

Neural Inactivation of Sex Pheromone Production in Mated Females of the Silkworm Moth, *Bombyx mori*

Authors: Ichikawa, Toshio, Shiota, Tomomi, and Kuniyoshi, Hisato

Source: Zoological Science, 13(1) : 27-33

Published By: Zoological Society of Japan

URL: <https://doi.org/10.2108/zsj.13.27>

The BioOne Digital Library (<https://bioone.org/>) provides worldwide distribution for more than 580 journals and eBooks from BioOne's community of over 150 nonprofit societies, research institutions, and university presses in the biological, ecological, and environmental sciences. The BioOne Digital Library encompasses the flagship aggregation BioOne Complete (<https://bioone.org/subscribe>), the BioOne Complete Archive (<https://bioone.org/archive>), and the BioOne eBooks program offerings ESA eBook Collection (<https://bioone.org/esa-ebooks>) and CSIRO Publishing BioSelect Collection (<https://bioone.org/csiro-ebooks>).

Your use of this PDF, the BioOne Digital Library, and all posted and associated content indicates your acceptance of BioOne's Terms of Use, available at www.bioone.org/terms-of-use.

Usage of BioOne Digital Library content is strictly limited to personal, educational, and non-commercial use. Commercial inquiries or rights and permissions requests should be directed to the individual publisher as copyright holder.

BioOne is an innovative nonprofit that sees sustainable scholarly publishing as an inherently collaborative enterprise connecting authors, nonprofit publishers, academic institutions, research libraries, and research funders in the common goal of maximizing access to critical research.

Neural Inactivation of Sex Pheromone Production in Mated Females of the Silkworm Moth, *Bombyx mori*

Toshio Ichikawa, Tomomi Shiota and Hisato Kuniyoshi¹

Department of Biology, Faculty of Science, Kyushu University, Fukuoka 812-81
and ¹Department of Applied Biological Chemistry, Faculty of Agriculture,
The University of Tokyo, Tokyo 113, Japan

ABSTRACT—Pheromone content in the pheromone gland of the female moth, *Bombyx mori*, declines after mating with a time course closely resembling that of decapitated females. The inactivation of pheromone production after mating was prevented when the ventral nerve cord or a pair of peripheral nerves (N4) extending from the terminal abdominal ganglion was severed before mating. In contrast, the post-mating inactivation of pheromone production was not prevented when the ventral nerve cord was transected 1 hr after the initiation of mating. Although females produced only a small amount of pheromone when the connection between the brain and the suboesophageal ganglion (SG) was cut at an early pupal stage, mating did not induce a significant decline of pheromone production in these females. The results suggest that inactivation of pheromone production is mediated by a neural signal, originating from a peripheral receptor, that is sent via the ventral nerve cord to the brain-SG complex to suppress activity of the neurosecretory cells responsible for the release of pheromonotropic neuropeptides, such as a pheromone biosynthesis activating neuropeptide (PBAN).

INTRODUCTION

Female moths release a species-specific male attractant from a glandular area at the abdominal tip. Production of the sex pheromone in many species of moths is under control of a pheromone biosynthesis activating neuropeptide (PBAN) or PBAN-like factors (Raina, 1993). PBANs have been isolated and identified from extracts of the brain-SG complex from *Helicoverpa zea* (Raina *et al.*, 1989), *Bombyx mori* (Kitamura *et al.*, 1989, 1990) and *Lymantria dispar* (Masler *et al.*, 1994). In a few species of *Helicoverpa* moths, PBAN appears to be transported from the SG through the ventral nerve cord to the terminal abdominal ganglion to stimulate aminergic neurons that innervate the pheromone gland to activate pheromone production (Christensen *et al.*, 1991; Teal *et al.*, 1989). However, in many other species of moths, PBAN or PBAN-like factors seem to be released into the haemolymph and act directly on the pheromone gland to induce the biosynthesis of pheromone (Fonagy *et al.*, 1992; Jacquin *et al.*, 1994; Jurenka *et al.*, 1991; Raina *et al.*, 1987; Soroker and Rafaeli, 1989; Tang *et al.*, 1989).

Mating in many species of moths causes a transient or permanent suppression of pheromone production (Jurenka *et al.*, 1991). In a gypsy moth and two tortricid moth, suppression of pheromone production after mating seems to be induced by a neural signal that originates from the abdomen and run up the ventral nerve cord (VNC) to inhibit the release of PBAN, because transection of the ventral nerve cord fails to induce the post-mating inactivation of pheromone production (Foster, 1993; Foster and Roelofs, 1994; Giebertowicz *et al.*, 1991; Jurenka *et al.*, 1993). In a few species of *Helicoverpa*

moths, factors originating from the testis or the accessory gland of the male reproductive system and acting through the mated female's haemolymph, are implicated in suppressing pheromone production (Raina, 1989; Ramaswamy *et al.*, 1994). Recently, a polypeptide with such pheromonostatic activity has been identified in *Helicoverpa zea* and this peptide requires an intact VNC for pheromonostatic action (Kingan *et al.*, 1993, 1995).

