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Physiological control of calling, pheromone release, and pheromone production in the female gypsy moth, Lymantria dispar.

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PHYSIOLOGICAL CONTROL OF CALLING, PHEROMONE RELEASE, AND PHEROMONE PRODUCTION IN THE FEMALE GYPSY MOTH, *LYMANTRIA DISPAR*

A Thesis Presented

By

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AND PHEROMONE PRODUCTION IN THE FEMALE GYSPY MOTH,

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PHYSIOLOGICAL CONTROL OF SEX PHEROMONE COMMUNICATION

Introduction

Anecdotal evidence for insect chemical communication dates as far back to the 17th century when Butler (1609) noted that honeybees were inclined to sting previously stung areas. Later, in the early 20th century, Fabré (1916) showed that odor perception was involved in the communication system used by male moths to locate conspecific females. Increased scientific interest in insect chemical communication, however, did not occur until relatively recently.

Pheromone was a term originally coined by Karlson and Lüscher (1959) to denote chemical(s) secreted by one individual which elicit(s) a specific response from another member of the same species. The nature of the response prompted the classification of pheromones into 2 broad categories. Releaser pheromones trigger a programmed behavioural reaction by the receiver, whereas primer pheromones cause a physiological change in the receiver such that subsequent reactions to environmental stimuli are different from individuals which were not primed (Wilson and Bossert, 1963). Further subclassification of pheromones pertains to their primary function, e.g. alarm pheromones, aggregation pheromones, oviposition-deterrent pheromones, sex pheromones, etc.

Chemicals which act as sex pheromones are synthesized either by modification of plant-derived compounds as in the bark beetles (Hendry et al., 1980; Fish et al., 1984) or de novo from simple precursors as in the moths (Bjostad and Roelofs, 1981; Bjostad and Roelofs, 1983). Biosynthesis in those instances described occurs in specialized epithelial tissues which histologically show characteristic features of exocrine glands (Percy and Weatherston, 1971). During sex pheromone emission, many species also exhibit a behavior which has been termed "calling" and typically involves a particular stance and exposure of the pheromone gland region.

Physiological control of processes which result in pheromone emission into the air can involve one factor which simultaneously controls pheromone release (from glandular cells), calling, and pheromone production or it may involve separate control mechanisms for each or some permutation thereof. In the past calling, pheromone release, and pheromone production were not well distinguished and one mechanism was believed to control all three processes. Recently, however, a hypothesis for separate control mechanisms is favored. Documentation of diel periodicities for calling behavior, pheromone emission rate, and glandular titer of pheromone in several species of moths has shown that calling females can emit wide ranges of pheromone over the course of a day (Bjostad et al., 1980; Charlton and Cardé, 1982; Pope et al., 1982; Haynes et al., 1983; Pope et al., 1984) and pheromone titer within the gland can exhibit high levels during periods of non-calling (Shorey and Gaston, 1965; Webster and Cardé, 1982).

Three possible physiological control mechanisms exist: endocrine, neural, and neuroendocrine. Available evidence for or against each of these will be presented for the insect groups from which work has been done, i.e. the cockroaches, Musca domestica, the beetles, and the moths. Unless specified otherwise, pheromone release refers generally to pheromone emission into the air and not the specific mechanisms required for release of pheromone from the glandular cells.

Endocrine Control

Cockroaches

Endocrine control of sex pheromone release by the corpora allata (CA) has been the favored hypothesis primarily due to the works of Barth and others in several species of cockroaches (Engelman, 1960; Barth, 1961b, 1962; Roth, 1962; Roth and Barth, 1964; Barth, 1965; Bell and Barth, 1970). In the majority of these experiments, however, assessment of sex pheromone release was determined using response bioassays of conspecifics since analytical techniques such as GLC quantification were not yet feasible. As a result, misinterpretation of the data has not been infrequent.

Two such examples involve Leucophaea maderae and Nauphoeta cinerea. Engelman (1960) was the first to demonstrate that 70% of the L. maderae females allatectomized shortly after the imaginal molt failed to mate with males. Reimplantation of CA into allatectomized

females restored normal female sexual behavior. Engelman (1960) interpreted failure to mate in allatectomized L. maderae as lack of female receptivity. Based on experiments in Byrsotria fumigata, Barth 1961a) suggested that Engelman's conclusion (1960) of the effect of allatectomy on female receptivity may have been incorrect. Instead, Barth (1961a) proposed that failure to mate was due to effects of alltectomy on pheromone release and not due to differences in female receptivity.

When allatectomy experiments were repeated in L. maderae (Roth and Barth, 1964), another discrepancy arose because Roth and Barth found that allatectomy did not prevent mating. Explanations besides strain differences specified the type of situation in which operated females were allowed to mate with males. In Engelman's study (1960), only 2 pairs of individuals were in each cage, whereas in Roth and Barth's study (1964) several pairs were in a cage. Since tactile stimulation in the absence of females was enough to elicit male courtship behavior (wing-raising display) (Barth, 1961a), Roth and Barth (1964) believed that the contact in the crowded cage stimulated male courtship behavior and resulted in successful mating by allatectomized females despite reduced pheromone release. Mating can only occur if the female showed her receptive state by feeding on male tergal secretions after he raised his tegmina. Although Roth and Barth (1964) never quantitatively substantiated that allatectomy caused reduced pheromone release, they did show that allatectomy had no influence on female receptivity in L. maderae.

