN-injected females were injected with 1 equivalent from each fraction. Each value is the average ± SD of three or four determinations. We have found by immunoassay of fractions from RPLC of MasFLRFamide that the UV trace may not accurately reflect low levels of peptides in fractions trailing the absorbing peak; thus, activity in fractions 23.0 and 23.5 (*Lower*) probably reflects this property of our detector as well as the extreme sensitivity of neck-ligated females to PSP injections. The earlier-eluting of the two peptides (*Lower*) was subjected to amino acid sequencing. mAU, absorbance units × 10^{-3}.

Sequencing of PSP-1. Initial attempts to sequence the peptides by automated Edman degradation yielded no signal in the first cycle, indicating that the N termini were blocked. The earlier-eluting of the two PSPs (PSP-1) was then sequenced by a combination of mass spectrometry and automated Edman degradation of proteolytic fragments (ref. 16; ¶). Digestion with trypsin produced molecular ions [(M+H)$^+$] of 2611 (fragment f1), 616 (f2), 985 (f3), 1478 (f4), and 1011 (f5) Da. f4 and f5 were analyzed by CAD MS and the identities of leucine and isoleucine in f5 were determined by automated Edman degradation. Incomplete trypsin digestion of the intact peptide followed by reduction and carboxymethylation revealed that f4 and f5 were part of a single larger fragment containing a disulfide linkage. Methyl esterification and quadrupole Fourier transform MS (16, 17) with unit resolution established f5 as the amidated C-terminal peptide. f2 and f3 were sequenced by CAD MS after isolation by RPLC, and the leucines in f3 were determined by automated Edman degradation. Incomplete trypsin digestion and ESMS revealed that f1 and f2 were part of a larger peptide; because direct attempts to sequence f1 by automated Edman degradation showed the N terminus to be blocked, f1 was established as the N-terminal fragment, with f2 and f3 following.

To determine the sequence of f1, the fragment was first digested with endoproteinase Asp-N. This treatment produced (M+H)$^+$ values of 1675 (f6), 698 (f7), 1516 (f8), and 955 (f9) Da. Sequence analysis by CAD MS of these four peptides established f7 as a fragment of f6 and f9 as a fragment of f8. Automated Edman degradation of f9 established the existence of leucines. A 15-min digestion of the intact peptide with Asp-N followed by matrix-assisted laser desorption time-of-flight MS$^\parallel$ established f9 as being at the C terminus of f1. Thus, f7 is the N-terminal fragment of f1 and the intact peptide; CAD MS established the sequence of this fragment with the isoleucines being deduced by amino acid analysis. Position 6 of this fragment contained a 60:40 mixture of asparagine and aspartic acid, suggesting that this position may be sensitive to deamidation. Digestion of f1 with pyroglutamate aminopeptidase resulted in the loss of 111 Da (although with only 5% yield), confirming the assignment of the first amino acid. An overlap in the sequence of the end of f6 with the beginning of f8 establishes the order of Asp-N fragments in f1. The complete sequence of PSP-1 is shown in Fig. 2.

The later eluting of the two PSPs (Fig. 1) was shown by ESMS to have a molecular mass identical to PSP-1; moreover, cleavage with trypsin resulted in fragments with masses identical to those of PSP-1; as with PSP-1, it contains a disulfide bond, an N-terminal pyroglutamic acid, and a C-terminal amide (data not shown). We have not yet determined how the later-eluting peptide differs from PSP-1. PSP-1 has no homology with the *Drosophila melanogaster* "sex peptides" (18). A homology search in the data bases GenBank 83.0 and Swiss-Prot 28.0 revealed no matches with >21% identity spanning the length of PSP-1. If the overlap was limited to 15–26 amino acids, matches containing between 53% (15 amino acids) and 31% (26 amino acids) identity were revealed (data not shown).

Biological Activity. In the neck-ligated female bioassay, PSP-1 potently evoked the depletion of sex pheromone (Fig. 3). Here, 0.3 equivalent (uncorrected for recovery of peptide in the purification; 23 ng of protein; ≈3.5 pmol of PSP-1) was the minimum dose required for almost complete depletion of pheromone. This finding suggested to us that PSP-1 (together

¶Bodnar, W. M., Hunt, D. F., Shabanowitz, J., Michel, H. P. & Kingan, T. G., Proceedings of the 40th ASMS Conference on Mass Spectrometry and Allied Topics, May 31–June 5, 1992, Washington, DC, pp. 1803–1804.

$^\parallel$Arnoltt, D., Shabanowitz, J. & Hunt, D. F., Proceedings of the 40th ASMS Conference on Mass Spectrometry and Allied Topics, May 31–June 5, 1992, Washington, DC, pp. 328–329.