"Bombykol" is the first insect sex pheromone to be identified in the silkworm moth, *Bombyx mori* (Butenandt *et al.*, 1959). PBAN molecules have been isolated from the same species of moth (Kitamura *et al.*, 1989, 1990). Kawano *et al.* (1992) suggested that PBAN, along with four other neuropeptides, is generated from a common precursor polypeptide that is translated from a single species of mRNA. The four neuropeptides, including the diapause hormone (DH), like PBAN, share a conserved pentapeptide amide at the C-terminal and have substantial pheromonotropic activity (Sato *et al.*, 1993). Twelve neurosecretory cells expressing the gene for the precursor protein are aggregated into three clusters on the ventral midline of the SG and occupy the mandibular (anterior), maxillary (medial) and labial (posterior) neuromeres of the ganglion (Sato *et al.*, 1994). The same clusters of neurosecretory cells are immunoreactive to antisera raised against PBAN or DH (Ichikawa *et al.*, 1995). The result of a surgical experiment indicated that potent pheromonotropic activity was assigned to the medial neurosecretory cells of SG (Ichikawa *et al.*, 1996). The intracellular injection of a fluorescent dye into the medial cells revealed that they had dendritic branches in the anterior part of SG and send an axon to a neurohaemal organ, the corpus cardiacum, via a branch of a maxillary nerve (Ichikawa *et al.*, 1995).

We report here that mating suppresses pheromone production in female *Bombyx mori* and that this suppression

Accepted October 12, 1995

Received July 25, 1995

may be induced by a neural signal ascending the ventral nerve cord to the brain-SG complex to trigger a neural mechanism for the inactivation of neurosecretory cells.

MATERIALS AND METHODS

Animal

Commercial races of *Bombyx mori* (Tokai x Fuyo) were used in this study. The eggs were incubated at 25°C under continuous illumination throughout embryogenesis, and larvae were reared on an artificial diet at 25–26°C under 16-hr light/8-hr dark photocycle. Pupae and adults were placed under the same conditions. A female was usually mated with an 1–2 day-old male about 7 hr after the onset of the second photophase when the pheromone titer is maximal (Ando *et al.*, 1988). Because a male usually mates with a female for more than 24 hr continuously, if the mating is not disturbed, the couple was artificially separated 4 hr after the start of mating, unless otherwise noted. Presence of a spermatophore formed in the bursa copulatrix of the mated female was usually examined and sometimes its diameter was measured.

HPLC analysis of pheromone titer

The pheromone glands of females were extruded and excised using a pair of scissors. Individual glands were placed in 500 μ l of hexane for 10 min and the extracts were injected onto an HPLC column (Nucleosil 5NO₂, Chemco Pak) that was installed in an HPLC system (Hitachi L-6200). Chromatographic conditions were the same as described by Arima *et al.* (1991). Pheromone titer was monitored with an absorbance at 230 nm.

Surgical procedures

Surgery was performed on 2-day old females anesthetized in crushed ice for 10–15 min. A drop of melted wax was put on the operation area and the wax was peeled off to remove scales. After all surgical operations had been completed, the wounds were sealed with melted wax.

The ventral nerve cord of a virgin female was transected at the neck or the abdomen. A small incision was made on the ventral side of the neck or the fourth abdominal segment and the ventral nerve cord was cut using micro-scissors. Peripheral nerves extending from the terminal ganglion were exposed by making a incision on the fifth abdominal segment and visualized with a drop of 1% methylene blue. An excess of the dye was removed with a piece of filter paper. All or particular pairs of the nerves were severed. For sham-operated controls, females were treated similarly except for the severance of the nerve cord or peripheral nerves.

Connection between the brain and SG was cut when the animal was at a pupal or adult stage. A small window was made on the ventral side of the head cuticle of a female pupa (<24 hr after pupation) and circumoesophageal connectives between the brain and SG were bilaterally transected, or left intact in case of the sham-operated control. The brain-SG complex of an adult female was exposed by removing part of the cuticle in front of the head and the tracheae under the cuticle. The brain-SG connection was cut at the level of the ventral surface of oesophagus, using micro-scissors, or left intact in case of the control.

To transect of the seminal duct, a small incision was made on the ventral side of the 6th abdominal segment of a virgin female and the duct was exposed by pulling up the bursa copulatrix after moving aside ovarian tubes over the bursa copulatrix. The duct was transected at the site near the bursa copulatrix or was left intact for the control.

Virgin females subjected to surgical procedures were kept at 25°C for 1 hr and mated with 1–2-day old males. After 4 hr, conjugating partners were separated and the females were left for a further 20 hr, after which their pheromone glands were excised. After excising of the glands, the moths were fix in 70% ethanol and all tissues were

examined for success or failure of the operation.

For transection of the ventral nerve cords of mated females, the moths were separated from their mates at 30 min, 1 or 2 hr after the start of copulation and then they were placed in crushed ice for 5–10 min for purposes of anesthetization. The ventral nerve cord was transected at the site between the 5th and 6th abdominal ganglia, or left intact for the sham-operated control. Pheromone titers of these females were determined 22–23.5 hr later.

When the penis was removed, using forceps, mating behavior of such a moth with no penis did not differ from that of an intact male and the former usually copulated with a female for more than 24 hr, as did the latter, when mating was not interrupted. Any female mating with a penisless male had no spermatophore in its bursa copulatrix.

PBAN injection

PBAN-I of *Bombyx mori* was synthesized in the laboratory of The University of Tokyo (Kitamura *et al.*, 1989). It was dissolved in saline at appropriate concentrations and 10 μ l of the solution was injected into the abdomen of mated or decapitated females. Pheromone was extracted from the glands 3 hr after injection.