The evidence for CA control of pheromone release in female N. cinerea is weak and was based on the correlation of the onset of female receptivity with the onset of CA activity (determined by oocyte length) (Roth, 1962). Roth (1962) stated that allatectomy adversely affected pheromone release although as Roth and Barth (1964) noted subsequently, allatectomies were never actually performed. As in L. maderae, allatectomy did not prevent successful mating in N. cinerea, but it did cause a 5 day delay of the time when mating began compared to controls (Roth and Barth, 1964). Since tactile stimulation in N. cinerea also elicited male courtship behavior in the absence of females (Barth, 1961a), the influence of the CA on pheromone release remains unclear. Female receptivity, however, seems unaffected by allatectomy.

It should be noted that Blomquist and Dillwith (1983) improperly cited Periplaneta americana and Blaberus discoidallis as two other species for which the CA influences pheromone production. Evidence for the former came from a personal communication by Yamamoto (Barth, 1965) and unpublished observations by Roufa and Barth (Barth and Lester, 1973) which to my knowledge have not been published. Information for B. discoidallis was also unpublished by Black and Barth (Barth and Lester, 1973).

Despite these incongruities, substantial evidence for CA control of sex pheromone release has been presented for B. fumigata (Barth, 1961b; Barth, 1962; Roth and Barth, 1964; Bell and Barth, 1970). Unlike L. maderea and N. cinerea, B. fumigata males required female sex pheromone stimulation and did not display courtship behavior after

receiving only tactile stimulation (Barth, 1961a). After removing the CA from B. fumigata females 1 to 3 days following the imaginal molt, 85.6% of the females failed to release pheromone compared to 9.6% in the unoperated controls (Barth, 1961b). Pheromone release was assayed by measuring the male wing-raising response to filter papers which lined the test female's cage. Reimplantation of CA renewed pheromone release in 12 of 27 females (Barth, 1962).

To examine the effect of allatectomy on female sexual receptivity, allatectomized females were placed with males into cages which were lined with a filter paper known to contain pheromone (Barth, 1962) or pheromone was smeared onto allatectomized females by vigorously rubbing them with control females (Roth and Barth, 1964). Mating proceeded normally in both cases indicating that allatectomy did not affect other aspects of female sexual behavior.

A suggested mechanism for juvenile hormone (JH) control of pheromone release was provided by administering increasing doses of synthetic JH and farnesyl methyl ether to allatectomized females (Bell and Barth, 1970). Lower doses (1 ug applied once or 1 ug once then again 14 days later) enhanced pheromone release whereas a high dose (2 ug once then again 14 days later) reduced pheromone production. Concurrent observations on yolk deposition and colleterial gland activity suggested that titers of JH required for maximal egg development and ovulation were inhibitory for pheromone production. This hypothesis may also explain the lack of pheromone release which occurred after mating (Barth and Bell, 1970).

An interesting approach to CA involvement in pheromone release determined the effects of allatectomy in two strains of Pycnoscelus (Barth, 1965) which have since been segregated into 2 closely related species, P. indicus which is bisexual and P. surinamensis which is parthenogenetic (Barth and Lester, 1973). Failure to release sex pheromone was found in most bisexual females allatectomized shortly after the imaginal molt, and continued pheromone production occurred in allatectomized parthenogenetic females. In two Lepidoptera, Antheraea pernyi (Barth, 1965) and Galleria mellonella (Röller et al., 1963) which eclose with fully developed eggs and have a brief adult life, allatectomy also proved to have no effect on pheromone release in field tests of male attraction.

These findings led Barth (1965) to propose a hypothesis, i.e. selection for endocrine control of pheromone production may only be expected in those species for which the adult exhibits multiple reproductive cycles during which there are periods when successful mating is not possible. As a corollary, these adults typically feed and are long-lived. The generality of Barth's hypothesis, however, remains to be determined.

Musca domestica

In the housefly, M. domestica, female sex pheromone was produced from modified cuticular lipids (Blomquist and Dillwith, 1983) of the abdominal integument (Dillwith et al., 1981). Production of pheromone started about 2 days after adult eclosion when vitellogenesis begins.

Dillwith et al. (1983) demonstrated that a factor released from vitellogenic ovaries was responsible for pheromone production by using GLC and radio-GLC analysis of hexane-extracted flies. Females which were ovariectomized within 12 hrs of eclosion failed to produce detectable amounts of pheromone. Furthermore, comparison of the relative incorporation of (1-^{14}C)-acetate into alkane and alkene fractions showed that the cuticular composition of these ovariectomized females resembled the pattern seen in pre-vitellogenic females. Before vitellogenesis, a greater amount of the radioactive label was incorporated into the alkene fraction (64%), whereas during vitellogenesis an increasing amount of the label was found in the alkane fraction until post-vitellogenesis when approximately 76% of the label was in the alkane fraction. These trends apparently reflected the biosynthetic pathway of the pheromone blend based on unpublished data of Blomquist and Dillwith (cited from Dillwith et al., 1983).

Pheromone analysis of ovariectomized flies previously receiving implants of previtellogenic ovaries showed that synthesis of (Z)-9-tricosene, the principal pheromone component, had begun 3 to 4 days later. Although evidence for synthesis of the two other pheromone components, (Z)-9,10-epoxytricosane and (Z)-14-tricosen-10-one was not verified, Dillwith et al. (1983) did find that incorporation of the radioactive label into alkane and alkene fractions followed the pattern observed during vitellogenesis.

After oviposition, female M. domestica continued to produce some pheromone. Ovariectomy of females after oviposition did not cause any

significant difference in the amount of (Z)-9-tricosene compared to controls. Dillwith et al. (1983) interpreted these results to mean that although vitellogenic ovaries were required for the initiation of pheromone production, they were not required to maintain it. Since, female M. domestica are monocoitic, continued pheromone production after oviposition is unusual. Dillwith et al. (1983) proposed, however, that it would ensure successful mating by all females in a population.

