J. exp. Biol. ***183****, 61–76 (1993)*
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MALE ACCESSORY GLAND FACTORS ELICIT CHANGE FROM 'VIRGIN' TO 'MATED' BEHAVIOUR IN THE FEMALE CORN EARWORM MOTH *HELICOVERPA ZEA*

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Accepted 30 June 1993

Summary

After mating, the females of many species of moths become depleted of sex pheromone, calling behaviour is terminated, and they become transiently or permanently unreceptive to additional matings. In the corn earworm moth, *Helicoverpa zea*, we have found that the male accessory gland/duplex is required for evoking the post-mating depletion of sex pheromone but apparently not for the cessation of calling. The latter change requires the receipt of a spermatophore or a chemical messenger derived from non-accessory gland/duplex sources. Desalted extracts of combined accessory glands and duplexes caused a depletion of pheromone in injected females. Proteinaceous components in extracts purified by fractionation in cation-exchange cartridges and by reverse-phase high-performance liquid chromotography retain their pheromonostatic activity. In addition, this fractionated material shuts off calling behaviour and prevents mating in injected females, raising the possibility that redundant mechanisms exist in eliciting the different components of 'mated' behaviour.

Introduction

Female insects exhibit a wide variety of mating strategies. In many species, the females mate only once or exhibit a low incidence of remating. Others are sexually unreceptive for a short time after mating, while still others show no apparent decrease in receptivity with each mating (Thornhill and Alcock, 1983). Moths exhibit the first two of these three patterns of mating behaviour. Field and laboratory studies reveal, for instance, that females of the sugarcane borer *Diatraea saccharalis* (Pyralidae; Perez and Long, 1964), the stem borer *Chilo partellus* (Pyralidae; Unnithan and Paye, 1991) and the gypsy moth *Lymantria dispar* (Lymantridae; Doane, 1968; Taylor, 1967) show a high incidence of single matings. Moths that show multiple matings, but apparently only once per diel cycle, include the spruce budworm *Choristoneura fumiferana* (Tortricidae; see

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Key words: insect, reproduction, seminal fluids, monogamy, moth, *Helicoverpa zea.*

Retnakaran, 1971) and the noctuids *Helicoverpa zea* (Callahan, 1958; Stadelbacher and Pfimmer, 1973) and the red bollworm *Diparopsis castanea* (Marks, 1976). Although the adaptiveness of monogamy is apparent if adequate sperm are obtained, it is assumed that the polyandrous female realizes some enhanced fitness to offset the costs of multiple mating. For instance, single matings in lepidopterans lead to increased egg maturation (Benz, 1969; Hendricks *et al.* 1970; Riddiford and Ashenhurst, 1973; Thibout, 1979; Sasaki and Riddiford, 1984; Satyanarayana *et al.* 1991), and this effect may be extended by second matings (Hendricks *et al.* 1970); in *D. castanea*, second matings enhance fertility in the latter half of life (Marks, 1976). Despite the uncertainty in selective pressures at work in mating strategies, the physiological mechanisms associated with the expression of 'virgin' and 'mated' behaviours should be accessible to experimentation.

Mating in moths generally depends upon the expression of several behaviours. In females, these behaviours include the production of volatile sex pheromones, the emission of pheromones from the producing tissue *via* 'calling behaviour', which leads to attraction of potential mates, and the receptivity to (acceptance of) males that attempt mating. In several families of moths, it has been shown that mating leads to a permanent or transient suppression of these behaviours (Perez and Long, 1964; Snow *et al.* 1972; Webster and Carde, 1984; Coffelt and Vick, 1987; Raina *et al.* 1986; Raina, 1989; Mbata and Ramaswamy, 1990; Giebultowicz *et al.* 1991*b*).

In principle, mating may lead to, or be accompanied by, either mechanical or chemical stimuli within the female's reproductive tract. In the case of calling and sexual receptivity, overt aspects of these behaviours include stereotypic patterns of motor activity; hence, the changes in behaviour that follow mating could be mediated by an action on the controlling elements in the central nervous system (CNS) or directly at effector organs (muscle). Similarly, the effects of mating on the production of sex pheromone could be mediated directly at the glandular tissue producing pheromone or through the CNS to the extent that neural or neurohormonal mechanisms regulate production of pheromone.

Male insects possess a diversity of secretory epithelia associated with the reproductive tract that produce structural components of the spermatophore as well as soluble components contained therein (reviewed by Gillott, 1988). Some soluble components may act as chemical messengers in evoking changes in reproductive behaviour of females; this has been documented most notably in dipterans (Manning, 1962). Early implantation experiments in houseflies and mosquitoes showed that the majority of changes are attributable to chemical stimuli from the secretory ejaculatory duct (Riemann *et al.* 1967) or accessory reproductive gland (Craig, 1967), respectively. Gypsy moths depend on the adequate transfer of sperm or testicular secretions to prevent resumption of pheromone production and calling after mating (Giebultowicz *et al.* 1991*b*). The decline of pheromone after mating in *H. zea*, however, appears not to depend on the presence of testes (Raina, 1989). Preliminary studies from our laboratory demonstrated that accessory gland extracts cause the depletion of pheromone when injected into virgin females (Raina *et al.* 1990; Bird *et al.* 1991).