Statistical analyses

Means were compared by Mann-Whitney *U*-tests. All differences, unless otherwise stated, are reported to $P \leq 0.05$.

RESULTS

Decline of pheromone titer after mating and decapitation

Females were mated or decapitated at about midpoint of the second photophase and pheromone titers were determined at different times after the start of mating, or decapitation. Pheromone titers in mated females declines to a level lower than 3% of the initial value after 24 hr and declines still further after another 24 hr (Fig. 1). There was no significant difference between mean titers of mated and decapitated females at any time after mating or decapitation. Thus, mating may induce a complete shut down of the release of pheromonotropic factors into the haemolymph at an early stage of copulation.

A male of *Bombyx mori* usually copulates with a female for more than 24 hr, if copulation is not artificially interrupted.

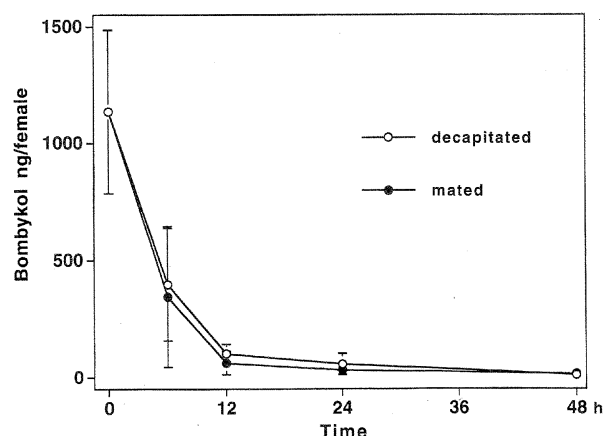


Fig. 1. Decline of pheromone titers of females after decapitation, or the start of mating. Each point represents the mean pheromone titer of 10 females and the standard error (SE) of the mean (bar).

To estimate the duration of copulation necessary for inactivation of pheromone production, couples were forcibly separated at different times after the initiation of copulation. The mean pheromone titer of females copulated for 1 hr was not significantly different from that of females copulated for 24 hr (Fig. 2). Pheromone titers of individual females forcibly separated from their mates at 30–40 min were highly variable: some females have a low titer (<50 ng/female) comparable to that of females mated for 24 hr and some have a high titer (>1000 ng/female) comparable to that of virgins. Examination of the bursa copulatrix at 30–40 min revealed that a spermatophore was formed in this organ. Mean diameter of the spermatophore in the females with a low pheromone titer (2.50 ± 0.33 mm, $n=25$) was not significantly different from that in the females with a high titer (2.46 ± 0.24 mm, $n=10$).

Role of the brain, ventral nerve cord and peripheral nerves

Neural inactivation of pheromone production after mating was examined following transecting the ventral nerve cord (VNC) of the females. Mean pheromone titer of virgin females determined 24 hr after transection was somewhat lower than that of sham-operated virgins, irrespective to the sites of the transection (between SG and the 1st thoracic ganglion or the

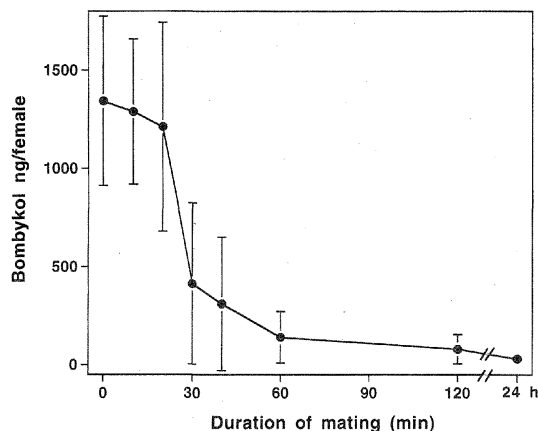


Fig. 2. Effect of the duration of mating on the pheromone titer. Copulation was artificially interrupted at various times and pheromone titers were determined at 24 hr after the time of copulation. Mean $\pm$ SE are shown for each point. $N=10$.

Table 1. Effect of transection of the ventral nervous system of females on pheromone titers

operation	virgin (mean $\pm$ S.E., ng)	mated (mean $\pm$ S.E., ng)
sham	1432 $\pm$ 352a	32.0 $\pm$ 25.4b
¹ SG-T1 cut	931 $\pm$ 210a	853 $\pm$ 327a
² A5-6 cut	956 $\pm$ 224a	943 $\pm$ 270a
³ Br-SG cut	26.4 $\pm$ 12.3b	21.5 $\pm$ 9.4b

The ventral nerve cord was cut between SG and the 1st thoracic ganglion(¹) or between the 5th and 6th abdominal ganglia (²). ³Connection between brain and SG was cut. $N=10$ for all operations. Means followed by the same letter are not significantly different ($P \leq 0.05$, Mann-Whitney U -test).

5th and 6th abdominal ganglia) (Table 1). However, there was no significant difference between titers of pheromone from virgin and mated females with transected-VNC, thereby indicating that the VNC is involved in transmission of a signal for inactivation of pheromone production.