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|——————————— f1 ———————————|
           10              20
pEIINNNDYHDDHHGDQPTLLLR-
           D

|— f2 —|——— f3 ———|
              30
SQPERMRPLLLAR-

|———————— f4 ————————|——— f5 ———|
         40              50
DAFGGECPPSSFHKLKNWCHIVa
      |                   |
      S———————————————————S
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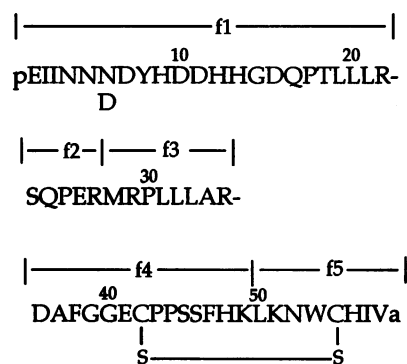
FIG. 2. Amino acid sequence of PSP-1. Sequence was determined by a combination of microcapillary RPLC/ESMS and automated Edman degradation of proteolytic fragments. Fragments shown were produced by trypsin digestion.

with the second PSP; Fig. 1) could account for the rapid depletion in pheromone typical of mated females (10). However, when PSP-1 was tested in intact females, activity was considerably lower, requiring 3 equivalents to achieve a 50% reduction in pheromone 2 h after injection (Fig. 4). Thus, it appears that injection of PSP-1 at this dose does not mimic the normal response to mating and that the head limits expression of activity in intact females.

If the transfer of PSP accounts for part of the normal postmating pheromonostasis, then some aspect of mating other than the transfer of seminal fluids is apparently required to reveal the full expression of this activity. We showed earlier (12) that females mated to RG males received a spermatophore (empty) but failed to become fully depleted of pheromone. It seemed possible, then, that any requirements in addition to the PSPs could be fulfilled by the empty spermatophore transferred by RG males. As a test of this idea, we injected saline or 1 equivalent of PSP-1 (an upper limit, since losses during the purification could not be accounted for) into RG-mated females immediately after separation of the mating
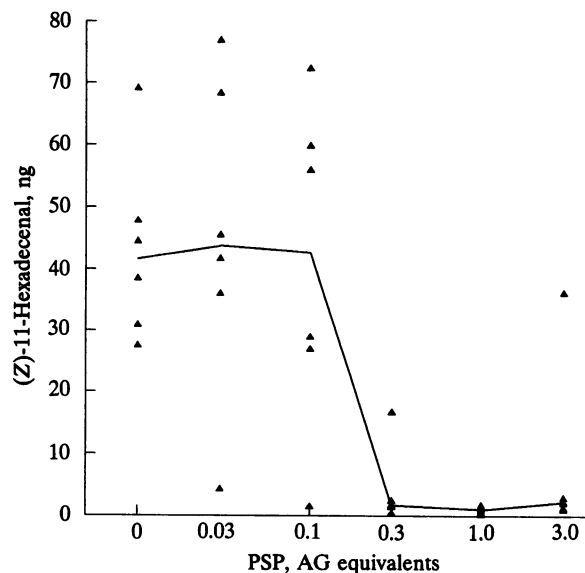

FIG. 3. Dose effectiveness of *H. zea* PSP-1 in neck-ligated females. The depletion of pheromone was measured in females that were neck-ligated 16 h earlier and injected with 1 pmol of PBAN 1 h before receiving an injection of PSP-1 in saline containing 10 μg of bovine serum albumin AG equivalent is the amount of PSP-1 recovered (no correction for losses in the purification) from the accessory glands and duplex of one insect. Individual values of six determinations at each dose (▲) are shown. Line represents median of these values.

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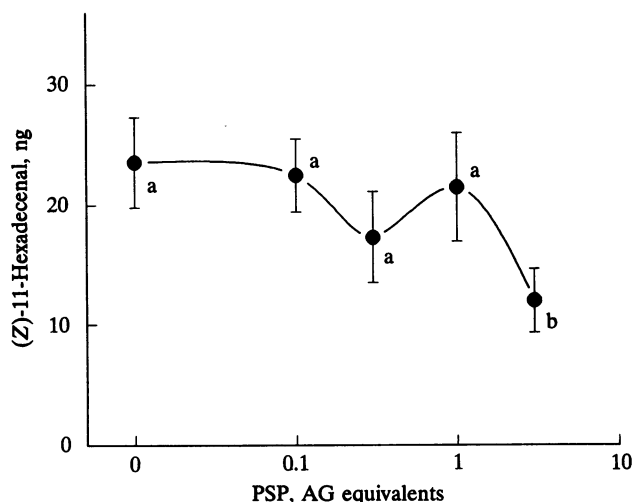
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FIG. 4. Dose effectiveness of *H. zea* PSP-1 in intact virgins. Females in the second scotophase after emergence (d2) were injected with various doses of PSP-1. Values shown are averages ± SEM ($n = 12$) for each dose. Values with same letter are not significantly different. At 3 accessory gland (AG) equivalents $P = 0.03$ (one-tailed Mann–Whitney U test).

pair. Two hours later, PSP-injected females were reduced in their pheromone with respect to saline-injected females (Fig. 5). Control values in this experiment were ≈2.4-fold lower than in our earlier study with similarly mated females that did not receive the injection (12); we have observed that the disturbance of handling and injection of saline can slightly lower pheromone levels in females. Therefore, while 1 equivalent of purified PSP-1 was apparently without effect in intact females (Fig. 4), it evokes ≈75% depletion of pheromone in RG-mated females.