To begin a search for the behavioural effects of chemical messengers in the seminal fluids of Lepidoptera, we have fractionated extracts of male accessory glands of *H. zea*,

and we describe their actions in evoking the different components of the 'mated' state in females. Some of our findings have appeared in abstract form (Kingan *et al.* 1991).

Materials and methods

Animals and dissections

Helicoverpa zea (Boddie) were reared on an artificial diet in the laboratory under a reversed 16 h:8 h (light:dark) photoperiod (Raina *et al.* 1986) and temperatures of 25˚C and 21 ˚C, during photo- and scotophase, respectively. Lights off was at 08:00h, local time. Male and female moths were fed 10% sucrose and maintained in separate chambers until use.

The combined accessory glands and duplexes (see Fig. 1 and Callahan and Cascio, 1963) were dissected in *Heliothis virescens* saline (Bindokas and Adams, 1988; also used with samples for bioassay); the excess saline was blotted, and the tissue was frozen in batches of 40–50 sets for storage at −70˚C.

Surgical procedures

Males were selected for surgery in early photophase on the day of, or the day after, their emergence (d1 or d2). Insects were anaesthetized for 2–3min under carbon dioxide. Scales were removed from the dorsolateral region of the mid-abdominal segments with adhesive tape and the insect was submerged under saline (Weevers, 1966). A transverse incision was made dorsolaterally in the third abdominal segment, and a small amount of fat body was removed. The fused testes were located and removed along with the upper vas deferens and seminal vesicles; the accessory glands and the duplex were then removed and cut from the primary simplex just below the union of the paired duplex (Fig. 1). The remaining simplex was tucked back into the abdominal cavity, the insect was removed from the saline, and the wound was blotted dry and sealed with beeswax. We refer to this procedure as radical gonadectomy (RG) and the operated males as RG males. In the first 1–2h of the following scotophase, RG and control males were mated to d2 females. Mating pairs were checked at 10min intervals; upon separation (approximately 90min), the females were segregated, and their ovipositors were extracted 2h later in 5 μl of heptane containing 20ng of dodecanyl acetate as an internal standard. The major component of sex pheromone blend from *H. zea*, *Z*11-hexadecenal, was quantified by capillary gas chromatography (GC) as previously described (Raina and Kempe, 1992); females were then checked for the presence of a spermatophore. Values of pheromone for the few females that did not have a spermatophore were excluded from the calculation of an average. Other mated females were retained in cups for the remainder of the scotophase and observed for calling behaviour at 30min intervals; at the end of the scotophase, they were checked for the presence of a spermatophore. Differences between experimental and control groups were analyzed by the non-parametric Kruskal–Wallis test.

Extraction and partial purification

Accessory glands and duplexes were homogenized in batches of approximately 300 sets with a Polytron (Brinkmann Instruments, Westbury, NY) in a 20-fold excess (v/w) of

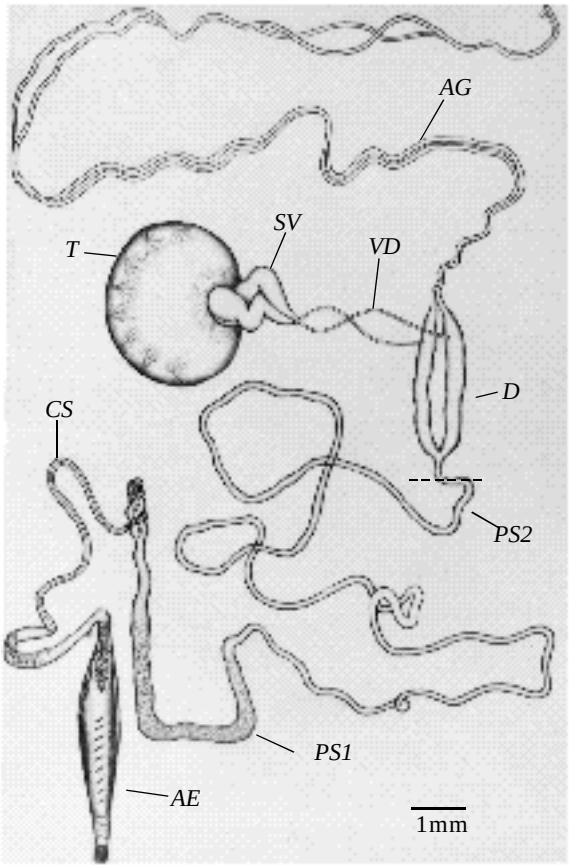

Fig. 1. The reproductive system of male *Helicoverpa zea*. *AE*, aedeagus, containing the eversible endophallus; *AG*, accessory glands; *CS*, cuticular segment of simplex; *D*, duplex; *PS1*, first secretory area of primary simplex; *PS2*, second secretory area of primary simplex; *SV*, seminal vesicle; *T*, fused testes; *VD*, vas deferens. The dashed line caudad to the duplex indicates the cut for production of RG males.