It is important to know whether the signal is needed to be sent to the brain for action. When the central nervous system of adult virgin females was transected between the brain (Br) and SG, the transection itself decreased pheromone titers of the females to the same level as seen in decapitated virgin females 24 hr after the operation (Table 1). When the brain of a female was isolated from the SG at an early pupal stage, the virgin female moth produced a small amount of pheromone (98 ± 48 ng/female, $n=10$), about one tenth of the sham-operated virgin female (950 ± 240 ng/female, $n=10$). Mean pheromone titer of mated females (102 ± 85 ng/female, $n=10$) did not significantly differ from the mean of virgin females with isolated brains.

There are nine pairs of peripheral nerves (N1-N9) extending from the terminal abdominal ganglion: the N1-N4 innervate female reproductive organs (Nishimura *et al.*, 1959). To trace the origin of such an inhibitory signal, the decline in the pheromone titer by mating was examined after severing the N1-N4. In contrast to the severance of N1-N3, bilateral severance of N4 selectively blocked transmission of the signal mediating the post-mating inactivation of pheromone production, as did severance of all the nerves (N1-9) (Fig. 3). The N4 extends posteriorly from the terminal ganglion and appears to innervate the oviduct and the muscle situated at the basal part of ductus bursae (Nishimura *et al.*, 1959). Because N5-N9 innervate only segmental muscles, effects of severance on the decline of pheromone titer was not examined.

We then asked whether an intact ventral nerve cord is needed to maintain an inactive state of pheromone biosynthesis of mated females. Mating moths were forcibly separated 30 min, 1 hr, or 2 hr after the start of copulation, and then ventral nerve cords of females were immediately transected after anesthesia had been induced by ice for 5–10 min. Mean pheromone titer of these females was not significantly different from that of sham-operated females (Fig. 4). Although pheromone titers of individual females copulated for 30 min were highly variable, the distribution profile of pheromone titers of VNC-transected females resembled that of sham-operated females. This means that the inhibitory signal is usually sent and received between 30 min and 1 hr after the start of copulation and that once the signal is received, it is no longer needed to maintain an inactive state of pheromone production.

PBAN injections into decapitated or mated females

Mated females injected with various amounts of PBAN did not have significantly different titers from decapitated virgins injected with the same amount (Fig. 5). Thus, mating did not reduce susceptibility of the pheromone glands to the pheromonotropic peptide.

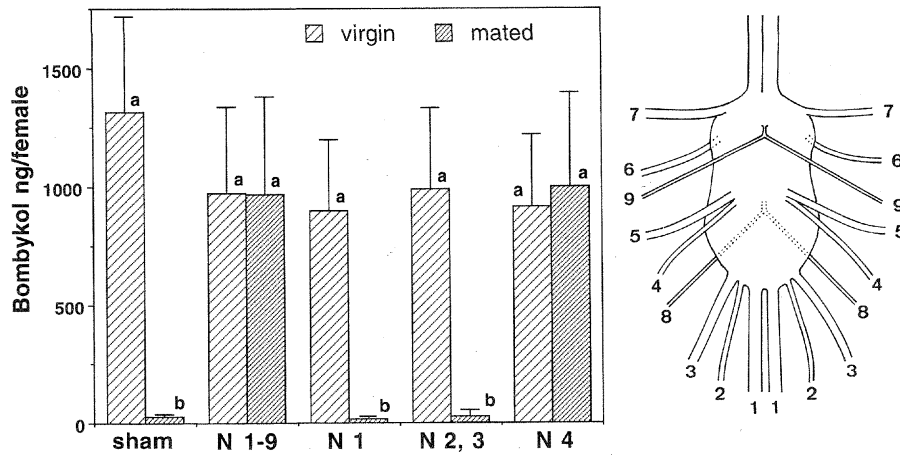


Fig. 3. Pheromone titers of virgin and mated females in which all or particular pairs of the peripheral nerves extending from the terminal ganglion were severed. Mean $\pm$ SE are shown for each bar. N=10. Numerals in the inset indicate the number of nerves (N1-9). Different letters (a, b) indicate statistically different groups ($P\leq 0.01$, Mann-Whitney *U*-test).

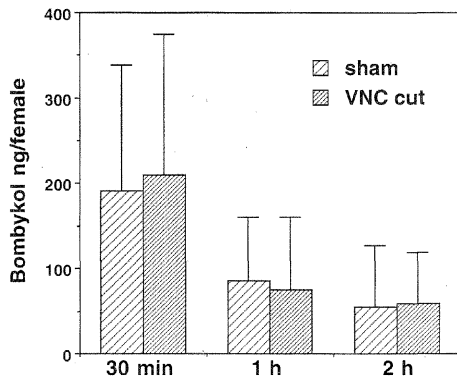


Fig. 4. Effect of transection of the ventral nerve cord or sham-operation of mated females on suppression of pheromone production. Females were sham-operated (sham) or their ventral nerve cords were cut (VNC cut) at 30 min, 1 hr, or 2 hr after the start of mating. Pheromone glands were extracted at 24 hr after the start of mating. Mean $\pm$ SE are shown for each bar. N=8.