Ecdysone may be the endocrine factor involved since it has been described as an ovarian hormone in other Diptera (Hagedorn, 1981). Moreover, in M. domestica, implantation of an ovaries about to undergo vitellogenesis or injections of 20-hydroxyecdysone into males induced female sex pheromone production by the male recipients (Blomquist et al., 1984). Further work, though, will have to be done to show that vitellogenic ovaries in M. domestica indeed secrete ecdysone at the appropriate time and that the abdominal epithelium is a target. Moreover, since Dillwith et al. (1983) used relative incorporation of the radioactive label in alkene and alkane fractions as one of their indicators for pheromone production, more detailed relationships which relate this index with pheromone biosynthesis should be explained.

Saturniidae (Lepidoptera)

At one time, endocrine control of calling by intrinsic secretory cells of the corpora cardiaca (CC) in Antheraea polyphemus and Hyalophora cecropia was purported to occur. Riddiford and Williams

(1971) observed that females of both species which had their CA removed in the pupal stage called normally as adults. If both the CC and CA were extirpated, however, a significant reduction in calling was noted. Since reimplantation of CC-CA complexes did not restore calling in either species, they concluded that intrinsic cells of the CC, which apparently were destroyed after contact with the brain was severed, released a substance responsible for calling.

Work by Sasaki et al. (1983) in the same 2 species failed to substantiate the findings of Riddiford and Williams (1971). Furthermore, in none of the Lepidoptera investigated to date, Lymantria dispar (Hollander and Yin, 1985), Manduca sexta and Utetheisa ornatrix (Itagaki, 1984), G. mellonella (Roller et al., 1963), and A. pernyi (Barth, 1965) has a role of the CA and/or CC been found for calling or pheromone release.

Endocrine, Neuroendocrine, and Neural Control

Beetles

In the bark beetles, investigation has shown that there is both endocrine and neural control mechanisms involved in pheromone production. Male Ips paraconfusus (formerly I. confusus) produced and began releasing pheromone approximately 4 to 6 hrs after entering host pine logs (Borden et al, 1969). The components of the male pheromone blend depended on host monoterpenes, myrcene and (-)-alpha-pinene, as precursors for pheromone synthesis (Hendry et al., 1980; Fish et al.,

1984). Modifications of these host-derived terpenes occurred in the hindgut and Malpighian tubule region, and pheromone was released through defecation.

Initial experiments by Borden et al. (1969) implicated JH in pheromone production. Topical application of synthetic JH (100 ug) to the abdominal venter induced pheromone production in males 24 hrs after JH application equivalent to that produced by males boring in host pine logs. Measurement of production was determined by female response to extractions of hindgut and Malpighian tubule regions. The minimal time after JH application before pheromone levels elicited female response was 18 hrs. Males boring in pine logs, however, showed some pheromone production as soon as 3 hrs after entering the wood.

Using GLC analysis of hindgut extractions, Hughes and Renwick (1977) performed a series of 5 experiments which demonstrated that there are several factors, endocrine, neuroendocrine, neural, and availability of host volatiles, which mediate pheromone production. Following topical application of JH (50 ug) to the mesosternum of newly-emerged males, they found increased production of 3 of the 4 pheromone components, ipsenol, ispdienol, and 2-phenylethanol, but no production of cis-verbenol, the remaining component. These beetles were held 18 hrs without contact with host volatiles before extraction of the hindgut. Exposing treated beetles to myrcene vapors for 22 hrs greatly enhanced the amount of pheromone production. Thus, JH induced pheromone production but production was further enhanced by the presence of myrcene.

Forcible distention of the male midgut and foregut regions followed by myrcene vapor exposure for 20 hrs also resulted in pheromone production at levels greater than that found in males fed 24 hrs on pine and substantially higher than males merely exposed to myrcene. Hughes and Renwick (1977) postulated that neural inhibition of JH release was removed after feeding by sensory feedback information from gut stretch receptors.

Implication of a neuroendocrine factor which they called "brain hormone" (BH) arose from experiments which examined the effects of decapitation, JH treatment, and gland implants on pheromone levels. Implants of CC into intact beetles resulted in significantly higher levels of pheromone than CA implants. Male beetles which were decapitated produced very little pheromone but significantly more was produced following CC implants. Furthermore, topical application of JH to decapitated insects did not increase pheromone production compared to decapitated controls. Hughes and Renwick (1977) concluded that once inhibition of JH release was lifted by gut distention, JH in turn stimulated release of a neurosecretion from the CC via the brain which was responsible for activating the enzymatic machinery for pheromone production from host volatiles.

This is a promising hypothesis and may apply to other beetles such as pheromone production by female Tenebrio molitor. Menon (1970) showed that decapitation resulted in about 25% decrease in male response to female extracted pheromone and a 50% decrease if both the brain and CA were removed. Topical application of JH restored

pheromone production in both allatectomized and decapitated females. Further work, however, will be needed to establish if similarities in control of pheromone production and release exist between I. paraconfusus and T. molitor.