the following mixture: 1mol l^{-1} hydrochloric acid, 5% formic acid, 1% trifluoroacetic acid (TFA) and 1% sodium chloride (Bennett, 1986). The homogenate was centrifuged at 12000 ***g*** for 15min at 4˚C and the supernatant was decanted through cheesecloth to remove fatty material. The pellet was similarly extracted twice more in a 10-fold excess of aqueous acids, and the three supernatants were pooled. The supernatant was then passed through two C18 Sep-Pak cartridges (Millipore Corp., Milford, MA), in tandem; acids and salts were rinsed through with 10% acetonitrile (AcN) containing 10mmol l^{-1} ammonium acetate (AmAc), pH5.0, and proteinaceous material was eluted with 40 % AcN/20mmol l^{-1} AmAc, pH5.0. This material was then diluted with an equal volume of water and applied to an Accell CM Sep-Pak cartridge (Millipore Corp.) which had previously been rinsed with 10ml of 20% AcN/10mmol l^{-1} AmAc/1mol l^{-1} NaCl followed by 20ml of 20% AcN/10mmol l^{-1} AmAc. The cation-exchange cartridge was rinsed successively with 10ml each of 20% AcN/10mmol l^{-1} AmAc, 20 %

AcN/10mmol l^{-1} AmAc/50mmol l^{-1} NaCl and 20% AcN/10mmol l^{-1} AmAc/500mmol l^{-1} NaCl. The first rinse was pooled with the unretained material, and the three rinses were diluted with equal volumes of water. The rinses were then desalted on C18 Sep-Pak cartridges; the adsorbed material was eluted with 40% AcN/0.1% TFA. Samples of each batch were dried by vacuum centrifugation for bioassay. The remaining material eluted from the cation-exchange cartridge with 500mmol l^{-1} NaCl was reduced in volume by approximately half by vacuum centrifugation. It was then fractionated by reverse-phase high-performance liquid chromotography (HPLC) in a wide-pore C4 column (Vydac, 4.6mm×25cm, The Separations Group, Hesperia, CA) using a gradient of 2-propanol in 0.1% TFA, beginning at 10% 2-propanol and increasing at 0.5 % min^{-1}. 1 min fractions were collected, and samples were prepared for bioassay.

Bioassays for pheromonostatic activity

Two bioassays were used. First, samples were injected in 10 μl of saline into intact d2 virgins; 2h later, the ovipositor tip was removed, cleaned and extracted with 5 μl of heptane containing internal standard; pheromone was quantified by GC. The basis for the second bioassay lies in the ability of active components to cause the depletion of sex pheromone accumulated in response to injections of pheromone-biosynthesis-activating neuropeptide (PBAN; Raina *et al.* 1990). Females were ligated between the head and thorax 1 or 2 days after emergence. The following morning, neck-ligated females were injected with 2pmol of PBAN (provided by Dr Thomas Kempe or purchased from Peninsula Labs, Belmont, CA), and 1h later they received an injection of material to be tested for pheromonostatic activity. 2h later, ovipositors were extracted and pheromone was quantified. Values in experimental and control groups were compared using two-tailed Mann–Whitney tests of medians.

Protease digestion

Factors from the accessory glands and duplex, partially purified by HPLC (see above), were digested with trypsin (Sigma Chemical Co., St Louis, MI) as follows. A sample was resuspended in 0.1mol l^{-1} Tris–HCl, pH8.5, to yield 0.26 μg protein μl^{-1}; protein was quantified with BCA protein reagent (Pierce, Rockford, IL). Trypsin was then added in 1/10vol of Tris–HCl, in an amount equal to 1/25 by weight of the sample protein; digestions proceeded for 18h at 37° C. All reaction mixtures were neutralized with approximately 0.5vol of 0.1mol l^{-1} HCl and then held in boiling water for 5min. Virgin females, decapitated in the previous photophase, were injected with 4pmol of PBAN in 10 μl of saline followed 1h later by the treated factor from 0.25 accessory glands/duplex. Pheromone was extracted and quantified as above. Experimental groups were compared using the Mann–Whitney tests of medians.

Bioassay for anti-calling activity

'Calling' is the extrusion of the ovipositor usually with wing fanning. Calling individuals were selected in early scotophase from cages of d2 virgin females. In our colony, even d3 females show some incidence of 'preoviposition' behaviour in which the extrusion of the ovipositor is accompanied by downward curvature of the abdomen and

spotting of the substratum without deposition of an egg; because this behaviour made scoring of calling more difficult, only d2 females, which do not show this behaviour, were used. Control females were injected with 5–10 μl of saline, while test females received an injection of an equal volume of material fractionated from extracts by HPLC as described above. Calling was scored at 15min intervals in dim red light for 90–120min after injection. The incidence of calling (fraction of females calling *versus* time post-injection) in the different treatment groups was analyzed in the statistical analysis program SAS (SAS Institute, Inc., Cary, NC) by comparisons of maximum likelihood values of regression parameters, slope and intercept, after logit transformation of calling data.