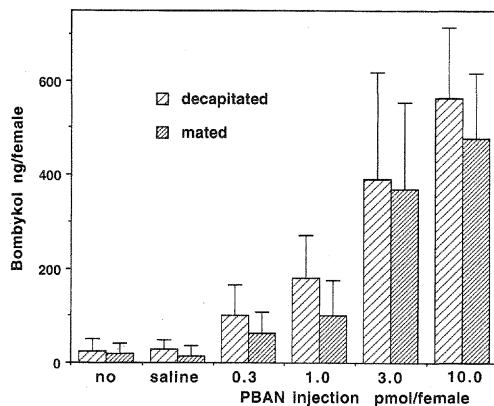


Fig. 5. Comparison of the mean titers of pheromone in mated or decapitated virgin females injected with different amounts of PBAN. No or 10 μ l of saline or PBAN solution was injected into each female 24 hr after decapitation or the beginning of mating. Pheromone glands were extracted 3 hr after injection. Mean $\pm$ SE are shown for each bar. N=8-10.

Effects of a physical act of copulation on pheromone titer

There are many mechanosensitive hairs on the anal papillae and cuticular plates. Because mechanical stimulation of these hairs during copulation may trigger mechanisms of the post-mating decline of pheromone titer, females were mated with a male whose penis was surgically excised prior to mating. Females mating with such a penis-less male for 1 hr maintained a pheromone titer comparable to the titer of virgin females, while females mating with an intact male for the same length of time had a low titer (Fig. 6). However, the pheromone titer of females mating with a penis-less male gradually decreased as the duration of copulation increased.

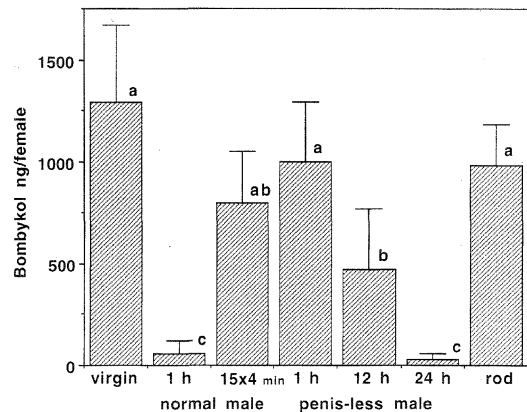


Fig. 6. Mean titers of pheromone in females that were mated with normal or defective males, or that receive mechanical stimulation of the bursa copulatrix. Females were mated for 1 hr, with a normal male (1 hr) or 4 different males (15x4 min), or mated for different periods of time (1 hr, 12 hr or 24 hr) with a (penis-less) male whose penis was excised before mating. A thin glass rod was inserted into the bursa copulatrix for 1 hr to mimic introduction of a penis into the bursa (rod). Mean $\pm$ SE are shown for each bar. N=6-10. Different letters (a-c) indicate statistically different groups ($P\leq 0.05$, Mann-Whitney *U*-test).

When females mated for 18 hr or 24 hr, their pheromone titers declined to the same level seen in females mating with an intact male for 1 hr (Fig. 6).

A female was successively mated with four different males, 15 min for each male. Although in such a female, the male genital organ was inserted in her bursa copulatrix for 1 hr, an high titer of pheromone continued to be produced (Fig. 6). The female had none or only a small spermatophore (<1.5 mm in diameter) in the bursa.

To mimic introduction of a penis into the bursa copulatrix, a thin glass rod (ca. 250 μ m in diameter) was inserted into the bursa of a female for 1 hr, and pheromone was extracted 23 hr later. Such a mechanical stimulation of the organ induced no significant drop in pheromone production (Fig. 6).

Effect of transection of a seminal duct on pheromone titer

The sperm ejaculated into the bursa copulatrix migrates to the spermatheca via the seminal duct and the oviduct (Omura, 1938). To determine whether migration of the sperm toward the spermatheca would induce a signal for inactivation of pheromone production, the seminal duct connecting the bursa copulatrix to the oviduct was cut prior to mating. Pheromone production in 6 of 10 females with a disconnected seminal duct was significantly inhibited by mating (>50 ng/female), though in 3 females, an high pheromone titer was maintained after mating (>1000 ng/female). The failure in inactivation of pheromone production in the latter may be due to injury of a peripheral neural pathway for inactivation of pheromone production, because a similar event sometimes occurred in sham-operated females. A female with a disconnected seminal duct, like an intact female, usually began to lay eggs after 4-hr mating. The lacking of pigmentation of these eggs after oviposition indicted that they were unfertilized. Thus, sperms and accompanying substances do not need to reach the seminal duct to induce the signal for inactivation of pheromone production.

DISCUSSION

Two forms of PBAN molecules (I and II) have been isolated from *Bombyx mori*: PBAN-I is a 33-residue peptide and PBAN-II is the same as PBAN-I except for the presence of an additional residue at the N-terminus (Kitamura *et al.*, 1989, 1990). cDNA encoding a precursor for PBAN-II contains sequences of four other neuropeptides, diapause hormone (DH) and three structurally-related neuropeptides (Kawano *et al.*, 1992; Sato *et al.*, 1993). DH and three peptides have a C-terminal sequence similar to that of PBAN and some have a significant pheromonotropic activity (Sato *et al.*, 1993). Thus, multiple species of peptides may be involved in the control of pheromone production of *Bombyx mori*. Although DH has a strong pheromonotropic activity (Sato *et al.*, 1993), it may not function as a pheromonotropic peptide in a female moth because DH in the SG appears to be almost depleted before adult emergence (Sonobe *et al.*, 1977).