There are, however, a few difficulties in the investigation of Hughes and Renwick (1977). The first applies to their method of GLC analysis. Rather than using an internal standard to quantify amounts of ipsenol, ipsdienol, etc., they merely reported peak heights found at the retention times for each pheromone component. In addition, they explained that there was an extreme degree of seasonal variation in the ability of male I. paraconfusus to produce pheromone after topical application of JH. This seasonal variation presumably accounted for the lack of pheromone observed after application of JH in one of their tables. This explanation, however, does not account for other anomalies. For example, implants of CC + CA from unfed males into newly-emerged males had a 2-fold effect in increasing pheromone levels compared to males receiving implants from either fed males or unfed females. Since their hypothesis claims that the CA only release JH following gut distention, one wonders why the CC + CA from unfed males was so active.

Moths

As I have indicated earlier, endocrine control of calling or pheromone release by the CA and/or CC has never been found (Röller et al., 1963; Barth, 1965; Sasaki et al., 1983; Itagaki, 1984; Hollander

and Yin, 1985) or demonstrated conclusively (Riddiford and Williams, 1971; Sasaki et al., 1983). Sasaki et al. (1983) was able to further rule out all endocrine involvement in calling by specially ligating the abdomen while leaving the nervous system intact. In these ligated females, percent calling was comparable with controls. Removal of ovaries from last instar L. dispar larvae also had no effect on calling or pheromone release (Hollander and Yin, 1985).

In the moths primary control mechanisms of calling and pheromone release appear to be neural. The details of nervous control, of calling, however, seem to vary with species. Hollander and Yin (1982) found that L. dispar females which were subjected to brain removal as pupae or received transection of the ventral nerve cord (VNC) anterior to the terminal abdominal ganglion (TAG), continued to call (79% and 81%, respectively) but only 0% and 6%, respectively, released pheromone to elicit a wing-fanning response from males. Severing the circumesophageal connectives produced similar results (Hollander and Yin, 1985). In sham-operated controls, on the other hand, over 95% called and over 60% released pheromone (Hollander and Yin, 1982). Removing the TAG or cutting the nerves posterior to the TAG resulted in no calling and no pheromone release in the former, and 8% calling and 8% pheromone release in the latter (Hollander and Yin, 1982).

Based on these results, Hollander and Yin (1982) postulated that calling was under nervous control of the TAG which contained a central pattern generator for calling. Pheromone release, on the other hand, was controlled by higher centers in the nervous system (i.e. the

brain). It was unclear from the context whether pheromone release in this case referred to those processes resulting in pheromone emission into the air or specifically from the glandular cells themselves. Nevertheless, inferences about control of pheromone release from this experiment may have been premature since they did not examine the amount of pheromone in the gland available for release.

Results from nerve transection experiments performed in 4 other species of moths, however, contrast with results reported by Hollander and Yin (1982). In H. cecropia and A. polyphemus, Sasaki et al. (1983) described calling behavior using a muscle transducer to record movements of the abdominal tip and found that removal of the first abdominal ganglion during the pupal stage eliminated calling in the adult. In M. sexta and U. ornatrix, Itagaki (1984) performed transection of the VNC at different levels of the abdominal nervous system, and in all cases, females were unable to call. Therefore, in these 4 species, calling was controlled by higher centers of the nervous system and not by the TAG alone. (Unfortunately, pheromone release and production in these 4 species were not examined and so no conclusions regarding their respective control mechanisms were made.)

In Platynota stultana, calling and pheromone production were controlled by factors in the head regions and JH was implicated in the switch from virgin to mated behavior (Webster and Cardé, 1984). After decapitation which removed influences from the brain and factors from the CA and CC, calling ceased and the glandular pheromone titer declined. Topical application of JH or JH analogues (10 ug) resulted

in the termination of calling, reduced pheromone titer, and induced oviposition comparable to mated females. The source of JH in mated P. stultana females, however, and details of its action on reproductive behavior require further investigation.

In H. cecropia, sperm reception into the bursa copulatrix induced the latter to release a factor which elicited oviposition and terminated calling (Riddiford and Ashenhurst, 1973). Presumably, the released factor acted on the nervous system to switch neural programs from virgin to oviposition behavior.

Neuroendocrine control of pheromone production has been proposed by Raina and Klun (1984) in Heliothis zea. The diel periodicity of pheromone titer was characterized by relatively small amounts of pheromone in the gland during photophase (2 to 8 ng) and much higher amounts during scotophase (Raina and Klun, 1986). Maximal titer (137 ng) occurred during the 3rd scotophase, 4 hrs after lights off (Raina and Klun, 1986). Gland-extractions during this time period from females which were neck-ligated 1 hr after adult eclosion showed a significant decrease in the pheromone titer (<1.0 ng) (Raina and Klun, 1984). Injection of saline extracts of brain homogenates into photophase females or neck-ligated females caused a significant increase in pheromone titer which was comparable to levels found in scotophase females (Raina and Klun, 1984). Since saline extracts of the CC had minimal effects of raising the pheromone titer, Raina and Klun (1984) concluded that the brain contained a neuroendocrine factor which activated increased pheromone production. This factor was

presumably stored in the brain during photophase and released into the hemolymph at the onset of scotophase.

Saline extracts of brain homogenates from 6 other species (H. phloxiphaga, the navel orangeworm, gypsy moth, granulate cutworm, fall cankerworm, and the bagworm) also had stimulatory effects on pheromone production in ligated H. zea females, Raina and Klun (1984) suggested that neuroendocrine control of pheromone production may occur in other Lepidoptera as well.

Concluding Remarks

Control of pheromone release, production, and calling is truly becoming much more complicated than previously recognized. Sources of endocrine factors appear to vary with insect groups. In the cockroaches, data support the CA as the control mechanism for pheromone release and production. Low titers of JH apparently stimulate pheromone release whereas high titers inhibit release. In M. domestica, hormonal control factors are derived from the ovaries. Although vitellogenic ovaries are responsible for the initiation of pheromone production, the ovaries are not required to maintain pheromone production.