We next wanted to know whether the action of mating could be mediated by a neural mechanism. The results showed that when the VNC is cut, females do not become depleted of pheromone after mating (Fig. 6). Thus, postmating pheromonostasis requires that an ascending or descending signal be transmitted via the central nervous system.

DISCUSSION

We have purified two PSPs from the seminal fluid-producing and storage tissues of the corn earworm moth. One of these
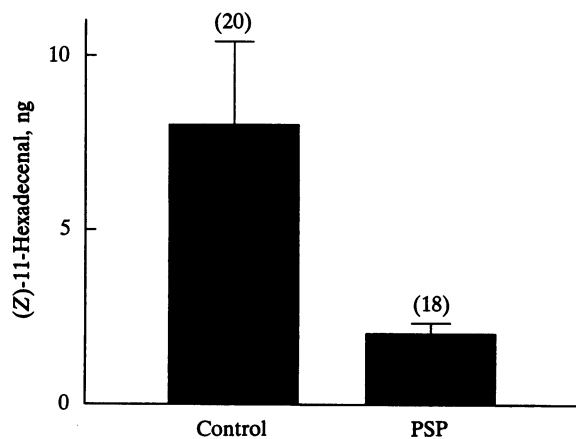

FIG. 5. Effectiveness of PSP-1 in RG-mated females. Females were mated on d2 with RG males and injected within 15 min after separation of the mating pair with saline alone or saline containing 1 equivalent of PSP-1. Values from the few females found not to have received a spermatophore were excluded from the calculations of average ± SD; *n* for each determination is shown in parentheses above the error bar.
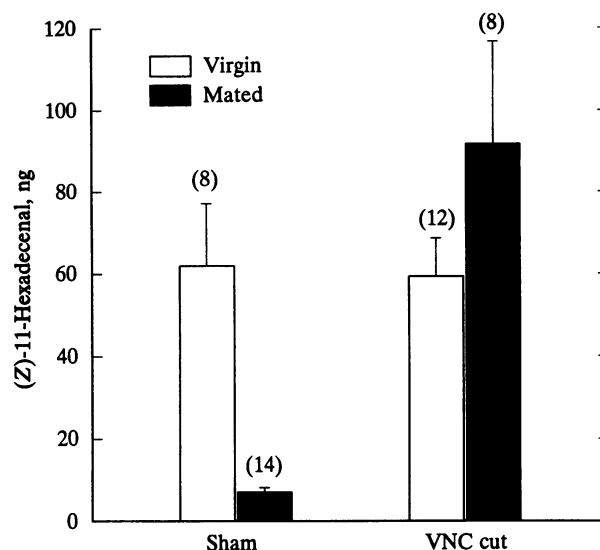

FIG. 6. Dependence of postmating pheromonostasis on intact VNC. *n* for each determination is shown in parentheses above the bar. Values shown are average ± SD.

peptides, PSP-1, has been fully characterized and shown to contain 57 amino acids and to be blocked at both N and C termini. The latter feature is characteristic of many neuropeptides and is thought to afford protection against amino- and carboxypeptidases. The second peptide, PSP-2, was isolated in amounts nearly equal to that of PSP-1. The PSPs have identical molecular weights and patterns of fragmentation with trypsin. How they differ has not been determined; in this regard, it may be important that PSP-1 was found to have a mixture of asparagine and aspartic acid in position 6. If PSP-1 is sensitive to deamidation at this position, it is possible that PSP-2 is the fully deamidated form, and that partial deamidation occurred in PSP-1 in handling during sequencing. The difference in their masses of 1 Da would not have been detected in our early determinations. The biological activity we describe for PSP-1 is unique among characterized polypeptides; thus, the significance of the limited homology of PSP-1 with sequences in the Swiss-Prot data base cannot be determined at this time.