Mating behaviour

To determine the effect of experimental treatments on the incidence of mating, females were injected with saline or HPLC-fractionated extract. They were then marked on opposite wings with a magic marker, to allow them to be distinguished, and placed as single pairs of control and test females in acrylic cages, 20cm×20cm×30cm (height), with a narrow-mesh wire screen on one side. Some test females were treated on their posterodorsal abdomen with 1 μl of the extract of virgin female ovipositors containing 30ng of *Z*11-hexadecenal. Immediately after pheromone treatment or 2h after injection, two virgin males were added to each cage; thus, both females had the opportunity to mate, and the males were provided with an initial choice. The incidence of copulation in control and test females was recorded at 10min intervals for 90min. At this time, females were checked for the presence of a spermatophore. A female was scored as mated if she remained *in copula* through at least two observation periods and a spermatophore was present. Comparisons of incidence of mating at the end of the observation period were made by χ^2 analysis; comparisons of times to onset of mating were made by the Mann–Whitney test of medians.

Results

The newly mated female becomes less active after separation from the male; while stationary, her wings remain back in a posture that conceals her abdomen. If mated females are paired with virgin males 1h after separation, they do not remate (0/10 *versus* 9/10 virgins mated). In fact, they elicited few mating attempts, an effect which was not reversed by application of pheromone to the abdomen of the mated female (0/7 *versus* 7/7 virgins mated). Photophase virgins are similarly unattractive to males (2/9 *versus* 7/9 scotophase virgins mated).

Effects of radical gonadectomy

Female *H. zea* become depleted of sex pheromone within 2h of separation of the mating pair; this response could not be attributed to spermatozoa, since males castrated during the larval stage still evoked this pheromonostasis (Raina, 1989). These findings suggested to us that pheromonostasis could be mediated (1) by neural or endocrine events in the female evoked by mechanical stimulation or (2) by the transfer of a non-testicular factor that itself is directly or indirectly pheromonostatic. In an attempt to distinguish

Table 1. *Pheromonostasis in females mated to experimental males*

Female treatment (*N*)	*Z*11-hexadecenal ± S.E.M. (ng)
Unmated (47)	39.2±3.7
Mated, intact male (42)	4.3±0.8[a]
Mated, RG male (24)	21.5±4.6[b]

Decline of pheromone after mating to intact or RG males. Unmated females were identical to mated females in age and time during scotophase at extraction of ovipositor.

[a]Significantly different from 'Unmated', $P=10^{-4}$; [b]significantly different from 'Mate, intact male', $P=10^{-4}$.

between these mechanisms, we mated RG males with calling females. RG males were significantly impaired in their ability to elicit the pheromonostatic response in females (Table 1). Mating to RG males caused only a twofold decrease in pheromone, while mating to intact males resulted in a 10-fold decline. Spermatophores found in females mated to RG males were normal in appearance but apparently empty (no spermatozoa or seminal fluid found). Therefore, pheromonostasis cannot be mediated solely by mechanosensory aspects of the presence of the aedeagus in the female during mating or the formation of the spermatophore. Together with our earlier results (Raina, 1989), these findings point to a possible role for soluble factors produced by the accessory glands and/or duplex.

Examination of calling behaviour yielded a different result (Table 2). In this smaller experiment, only three females resumed calling in the remaining scotophase. These females were found not to have received a spermatophore during mating (Table 2). Two females which did not resume calling, did not have spermatophores in their bursae, but they did have a 'white fluid', the nature of which we did not identify. Thus, calling can be distinguished from pheromone accumulation, in that the receipt of a spermatophore is sufficient to evoke the 'mated' state.

Partial purification of pheromonostatic factors

Preliminary studies showed that material with pheromonostatic activity could be extracted from accessory glands and recovered after fractionation by HPLC (Raina *et al.* 1990) or adsorption to cation-exchange cartridges (Bird *et al.* 1991). We subjected the desalted crude extract to a batch fractionation in cartridges containing a weak cation

Table 2. *Calling behaviour in females mated to RG males*

Group, mated female (*N*)	Number calling
RG-mate, no spermatophore (3)	3
RG-mate, no spermatophore/WF* (2)	0
RG-mate, with spermatophore (10)	0

Calling in females mated to RG males during remaining scotophase. Females were mated within 1 h of onset of scotophase and had separated with 5–6h remaining in the 8h scotophase.

*WF, white fluid only, found in bursa.