In the beetles, combined neural, neuroendocrine, and endocrine factors control pheromone production. Until a bark beetle feeds on host materials, neural inhibition of JH release presides. Following

gut distention, neural inhibition is lifted and release of JH stimulates subsequent release of a neuroendocrine factor from the brain-CC complex. It is this latter factor which activates pheromone production by the hindgut.

There is virtually no doubt that control of calling behavior in the moths which involves coordinated muscle action to protrude and retract the abdominal tip is neural. For female L. dispar, a mechanism for neural control of pheromone release has been proposed which is independent of the neural control of calling. Although there is no evidence for endocrine control of calling, pheromone release, or pheromone production, pheromone production during specific times of the day may be mediated by a neuroendocrine factor from the brain.

Subsequent chapters will re-examine the role of the CA and the effect of VNC transection on calling, pheromone release, and pheromone production in the gypsy moth. In addition, the diel periodicity of pheromone production will be described and the effects age and mating have on pheromone titer.

C H A P T E R I I

EFFECT OF ALLATECTOMY AND VENTRAL NERVE CORD SECTION ON CALLING, PHEROMONE EMISSION, AND PHEROMONE PRODUCTION IN LYMANTRIA DISPAR

Abstract

The underlying endocrine and nervous controls of calling in virgin female gypsy moths were investigated. Mean pheromone emission rates from adult females allatectomized as larvae (14.1 ng/hr) were not significantly different from sham-operated (16.4 ng/hr), anesthetized (13.1 ng/hr), or untreated controls (13.1 ng/hr). Females which received ventral nerve cord transection anterior to the terminal abdominal ganglion between 0 and 24 hrs after pupation showed significant reductions in both gland-extractable pheromone ($\bar{x}$ = 8.7 ng) and pheromone emission rates ($\bar{x}$ = 2.1 ng/hr) compared with controls ($\bar{x}$ = 25.0 ng and $\bar{x}$ = 13.1 ng/hr, respectively). Differences in calling behavior were also observed; in control females, the duration of ovipositor protraction was ca. 16.5 sec and retraction ca. 1.3 sec, in ventral nerve cord-transected females, the ovipositor was maintained in a full or partially protruded state for periods longer than 300 sec. Post-operative inspection of the terminal abdominal ganglion revealed fewer descending nerve branches from the ganglia of ventral nerve cord transected females compared to controls.

Introduction

Pheromone emission in most female moths is associated with a "calling" behavior expressed as extrusion and retraction of the ovipositor. In the gypsy moth, Lymantria dispar (L.) (Lepidoptera: Lymantriidae) as is typical of many Lepidoptera (Percy and Weatherston, 1971), the pheromone gland is located on the intersegmental membrane between abdominal segments VIII and IX (Hollander et al., 1982). The pheromone, commonly known as disparlure, has been identified as cis-7,8-epoxy-2-methyloctadecane (Bierl et al., 1970).

Although the influence of exogenous factors such as temperature and photoperiod on calling in moths is well documented (Sower et al., 1971; Kaae and Shorey, 1972; Cardé and Roelofs, 1973; Fatzinger, 1973; Nordlund and Brady, 1974; Baker and Cardé, 1979; Alford and Hammond, 1982; Webster and Cardé, 1982; Haynes and Birch, 1984; Conner et al., 1985; Webster, 1986), much less is known about the underlying physiological control of calling, pheromone release, and pheromone production.

Evidence for endocrine control of pheromone release by the corpora allata (CA) has been demonstrated for some cockroaches (Engelmann, 1960; Barth, 1961b, 1962; Roth, 1962; Barth, 1965; Bell and Barth, 1970) and beetles (Borden et al, 1969; Menon, 1970; Hughes and Renwick, 1977; Harring, 1978). Corpora allata control of calling and/or pheromone release, however, has not been substantiated in the Lepidoptera investigated: Galleria mellonella (Röller et al., 1963),

Antheraea pernyi (Barth, 1965), A. polyphemus, Hyalophora cecropia (Sasaki et al., 1983), L. dispar (Hollander and Yin, 1985), and Manduca sexta and Utetheisa ornatrix (Itagaki, 1984). In the latter 5 species, an essential role of the corpora cardiaca (CC) for calling also was discounted.

Nervous control of calling in moths has been substantiated, but the site of control within the nervous system may vary with species. Hollander and Yin (1982) found that L. dispar females which were either brainless or received transection of the ventral nerve cord (VNC) anterior to the terminal abdominal ganglion (TAG) called but did not release pheromone. They concluded that calling and pheromone release were separately controlled events; calling was controlled by the TAG which acted as a central pattern generator (CPG) for the behavior, and pheromone release was controlled by higher centers of the nervous system. In A. polyphemus and H. cecropia, removal of the first abdominal ganglion eliminated calling even though the remaining ganglia were intact (Sasaki et al., 1983). Similarly, Itagaki (1984) reported the cessation of calling after nerve transection at different points along the VNC of M. sexta and U. ornatrix. Results from these 4 species indicate that anterior portions of the nervous system, presumably the brain, regulate calling.