There are twelve neurosecretory cells near the ventral

midline of SG of *Bombyx mori* and these can be visualized by *in situ* hybridization using the cDNA for the common precursor polypeptide (Sato *et al.*, 1994) or by immunocytochemistry using an antiserum raised against PBAN or DH (Ichikawa *et al.*, 1995). They are aggregated into three clusters localized in the anterior (mandibular), medial (maxillary) and posterior (labial) neuromeres of the SG. Presence of the medial cluster of the cells are essential for a strong pheromonotropic activity (Ichikawa *et al.*, 1996). The medial cells have an axon terminal in the neurohaemal organ, the corpus cardiacum (CC) (Ichikawa *et al.*, 1995), thereby suggesting that the cells release pheromonotropic peptides from the CC into the haemolymph to activate pheromone production in the pheromone gland. When the central nervous system of virgin females was transected between the brain and the SG, their pheromone titer declined to the same level as that of decapitated females in 24 hr after transection (Table 1). Thus, the brain may have a neural mechanism that activates neurosecretory cells to release pheromonotropic peptides to maintain pheromone production of virgin females. A possible activation of biosynthesis of the peptides in the neurosecretory cells by the putative neural mechanism may be excluded, because the expression of the gene for DH/PBAN terminates before eclosion and does not occur in the adult (Xu *et al.*, 1995). Since the pheromone titer of virgin females changes with a daily rhythm: maximal at the photophase and minimal at the scotophase (Ando *et al.*, 1988), the putative neural mechanism may couple with a circadian clock.

The act of copulation and successful transfer of male gametes to a female moth causes inactivation of pheromone biosynthesis, maturation of oocytes and initiation of ovipositional behavior (Truman and Riddford, 1974). There are different mechanisms in the inactivation of pheromone production after mating (Foster and Roelofs, 1994; Ramaswamy *et al.*, 1994). In a few species of *Helicoverpa* moths, an humoral factor transferred from a male to its mate during copulation appears to act on the central or peripheral nervous system to inhibit the release or action of PBAN (Kingan *et al.*, 1993, 1995; Raina, 1989; Ramaswamy *et al.*, 1994). In a tortricid moth, a neural signal elicited in a peripheral receptor by mating seems to inhibit the release of a pheromonotropic peptide (PBAN) to inactivate pheromone biosynthesis in the pheromone gland, because (1) an intact ventral nerve cord is needed for such an inactivation of pheromone production, (2) the head of mated females contains a larger amount of pheromonotropic peptides than does the head of virgin females, and (3) sensitivity of the pheromone gland of mated females to injected PBAN does not differ from that of decapitated females (Foster, 1993). The results in the present study suggests that a similar neural mechanism may be involved in inactivation of pheromone production in *Bombyx mori* (Figs. 3 and 5, Table 1).

The release of pheromonotropic peptides seems to be irreversibly blocked by a neural signal induced by mating, because an intact ventral nerve cord of the female is no longer needed for inactivation of pheromone production 1–2 hr after

the start of copulation (Fig. 4). A similar finding was noted in an apple moth, *Epiphyas postvittana*, though its nerve cord is needed until 1–2 hr after termination of copulation (Foster, 1993). There likely exists a neural mechanisms in the brain-SG complex that maintains the neurosecretory cells an inactive state and the signal of mating is needed only for triggering such an inhibitory neural mechanism. If this is the case, the inhibitory neural mechanism can work in two possible ways: direct inhibition of neurosecretory cells or indirect inhibition through depression of the putative neural mechanism for activation of neurosecretory cells.

Mating changes behavioral patterns of a female cabbage white butterfly from acceptance of a male to rejection of remating (Obara *et al.*, 1975). A pair of stretch receptors has been found in the bursa copulatrix and afferent signals from the receptors seem to trigger such a behavioral change (Sugawara, 1979, 1981). What type of receptors are involved in the post-mating inactivation of pheromone production in moths remains to be determined. Although mechanical stimulus caused by introduction of male genitalia into the bursa copulatrix may trigger an initial, transient suppression of pheromone production in a female gypsy moth, another signal elicited by sperm migrating into the spermatheca seems to be essential for permanent suppression (Giebultowicz *et al.*, 1991). Involvement of the sperm, or an accompanying substance present in the female reproductive tracts, in the neural inactivation of pheromone production, has been suggested for another species of moth, *Epiphyas postvittana* (Foster, 1993). In *Bombyx mori*, the physical act of copulation may not provide a signal for rapid inactivation of neurosecretory cells that seems to occur at 30–60 min after the beginning of copulation, though it has a significant suppressive effect on the pheromone production when the act is prolonged (Fig. 6). Mechanical stretching of the bursa copulatrix may not induce a signal for the depression, because some females mated for 30–40 min had a large spermatophore yet their pheromone titers remained high.