Table 3. *Fractionation of pheromonostatic factors in cation-exchange cartridges*

Treatment	*Z*11-hexadecenal ± range (ng)
Control	47.2±4.0
0 mmol l^{-1} NaCl	120.6±15.0
100mmol l^{-1} NaCl	8.3±0.9
200mmol l^{-1} NaCl	3.4±0.6
500mmol l^{-1} NaCl	4.2±0.4
1000mmol l^{-1} NaCl	42.2±25.7

Pheromonostatic effects of successive eluates of Accell CM cartridge.
Females were injected with 5pmol of PBAN followed by 0.25 equivalent of the indicated eluate.
Control females received saline injections.
N=2 for each determination.

exchanger. All the active material was retained in low-salt eluate but eluted with increasing concentrations of NaCl (Table 3). Since active material eluted with each step in NaCl concentration up to 0.5mol l^{-1}, we routinely step-eluted bioactive material with buffer containing 0.5mol l^{-1} NaCl after rinsing through unretained components. The reason for the apparent pheromonotropism of the unretained material is unknown. The batch-fractionated material was next subjected to reverse-phase HPLC (Fig. 2). Initially,

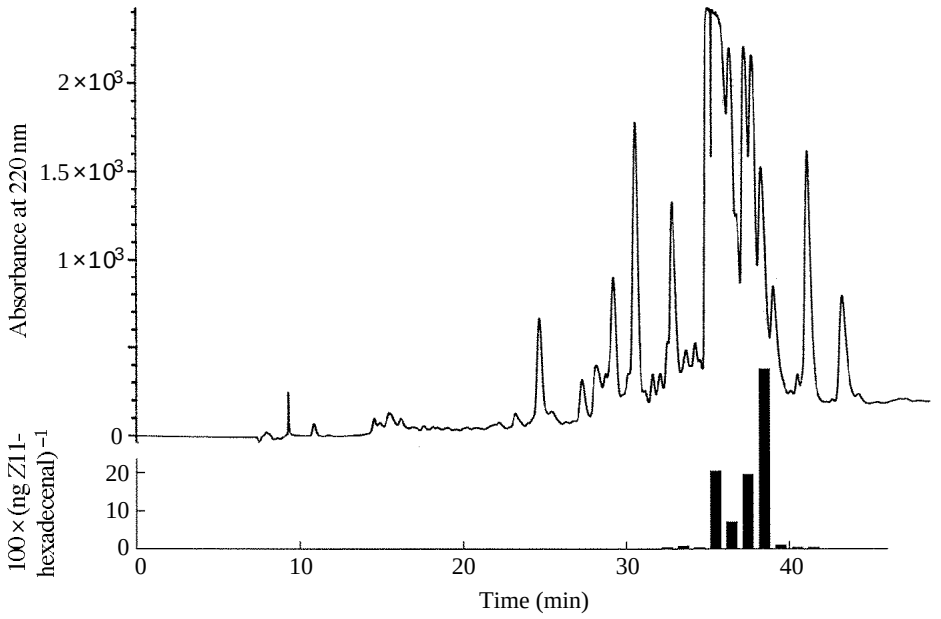

Fig. 2. Pheromonostatic response in females injected with one equivalent from individual fractions of a reverse-phase chromatogram of 30 accessory gland/duplex complexes (see Materials and methods). Note that values plotted are reciprocals of (ng *Z*11-hexadecenal)×100. Each bar represents the average of three or four determinations; error bars are omitted for clarity. Controls, mean 180.6ng *Z*11-hexadecenal. The trace shows absorbance at 220nm.

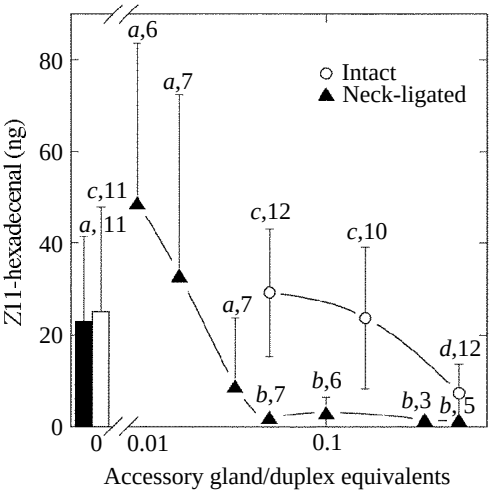

Fig. 3. Dose effectiveness of PSF (pooled fractions 35–38 from Fig. 1) in intact virgins and neck-ligated and PBAN-injected females. Values for control females, injected with saline alone, are shown as filled (neck-ligated) and open (intact) bars. Comparisons of experimental values were made with controls of that group; values with same letter are not significantly different; *b*, P=0.003; *d*, P=0.05. Number of determinations for each value is shown. Error bars represent ± S.D.

we pooled samples for bioassay from five adjacent 1min fractions beginning with minute 10. The only fractions to reduce pheromone by more than 90% of control values were contained in the pool of fractions 35–40 (data not shown). When individual fractions in and around this zone were assayed, we found that fractions 35–38 contained the majority of the pheromonostatic activity (Fig. 2). This zone of the chromatogram from subsequent preparations was pooled for tests in behavioural assays. We refer to this material as 'PSF' to indicate that it contains pheromonostatic factors.

Pheromonostatic activity of HPLC-fractionated extract

The active material from the reverse-phase separation, PSF, was tested for its dose effectiveness in causing the depletion of sex pheromone in neck-ligated and in intact virgins (Fig. 3). The neck-ligated and PBAN-injected females are more sensitive than intact virgins to the accessory gland factors; 0.05 equivalent was sufficient to deplete pheromone. In addition, it is evident that greater variability exists in the responsiveness of a group of insects to the low doses of PSFs than to the high doses. An examination of the raw data (not shown) shows that individual females may have different thresholds, above which the pheromonostatic response is often complete rather than graded. The partially purified factors were tested for sensitivity to a protease. Trypsin largely destroyed PSFs (Table 4). In this experiment, the factors were also found to be stable to boiling.