A neuroendocrine factor has been implicated in the control of pheromone production. The amount of gland-extractable pheromone from Heliothis zea females during scotophase was significantly higher than during photophase when female did not call (Raina and Klun, 1984; Raina

and Klun, 1986). Injection of saline extracts of brain homogenates into photophase or neck-ligated females, however, caused a significant increase in pheromone titer which was comparable to levels found in scotophase females (Raina and Klun, 1984). Since saline extracts of the CC had only minimal effects on raising pheromone titer, Raina and Klun (1984) concluded that the brain harbored a factor which activated pheromone production. Furthermore, release of the brain factor into the general circulation may occur in response to exogenous cues such as photoperiod.

In addition to those mechanisms which control calling and pheromone production in virgin females, other factors may coordinate the termination of calling, pheromone release, and pheromone production during the transition from a virgin to a mated status. In virgin Platynota stultana females, decapitation or exogenously applied juvenile hormone (JH) (10 ug) resulted in cessation of calling and a significant decline in pheromone production (Webster and Cardé, 1984). Although decapitation also eliminated oviposition, topical JH application induced egg deposition comparable to mated females.

In H. cecropia, increased oviposition after mating only occurred when the CC were intact (Truman and Riddiford, 1971). Implantation of an empty bursa (spermatophore removed) from a mated female into a virgin female induced an oviposition pattern similar to that observed in mated females (Riddiford and Ashenhurst, 1973). The oviposition response, however, was not observed in females mated to castrated males (Truman and Riddiford, 1971), or in virgin females which received

implants of an empty bursa (spermatophore removed) from females mated to castrated males (Riddiford and Ashenhurst, 1973). Thus, in H. cecropia sperm deposited in the bursa appears to precipitate release of a bursa factor which acts on the CC. The CC in turn mediates the oviposition response. Data on calling, pheromone release, and pheromone production, however, were not reported.

In many of these investigations, pheromone release was either not measured or was assessed by male response bioassays, and test females were scored simply (+) or (-) for calling. As the complexity of the endogenous control mechanisms is revealed, however, the need for more precise analysis is evident. Therefore, in the present study, we examine the effects of allatectomy and VNC transection immediately anterior to the TAG on parameters of calling behavior, pheromone emission rate, and pheromone titer in virgin L. dispar.

Material and Methods

Moths

Egg masses were supplied by the USDA Gypsy Moth Methods Laboratory, Otis AFB, MA. Larvae were reared on a modified wheat germ diet (Bell et al., 1981) in an environmental chamber held at 24 ± 2°C, 50% r.h., and 16h:8h light:dark cycle. Under these conditions, development to adult eclosion required approximately 6.5 wks. Pupal duration, periodicity of eclosion (monitored every 4 hrs), and records of females not tested due to mortality after the time of treatment or

abnormal adult eclosion were kept for all moths. Control treatments included sham-operated (SHAM), anesthetized-only (CO_2), and untreated (NORM) moths.

Analysis of variance showed no statistical differences ($P > 0.05$) in pupal duration or periodicity of eclosion among all treated and untreated insects. Mean pupal duration of all treatments ranged between 11.8 and 12.7 days. The peak in eclosion periodicity for all treatments occurred between 4 and 8 hrs after lights on which concurs with earlier findings for a wild population (ODell, 1978) and a lab-reared strain (Ma et al., 1982). In addition, Chi-squared analysis of each treatment with the normal moths indicated that the different treatments did not exert any significant effect ($P > 0.05$) on mortality or abnormal eclosion.

Surgical Manipulations

Allatectomy. Allatectomies were performed on last instar larvae 24-48 hrs after ecdysis. The technique employed for the operation was similar to that used by Hollander and Yin (1985). Briefly, anesthetized females (subjected to CO_2 gas and chilling) were immersed in modified Weever's saline (Carrow et al., 1981) and anchored dorsal side up in a silicone rubber-filled dish under a dissection scope. To prevent excessive hemolymph loss, a wire restrainer was positioned at the juncture between the cervix and the thorax.

After an incision was made along the midline of the cervical membrane, the spherical-shaped CA were found amidst tracheae and fat

body below the dorsal neck muscles and above the esophagus. The CC connect with the CA via a fine nerve. By severing this nerve, the CA were removed without damaging the CC. The cervical membrane was then pulled back into place, dried, and sealed with melted dental wax. In sham-operated controls, the procedure was identical except the CA were touched with forceps and left undamaged.

Nerve transection. The second experiment involved severing the VNC directly anterior to the TAG. Pupae were anesthetized and operated on at 4 different times: 0, 1, 2, or 24 hrs after pupal ecdysis. These 4 times were chosen to encompass the operation times performed by Hollander and Yin (1982). A small opening (2.0 mm X 2.0 mm) was made in the middle of sternum VI and covered with a drop of saline. Using forceps and a hooked minuten pin, the fat body was parted and the section of the paired VNC lying anterior to the TAG was gently lifted out the window. Because of the close proximity of the VNC to the cuticle, exposure of the TAG was possible without stressing other nerve branches. Thus, the TAG could be positively identified before severing the VNC. The nerve tissue was replaced below the fat body, the area dried and sealed with melted dental wax. Sham-operated controls received identical manipulations except that the VNC was not cut. Because of potential nerve regeneration, all moths tested were subjected to post-operative inspection of the TAG.

Calling Behavior

On the third day of eclosion prior to the pheromone collection period, females were gently transferred onto a supported section of balsa wood. After about 1/2 hr, the calling behavior of individual females was videotaped for 5 min using a Sony 1050 rotary shutter camera. Illumination was provided by a fiber optic light source positioned 3 cm from the abdominal tip. Recorded calling behavior was then viewed through a TV monitor. The calling behavior of 10 females/treatment was analyzed except in the case of VNC transection where 19 females were used.