We have attempted to address the mechanisms by which the PSPs could participate in postmating pheromonostasis. Our findings show that the normal depletion of pheromone in the mated female may be evoked by some physical and/or chemical aspect or component of the lower reproductive tract acting together with the seminal fluids. This component apparently requires an intact VNC for its action to be revealed. Recent studies with the lightbrown apple moth (7) and the redbanded leafroller (8) have shown that postmating pheromonostasis also does not occur in these species when the VNCs have been severed. The significance of these findings in moths may lie in events mediated by sensory innervation demonstrated in the bursa copulatrices of, for instance, *Manduca sexta* (4) and *Pieris rapae crucivora* (6). In *P. rapae* it appears that the presence of a spermatophore triggers activity in these proprioceptive neurons, causing the loss of sexual receptivity (5). Using methylene blue staining, we have identified a small number of neurons in *H. zea* at the base of the bursa copulatrix, near its juncture with the corpus bursa, that are similar in appearance to those of *P. rapae* (T.G.K., unpublished observation). In gypsy moths the signal for loss of attractiveness is apparently transmitted neurally from the spermathecal duct after the arrival of spermatozoa (3). It is possible, then, that mechanosensory events in those portions of the female's reproductive tract that contain the spermatophore and/or store spermatozoa evoke activity in ascending neurons of the VNC, signaling the female that she is mated. While this

suggestion is consistent with the results of the VNC transection experiment, a participation by descending signals is not excluded.

Mating may shut off the release of pheromonotropic peptides, either into the hemolymph or from presumptive release sites in the VNC (19). An intact VNC was previously reported to be essential for pheromone production in *H. zea* (20); we found, however, that there was no apparent diminution of the pheromone present in the VNC-transected virgins (Fig. 6). Thus, while the role of peripherally and centrally released PBAN in pheromone production is still uncertain, the transection experiment does show that ascending or descending neural signals are necessary for pheromonostasis. Whether these signals are also sufficient is not determined by this experiment. That the PSPs, together with neural signals, do play a role in normal pheromonostasis is supported by the finding that RG-mated females respond to 1 equivalent PSP-1 while intact females do not. Thus, the presence of a normally formed, albeit empty, spermatophore is permissive in revealing the action of PSP-1; a full pheromonostatic response is reconstituted in these experimental females. A more complete interpretation of the apparent synergism between the action of the spermatophore *per se* and the PSPs will be possible when the amount of these polypeptides that are transferred during a normal mating is determined.

The close temporal coincidence in the accumulation of pheromone with calling in virgin *H. zea* (21) and the finding that regulation of calling in the sphingid moth *M. sexta* (22) and accumulation of pheromonotropic peptides of *H. zea* (19) occurs in the fused brain/subesophageal ganglion raises the possibility that events leading to the initiation of these behaviors in virgins are coordinately regulated. After mating, secretions from the lower reproductive tract (likely to be from the ejaculatory duct; see ref. 23 for description of male reproductive tract), apart from the spermatophore itself, are sufficient to shut off calling while not causing the depletion of pheromone; thus, it appeared that the seminal fluids are not necessary in the cessation of calling (24). Moreover, while we found earlier that partially purified PSPs were effective in evoking the cessation of calling and sexual receptivity (12), we have recently found that 3 equivalents of PSP-1 have no effect on these behaviors (T.G.K. and A.K.R., unpublished observation). Together, these findings suggest that the components of virgin behavior may require somewhat different or incompletely overlapping sensory and/or chemical events for their cessation after mating.

While it is clear that temporary or permanent monogamy in moths, as in other groups of insects, is due to loss of attractiveness and sexual receptivity, only a few studies have provided insight into the mechanisms for this behavior. Only in *D. melanogaster* and related species have identified chemical components of the males' reproductive tract (paragonial glands) been implicated in regulating the postmating behavior of females. In these species, sex peptides are thought to block the female's sexual receptivity as well as to activate egg laying after mating (20). Thus, sex peptides are not known to affect female attractiveness in flies, which instead appears to be decreased after mating by the production in females of the male-predominant antiaphrodisiacs (25). In this regard, the action in *H. zea* of the seminal fluids in general (12) and PSPs in particular in regulating attractiveness of females represents an unusual mechanism for shutting off a component of virgin behavior in

insects. Remating may occur in the following scotophase, at which time the paternity of subsequent progeny would depend on the extent of sperm precedence. Nevertheless, this temporary monogamy may confer some fitness on the male, since egg laying can be activated within a few hours of copulation; females may then oviposit 36% of their eggs in the first 24 h after mating (6379 of 17,926 total eggs from 10 females of a lab-reared colony; A.K.R., unpublished observation).

The mechanisms by which PSPs exert their pheromonostatic effect have not been determined. It has been reported that the hemolymph of females becomes pheromonostatic after mating (10). Thus, PSP could act directly or indirectly on the glandular tissue through the hemolymph; depletion of pheromone could be affected by altering biosynthesis or degradation of pheromone itself or its biosynthetic precursors. These possibilities can be assessed experimentally.

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