Anti-calling activity of HPLC-fractionated extract

While the receipt of a spermatophore was shown above to be sufficient to shut off

Table 4. *Protease sensitivity of PSF activity in C4 35–38*

Treatment (*N*)	*Z*11-hexadecenal (ng)
Tris–HCl control (10)	20.2 ± 6.7^{b}
Trypsin control (11)	11.2 ± 4.0^{b}
PSF, not boiled (11)	1.61 ± 0.3^{a}
PSF (11)	1.6 ± 0.4^{a}
PSF + trypsin (12)	20.2 ± 6.5^{b}

All mixtures were boiled at the end of the incubation except for the one indicated. Amount assayed was 0.25equivalent PSF.

Values shown are mean ± S.E.M.

Values with same letter are not significantly different.

Trypsin destroys PSF activity in C4 35–38 (P=0.008).

calling, we wanted to consider the possibility that soluble factors transferred by the male could also participate in this response to mating. Accordingly, we tested PSF for its effect on calling behaviour. Control females recover and resume calling quickly after handling and injection (Fig. 4). During the period of observation, all control females called. Calling was completely suppressed in test females immediately after injection. The behaviour resumed in females injected with more dilute factor, and the incidence increased to a level nearly equal to that of control females by the end of the 2h observation period. The times to resumption of calling in the 0.05 and 0.16 equivalent groups cannot be distinguished from each other (P=0.14); at 0.5 equivalent, however,

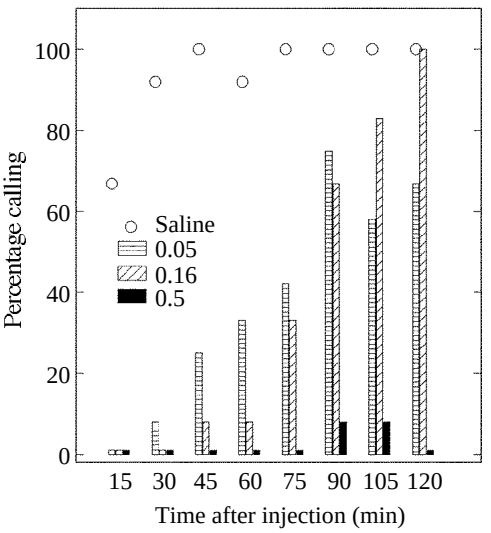

Fig. 4. Calling in females injected with varied doses (given in accessory gland/duplex equivalents) of partially purified PSFs. Scoring began 15min after injection. The incidence of calling was delayed in all groups of PSF-injected females with respect to saline-injected females ($P\leqslant10^{-4}$). N=12 for all treatment groups. The smallest bar denotes 0% calling.

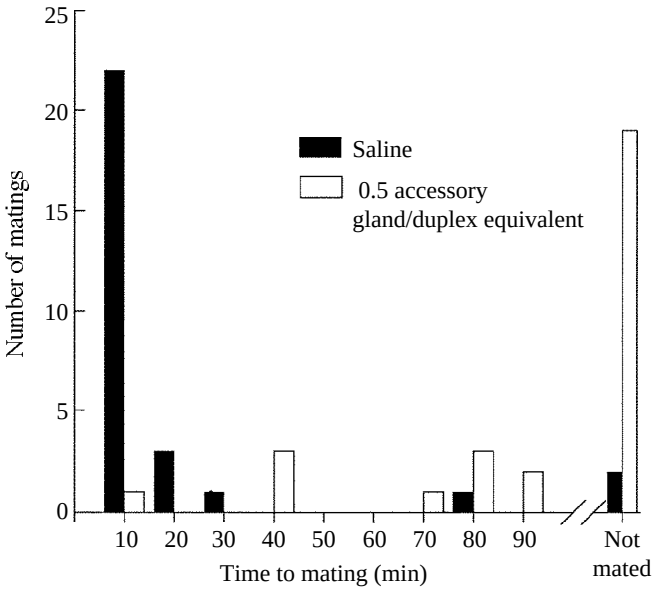

Fig. 5. Inhibition of mating in PSF/ACF-injected females. New matings were recorded at 10min intervals. Data were obtained from 29 pairs (1 saline-injected, 1 PSF-injected) of females.

only a single female (N=12) called. Non-calling females were inactive, and their wings remained back in a posture that covered their abdomens; in this regard, their behaviour mimicked that of a mated female. Thus, partially purified extracts, like PSFs, also contain anti-calling factor(s) (ACFs).