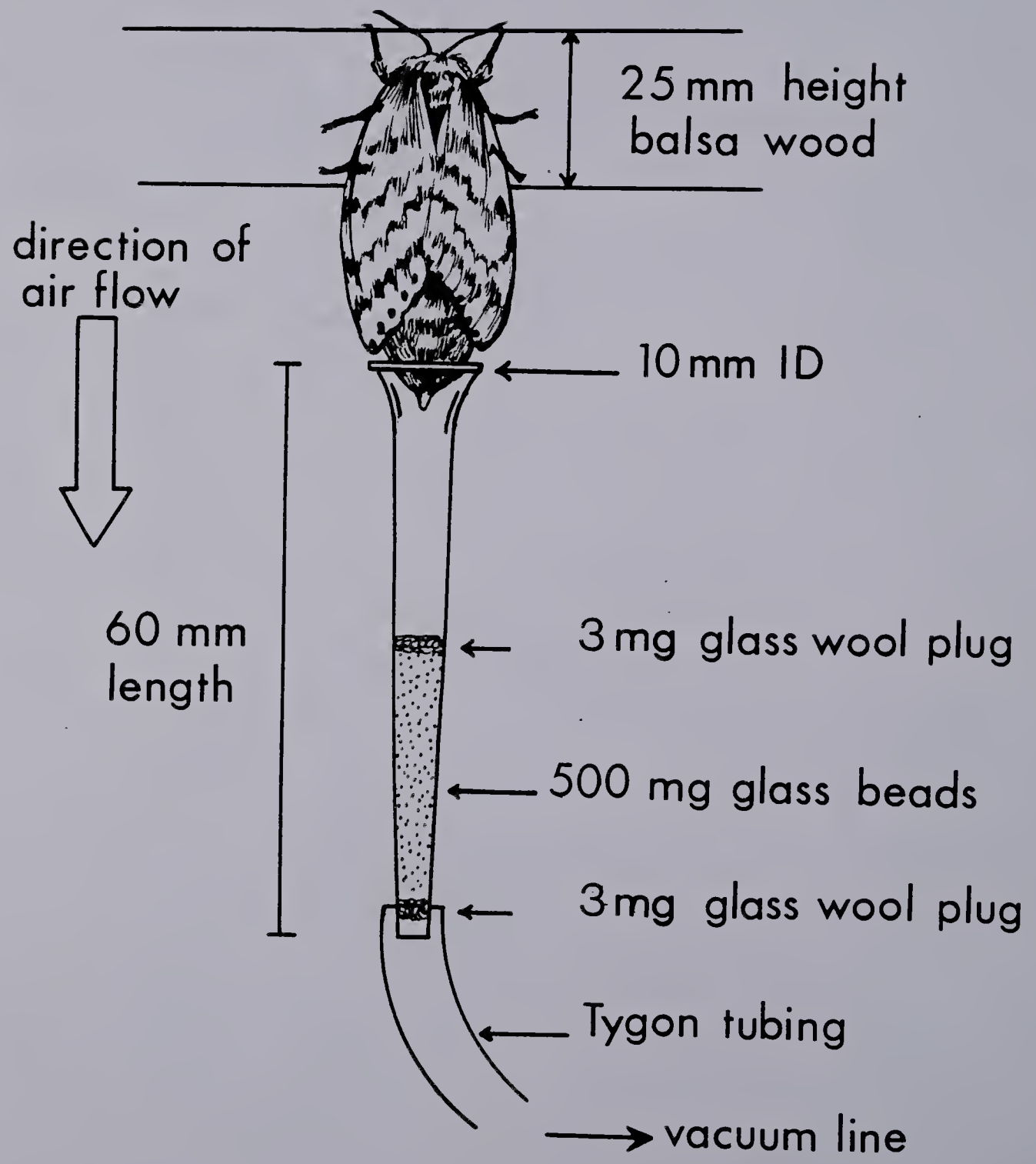
Four components of calling behavior were described: (1) the duration of ovipositor protraction, (2) the duration of ovipositor retraction, (3) the average number of pulses/min, and (4) the mean number of peristaltic waves/min. During ovipositor protraction, the pheromone gland which lies on the intersegmental membrane between abdominal segments VIII and IX (Hollander et al., 1982) was fully exposed. Peristaltic wave was a term used for the undulating motion which passed anterior to posterior over the intersegmental membrane bearing the pheromone gland.

Pheromone Collection Apparatus

Pheromone was collected from individual females using a system based on the glass-bead adsorption apparatus devised by Charlton and Cardé (1982). The collection tube (Fig. 1) contained 500 mg of 0.2 mm diam. glass beads secured by two 5 mg glass wool plugs. During the

Figure 1

Glass collection device used to entrain pheromone emitted by individual L. dispar females (modified from Charlton and Carde, 1982)


25 mm height
balsa wood
direction of
air flow
10 mm ID
60 mm
length
3 mg glass wool plug
500 mg glass beads
3 mg glass wool plug
Tygon tubing
vacuum line

1-hr collection period air was drawn through the tubes by a vacuum pump at a rate of 200 ± 20 ml/min. Temperature and humidity conditions were identical to those used during rearing.

Performance of the collection apparatus was appraised using standard solutions to calculate the relative loss of synthetic disparlure to the internal standard (IS), cis-9,10-epoxy-eicosane, for each stage of the procedure from sample collection to sample injection. The stages included: (1) a 1-hr sample collection, (2) hexane extraction of the collection tube followed by addition of the IS, (3) concentration of the eluent (250 ul) to ca. 0.8 ul under filtered N_2, and (4) sample injection for GLC analysis.