Omura (1938) described ejaculatory processes of *Bombyx mori*. Ejaculation of the semen begins at 5–10 min after the start of copulation and lasts for 25–30 min. Transparent fluid in the ejaculatory duct is first ejaculated for 5–10 min and then follows ejaculation of sperm which lasts for about 10 min. Finally, emission of semen without sperms occurs for about 10 min. The result of the present study indicates that suppression of pheromone production by mating usually occurs at 30–60 min after the beginning of copulation (Figs. 2 and 4). The critical time for the suppression of pheromone production seems to correspond to the final stage of the ejaculation of the semen, though there may be a racial difference in the time course of the ejaculation. If suppression of pheromone production is triggered by a factor transferred from the male during copulation, a possible candidate for such a factor may originate from the accessory gland, a peptide from which has a pheromonostatic activity in *Helicoverpa zea* (Kingan *et al.*, 1993, 1995). In a preliminary experiment, however, a large drop in pheromone titers did not occur in

females mated with males whose testes were removed at the earliest pupal stage but with accessory glands intact. In addition, an injection of haemolymph (50 μ l) from a mated female into a virgin female failed to induce a significant drop of pheromone production of the virgin (data not shown), although it could not be excluded that the factor may be present in the haemolymph for a short, specific period of time during copulation. A male of *Bombyx mori* appears to transfer a substance to its mate, a substance that can pass through the wall of the bursa copulatrix into the haemocoel and act on the nervous system to release ovipositional behavior from the female (Yamaoka and Hirao, 1977). It is possible that the same or a similar substance stimulates an unknown receptor that sends a mating signal to the CNS through the N4 (Fig. 3). Identification of the putative receptor and analyses of its physiological properties may aid in elucidating the localization and nature of the substance or any other stimulus triggering regulatory mechanisms of pheromone production after mating.

ACKNOWLEDGEMENTS

We thank M. Ohara for helpful comments on the manuscript. This work was supported in part by Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan.

REFERENCES

- Ando T, Hase T, Funayoshi A, Arima R, Uchiyama M (1988) Sex pheromone biosynthesis from 14 C-hexadecanoic acid in the silkworm moth. *Agric Biol chem* 52: 141–147
- Arima R, Takahara K, Kadoshima T, Numazaki F, Ando T, Uchiyama M, Nagasawa H, Kitamura A, Suzuki A (1991) Hormonal regulation of pheromone biosynthesis in the silkworm moth, *Bombyx mori* (Lepidoptera: Bombycidae). *Appl Entomol Zool* 26: 137–147
- Butenandt A, Beckmann R, Stamm D, Hecker E (1959) Über den Sexual-Lockstoff des Seidenspinners *Bombyx mori*. Reindarstellung und Konstitution. *Z Naturforsch (B)* 14: 283–284
- Christensen TA, Itagaki H, Teal PEA, Jasensky RD, Tumlinson JH, Hildebrand JG (1991) Innervation and neural regulation of the sex pheromone gland in female *Heliothis* moths. *Proc Natl Acad Sci USA* 88: 4971–4975
- Fonagy A, Matsumoto S, Uchiyama K, Orikasa C, Mitsui T (1992) Action of pheromone biosynthesis activating neuropeptide on pheromone glands of *Bombyx mori* and *Spodoptera litura*. *J Pest Sci* 17: 47–54
- Foster SP (1993) Neural inactivation of sex pheromone production in mated lightbrown apple moths, *Epiphyas postvittana* (Walker). *J Insect Physiol* 39: 267–273
- Foster SP, Roelofs WL (1994) Regulation of pheromone production in virgin and mated females of two tortricid moths. *Arch Insect Biochem Physiol* 25: 271–285
- Giebultowicz JM, Raina AK, Uebel EC, Ridgway RL (1991) Two-step regulation of sex-pheromone decline in mated gypsy moth females. *Arch Insect Biochem Physiol* 16: 95–105
- Ichikawa T, Hasegawa K, Shimizu I, Katsuno K, Kataoka H, Suzuki A (1995) Structure of neurosecretory cells with immunoreactive diapause hormone and pheromone biosynthesis activating neuropeptide in the silkworm, *Bombyx mori*. *Zool Sci* 12: 703–712
- Ichikawa T, Shiota T, Shimizu I (1996) Functional differentiation of neurosecretory cells with immunoreactive diapause hormone and pheromone biosynthesis activating neuropeptide of the moth,