Components of the seminal fluid prevent mating

If the pheromonostasis and inhibition of calling evoked by PSF are paradigms for events occurring in mated females, then similarly prepared extract should prevent mating in injected females. Accordingly, we injected intact virgins with PSF and tested their receptivity to mating attempts. PSF significantly inhibited mating (P<0.001) during the 90min observation period (Fig. 5). Moreover, those test females that did mate did so much later than saline-injected controls: test, median 70min; control, median 10min (P<0.001). Test females received few mating attempts. When mating attempts did occur, the female usually moved away, and the male was further deterred in his attempts. In some cases, the male persisted in courtship; this prompted kicking and vigorous wing-flapping by the female in an apparent unwillingness to accept a mate.

We have shown that factors contained in PSF cause the depletion of sex pheromone and turn off calling in virgin females, leading to the prevention of mating in injected females (Fig. 5). The failure to mate may depend on one or both of these changes, in addition to a direct effect on receptivity. In a test of the necessity for depletion of pheromone in the prevention of mating, we administered two injections, separated by 1h, to three groups of d2 virgins. Females receiving two injections of saline readily mated 1 h

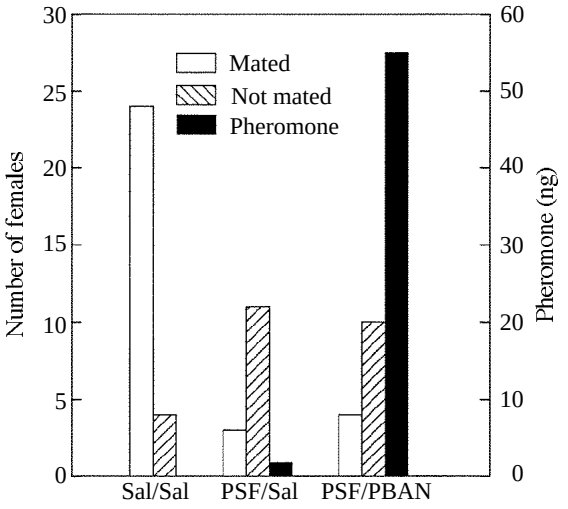

Fig. 6. Reversal of pheromonostatic, but not the anti-mating, actions of PSF by PBAN. Pheromone was quantified in females injected with PSF that did not mate over the course of the observations. Sal, saline.

after the second injection (Fig. 6). Only 21% of those receiving PSF injections mated, and the addition of an injection of 5pmol of PBAN did not increase the incidence of mating. PBAN did, however, reverse the pheromonostatic effects of PSF (mean 1.8ng *versus* 55ng of *Z*11-hexadecenal). Thus, the prevention of mating associated with injections of PSF appears to depend on events in addition to pheromonostasis; the anti-calling action and the assumption of a 'mated' posture apparently participate as well.

Discussion

Virgin females accumulate sex pheromone and call early in the scotophase (Raina *et al.* 1991). Mated females of *H. zea* are less effective than virgins at luring males to field traps (Snow *et al.* 1972), a finding that is explained by the suppressing effect that mating has on levels of sex pheromone (Raina *et al.* 1986; Raina, 1989). Moreover, calling does not resume after mating during the same scotophase. Thus, females apparently cease production of pheromone and, by not calling, avoid the unnecessary attraction of males for the 1–2h required for pheromone to disappear from the pheromone gland.

We have shown that males from which the testes, duplex and accessory glands have been removed (RG males) fail to elicit a normal post-mating decline in pheromone levels in females, despite the formation of a 'normal' spermatophore. Since castrated males do elicit pheromonostasis (Raina, 1989), the accessory gland/duplex complex seems to be the source of factor(s) required for the normal post-mating depletion of pheromone. This conclusion is supported by the observations that extracts of these seminal-fluid-producing tissues are pheromonostatic (Raina *et al.* 1990; Bird *et al.* 1991; Kingan *et al.* 1991; this report). The significance of the apparent reduction in pheromone in RG-mated females

from levels found in virgins is unclear; we cannot conclude, for instance, that this represents the contribution of the spermatophore, since the effect of mating *per se* could not be determined.

The cessation of calling differs from the depletion of pheromone, in that 'mated' behaviour was found in females receiving spermatophores from RG males. The latter finding apparently precludes a requirement for, but not the existence of, participation of the seminal fluids in cessation of calling. A precedent for this notion comes from work with the cabbage white butterfly. Here, mechanosensory impulses in sensory afferents innervating the bursa copulatrix are increased in frequency by the receipt of a spermatophore or artificial inflation, and they are an indicator of the ensuing 'mate-refusal posture' (Sugawara, 1979). Nevertheless, Obara (1982) showed that haemolymph from mated females of this species is active in inducing mate-refusal posture in virgins, showing that humoral factors may also participate. The reason(s) for redundant mechanisms and their relative roles in the switch to 'mated' behaviour is unknown.

The stereotypic changes from virgin to mated behaviour in female *H. zea* can be induced in virgins by proteinaceous material from fractionated extracts of male accessory glands and duplex. Bioassays after size-exclusion chromatography suggest that one or more pheromonostatic components between 2000 and 12000Da in molecular mass are present in extracts (T. G. Kingan, unpublished observation). In the course of an effort to purify and characterize PSFs, we found that partially purified PSFs also exibit anti-calling activity. Despite the similarity in the chromatographic properties of PSFs and ACFs, we cannot yet conclude that they are identical or even structurally related.

The target tissues and mechanisms of action for PSFs and ACFs are unknown. We cannot conclude, for instance, that, because factors are active when injected (this report) and because the haemolymph of mated females is pheromonostatic (Raina, 1989), factors must reach their target tissue in mated females *via* the haemolymph. Factors may act at sensory receptors in the female reproductive tract, as apparently happens with sperm or soluble testicular secretions in gypsy moths (Giebultowicz *et al.* 1991*a*). Regardless of these considerations, pheromonostasis could be mediated by one or more mechanisms including inhibition of release of PBAN from the producing neurons, by an action on the pheromone gland to decrease synthesis and/or increase degradation of *Z*11-hexadecenal or its precursors, or by limiting the supply of metabolic precursors to the gland. Interestingly, neck-ligated females are more sensitive to PSFs than are intact virgins. Given that PBAN, administered after PSFs, can reverse the pheromonostatic response, it is possible that the response in intact virgins is limited by the existing action of endogenous PBAN; in our bioassay with neck-ligated females, a single pulse (injection) of PBAN is administered.

Calling behaviour requires descending neural input *via* the ventral nerve cord in giant silkmoths and the tobacco hornworm moth (Sasaki *et al.* 1983; Itagaki and Conner, 1986) which apparently originates in the brain or suboesophageal ganglion and projects to the terminal abdominal ganglion (Itagaki and Conner, 1986). During normal mating, the cessation of calling may be triggered by mechanosensory and/or chemosensory events associated with the presence of a spermatophore in the bursa copulatrix, known to receive sensory innervation in, for instance, *Manduca sexta* (Stringer *et al.* 1985). In *M. sexta*,

(artificial) inflation of the bursa alone is insufficient to shut off calling (Sasaki and Riddiford, 1984), showing that events elicited by the spermatophore may arise because of the chemical messengers it contains. In *H. zea*, we cannot rule out mechanosensory events but, if cessation of calling is attributable to chemosensory events in the bursa, the chemical messengers apparently arise from spermatophore-producing structures as well as from those structures producing the seminal fluids. The incidents of calling and pheromone production are periodic in moths and generally occur in near synchrony, as reported in *H. zea* (Raina *et al.* 1986). It is possible, then, that these components of 'virgin' behaviour are triggered by a common neural centre in the brain or suboesophageal ganglion, and that after mating PSFs/ACFs act on this pathway. Whatever the mechanism(s), pheromonostasis is distinguished from cessation of calling by its additional requirement for transfer of seminal fluids.

The interpretation of the finding that PSFs/ACFs prevent mating is hampered by the inadequacy of the paradigm for sexual receptivity. Because injected females stop calling and become depleted of pheromone, they are unattractive to males; indeed, males make few mating attempts with these females despite approach and physical contact. Moreover, injected females assume a 'mated' posture in which their wings are back in a resting position which covers their abdomens. Agee (1969) has pointed out that the male corn earworm moth taps the female's ovipositor with his antennae just before making a mating attempt, an act which he is physically prevented from doing with mated and PSF/ACF-injected females. If a hierarchical organization of courtship behaviour exists in moths, as in *Drosophila melanogaster* (see references in Szabad and Fajszi, 1982), then the inability to complete precopulatory sequences could block mating attempts. Clearly, the failure to attempt mating is not for lack of pheromone in our paradigm, since a calling virgin female is in the same cage. We cannot rule out a requirement for the detection of a steep gradient of pheromone at close range; however, we were unable to induce males to make mating attempts with injected females whose posterodorsal abdomens were spotted with pheromone blend extracted from ovipositors. Nevertheless, males sometimes did attempt to mate with PSF/ACF-injected females but were nearly always unsuccessful; females moved away or kicked at persistent males. Thus, a third component of 'virgin' behaviour, sexual receptivity, may also be turned off in mated and injected females. Sexual receptivity has been shown in female fruit flies, by the use of genetic mosaics, to have a control site (focus) in the anterior dorsal brain (Tomkins and Hall, 1983).

The assignment of function to specific components of the seminal fluids will ultimately require their characterization. Moreover, if PSFs/ACFs act either directly or indirectly on the central nervous system *via* the haemolymph, then pheromone-depleting or anti-calling factors would appear in the haemolymph of mated females. In fact, pheromonostatic activity appears transiently in the haemolymph of mated females (Raina, 1989). A full understanding of reproductive changes after mating will require that we determine the relatedness of factors in mated haemolymph and accessory glands.

In summary, we have shown that the transient depletion of pheromone following mating is attributable to factors transferred from the male accessory gland/duplex. Thus, *H. zea* stands in contrast to *Lymantria dispar*, which has rudimentary accessory glands and in which pheromonostasis depends on the transfer of sperm or testicular secretions

(Giebultowicz *et al.* 1991*b*). The accessory glands and duplex of *H. zea* are a rich source of behaviourally active molecules whose actions can be studied in paradigms of mating behaviour.

We would like to thank Dr Jaga Giebultowicz for many helpful discussions and for a critical reading of the manuscript.

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