- Bombyx mori*. Zool Sci 13: 21–25
- Jacquin E, Jurenka RA, Ljungberg H, Nagman P, Lofstedt C, Descoins C, Roelofs WL (1994) Control of sex pheromone biosynthesis in the moth *Mamestra brassicae* by the pheromone biosynthesis activating neuropeptide. Insect Biochem Molec Biol 24: 203–211
- Jurenka RA, Fabrias G, Ramaswamy S, Roelofs WL (1993) Control of pheromone biosynthesis in mated redbanded leafroller moths. Arch Insect Biochem Physiol 24: 129–137
- Jurenka RA, Fabrias G, Roelofs WL (1991) Hormonal control of female sex pheromone biosynthesis in the redbanded leafroller moth, *Argyrotaenia velutinana*. Insect Biochem 21: 81–89
- Kawano T, Kataoka H, Nagasawa H, Isaogai A, and Suzuki A (1992) cDNA cloning and sequence determination of the pheromone biosynthesis activating neuropeptide of the silkworm, *Bombyx mori*. Biochem Biophys Res Commun 189: 221–226
- Kingan TG, Thomas-Laemont PA, Raina AK (1993) Male accessory gland factors elicit change from 'virgin' to 'mated' behaviour in the female corn earworm moth *Helicoverpa zea*. J exp Biol 183: 61–76
- Kingan TG, Bodner WM, Raina AK, Shabanowitz J, Hunt DF (1995) The loss of female sex pheromone after mating in the corn earworm moth *Helicoverpa zea*: Identification of a male pheromonostatic peptide. Proc Natl Acad Sci USA 92: 5082–5086
- Kitamura A, Nagasawa H, Kataoka H, Inoue T, Matsumoto S, Ando T, Suzuki A (1989) Amino acid sequence of pheromone-biosynthesis-activating neuropeptide (PBAN) of the silkworm, *Bombyx mori*. Biochem Biophys Res Commun 163: 520–526
- Kitamura A, Nagasawa H, Kataoka H, Ando T, Suzuki A (1990) Amino acid sequence of pheromone biosynthesis activating neuropeptide-II (PBAN-II) of the silkworm, *Bombyx mori*. Agric Biol Chem 54: 2495–2497
- Masler EP, Raina AK, Wagner RM, Kochansky JP (1994) Isolation and identification of a pheromonotropic neuropeptide from the brain-suboesophageal ganglion complex of *Lymantria dispar*: a new member of the PBAN family. Insect Biochem Molec Biol 24: 829–836
- Nishimura K, Furihata N, Ikeda H (1959) Effect of cutting operation of the nerve fibers from 9th ganglion on the oviposition of the moths *Bombyx mori* and *Antheraea pernyi*. J Seri Sci Japan 28: 10–16 (in Japanese with English summary)
- Obara Y, Tateda H, Kuwabara M (1975) Mating behavior of the cabbage white butterfly, *Pieris rapae crucivora* Biosduval. V. Copulatory stimuli inducing changes of female response patterns. Zool Mag 84: 71–76
- Omura S (1938) Studies on the reproductive system of the male of *Bombyx mori*. II. Post-testicular organs and post-testicular behaviour of the spermatozoa. J Fac Agri Hokkaido Imp Univ 40: 129–170
- Raina AK (1989) Male-induced termination of sex pheromone production and receptivity in mated females of *Heliothis zea*. J Insect Physiol 35: 821–826
- Raina AK (1993) Neuroendocrine control of sex pheromone biosynthesis in Lepidoptera. Annu Rev Entomol 38: 329–349
- Raina AK, Jaffe H, Kempe TG, Keim P, Blacher RW, Fales HM, Riley CT, Klun JA, Ridgway RL, Hayes DK (1989) Identification of a neuropeptide hormone that regulates sex pheromone production in female moths. Science 244: 796–798
- Raina AK, Jaffe H, Klun JA, Ridgway RL, Hayes DK (1987) Characteristics of a neurohormone that controls sex pheromone production in *Heliothis zea*. J Insect Physiol 33: 809–814
- Ramaswamy SB, Mbata GN, Cohen NE, Moore A, Cox NM (1994) Pheromonotropic and pheromonostatic activity in moths. Arch Insect Biochem Physiol 25: 301–315
- Sato Y, Oguchi M, Menjo N, Imai K, Saito H, Ikeda M, Isobe M, Yamashita O (1993) Precursor polypeptide for multiple neuropeptides secreted from the suboesophageal ganglion of the silkworm *Bombyx mori*: Characterization of the cDNA encoding the diapause hormone precursor and identification of additional peptides. Proc Natl Acad Sci USA 90: 3251–3255
- Sato Y, Ikeda M, Yamashita O (1994) Neurosecretory cells expressing the gene for common precursor for diapause hormone and pheromone biosynthesis-activating neuropeptide in the suboesophageal ganglion of the silkworm, *Bombyx mori*. Gen Comp Endocrinol 96: 27–36
- Sonobe H, Hiyama Y, Keino H (1977) Changes in the amount of the diapause factor in the suboesophageal ganglion during development of the silkworm, *Bombyx mori*. J Insect Physiol 23: 633–637
- Soroker V, Rafaeli A (1989) *In vitro* hormonal stimulation of [¹⁴C] acetate incorporation by *Heliothis armigera* pheromone glands. Insect Biochem 19: 1–5
- Sugawara T (1979) Stretch reception in the bursa copulatrix of the butterfly, *Pieris rapae crucivora*, and its role in behavior. J Comp Physiol 130:191–199
- Sugawara T (1981) Fine structure of the stretch receptor in the bursa copulatrix of the butterfly *Pieris rapae crucivora*. Cell Tissue Res 217: 23–36
- Tang JD, Charlton RE, Jurenka RA, Wolf WA, Phelan PL, Sreng L, Roelofs WL (1989) Regulation of pheromone biosynthesis by a brain hormone in two moth species. Proc Natl Acad Sci USA 86: 1806–1810
- Teal PEA, Tumlinson JH, Oberlander H (1989) Neural regulation of sex pheromone biosynthesis in *Heliothis* moths. Proc Natl Acad Sci USA 86: 2488–2492
- Truman JW, Riddford LM (1974) Hormonal mechanisms underlying insect behavior. Adv Insect Physiol 10: 297–352
- Yamaoka K, Hirao T (1977) Stimulation of virginal oviposition by male factor and its effect on spontaneous nervous activity in *Bombyx mori*. J Insect Physiol 23: 57–63
- Xu W-H, Sato Y, Ikeda M, Yamashita M (1995) Stage-dependent and temperature-controlled expression of the gene encoding the precursor protein of diapause hormone and pheromone biosynthesis activating neuropeptide in the silkworm, *Bombyx mori*. J Biol Chem 270: 3804–3808