Two approaches were used to evaluate the relative amount of pheromone lost during the 1-hr collection period. In both cases, comparisons of pheromone quantities were made before and after aeration to determine the amount lost. The first involved dispensing 25 ng pheromone directly onto the glass beads of the collection tube and aerating it for 1.5 hrs. The second approach entailed the construction of an artificial gland. Newly eclosed females (<1/2 hr old) which have not yet begun to call (Charlton and Cardé, 1982) do not have detectable pheromone (<0.1 ng) in the gland (Tang, unpublished). At this time, females were frozen, dried, and acetone-washed. A piece of tissue paper approximating the area of the intersegmental membrane bearing the pheromone gland (7.0 mm^2) was suspended at the tip of a minuten pin below a dead female to simulate a typical calling position. Synthetic disparlure (100 ng) was applied to the artificial gland and aerated for

1 hr above a collection tube. Efficiency was calculated as the amount of pheromone recovered by the collection tube divided by the amount of pheromone volatilized from the tissue paper.

Air-borne Collection from Females and Glandular Titer Determinations

Pheromone was collected for 1 hr from individual females 44-60 hrs old on the third day of eclosion. Due to the diel periodicity of pheromone emission, collections were made between 8 and 12 hrs after lights on, coincident with the peak period of release (Charlton and Cardé, 1982).

Prior to the collection period the moths were placed on a piece of balsa wood. Once they were settled, the wood was suspended above the collection apparatus (Fig. 1). Each collection tube was maneuvered such that the flared portion of the tube enveloped but did not contact abdominal segment VII. The glandular tissue was centered about 2 mm above the constriction of the collection tube. Air flow was started once calling recommenced, typically within 5 min for all control females. For each collection period, a blank was also run. Immediately following the collection period, tubes were extracted with hexane to collect 250 ul eluent and 25 ng IS added.

The amount of extractable glandular pheromone from each of these same females was determined on the following day 0-4 hrs before the onset of scotophase which corresponds to the peak in glandular titer periodicity (see Chapter III). The abdominal tip which included sclerite IX, the glandular tissue, and part of sclerite VIII, was

excised and extracted for 1 hr in an ampule containing 150 ul hexane and 25 ng IS. Both air-borne and gland samples were stored in vials with Teflon-lined caps at -15°C until analysis.

Sample Analysis

Samples were concentrated to ca. 0.8 ul under a filtered N_2 stream and injected onto a 2 m X 2 mm ID glass column packed with SP-2100 (3% loading on 100/120 Supelcoport; N_2 carrier, 30 ml/min). Quantification of FID peak areas was accomplished using an HP 3390 integrator. Under isothermal operation at 185°C, disparlure and IS eluted at 6.5 and 10.7 min, respectively. The lower analytical limit of the system was ca. 0.1 ng.

Results

Collection Efficiency

Table 1 describes the efficiency of pheromone recovery for our emission rate determination. Overall, the procedure was 74.7% efficient in recovery of air-borne pheromone. Step-wise analysis of the procedure showed that 23.9% of the pheromone was lost during 1-hr collection from the artificial gland and 1.4% was lost relative to the IS during concentration of the collection tube eluent. No notable losses were incurred during hexane extraction of the collection tube or during sample injection onto the GLC column. By comparing the losses of pheromone from the 2 types of air-borne collections, i.e. pheromone

Table 1. Pheromone losses incurred for each step of the procedure from air-borne collection to GLC analysis.*

	procedure	n	% loss (SE)
(1)a.	1-hr collection from artificial gland	5	23.9 (2.3)
b.	1.5 hr aeration after direct application of disparlure to glass beads of collection tube	5	0
(2)	extraction of collection tube	5	0
(3)	concentration of eluent (250 ul) to 0.8 ul under filtered nitrogen	5	1.4 (1.0)
(4)	sample injection onto GLC column	5	0

total pheromone loss = 25.3%

total pheromone recovery = 74.7%

* Pheromone quantities were calculated relative to the internal standard.

application to the artificial gland versus direct application onto the glass beads of the collection tube, we found that 23.9% of the pheromone was lost during aeration in the former case and 0% for the latter. This indicated that pheromone loss probably occurred near the tube opening, but once pheromone was adsorbed to the glass beads, further losses during the collection period did not occur.

All female data represent actual amounts and were not adjusted for the losses determined by our efficiency analysis.

Allatectomy

Mean pheromone release rates from females allatectomized during the last larval instar (14.1 ± 2.6 SE ng/hr) did not show any significant difference from sham-operated (16.4 ± 2.0 SE ng/hr), anesthetized (13.1 ± 1.7 SE ng/hr), or normal controls (13.1 ± 2.6 SE ng/hr) (Fig. 2). Results from blanks run during each collection period consistently revealed only minimal quantities (0.6 ± 0.2 SE ng/hr) of compounds collected with the same retention time as disparlure. Observation of calling behavior also appeared normal for all 4 treatments. Therefore, allatectomy appeared to have no influence on either calling behavior or pheromone emission rates.

Nerve Transection

Following transection of the VNC anterior to the TAG, female pheromone emission rate was significantly lower compared to controls (Fig. 3). For females treated 0, 1, 2, and 24 hrs after pupation, mean

Figure 2

Effect of allactectomy on mean pheromone emission rates of 2-day-old females as compared with control moths and blank air collections. Logarithmic transformation of the data was necessary to satisfy requirements of homogeneity of variance. Means followed by the same letter were not significantly different ($P > 0.05$) by the Student-Newman-Keuls multiple range test. Vertical bars denote standard errors of the mean.

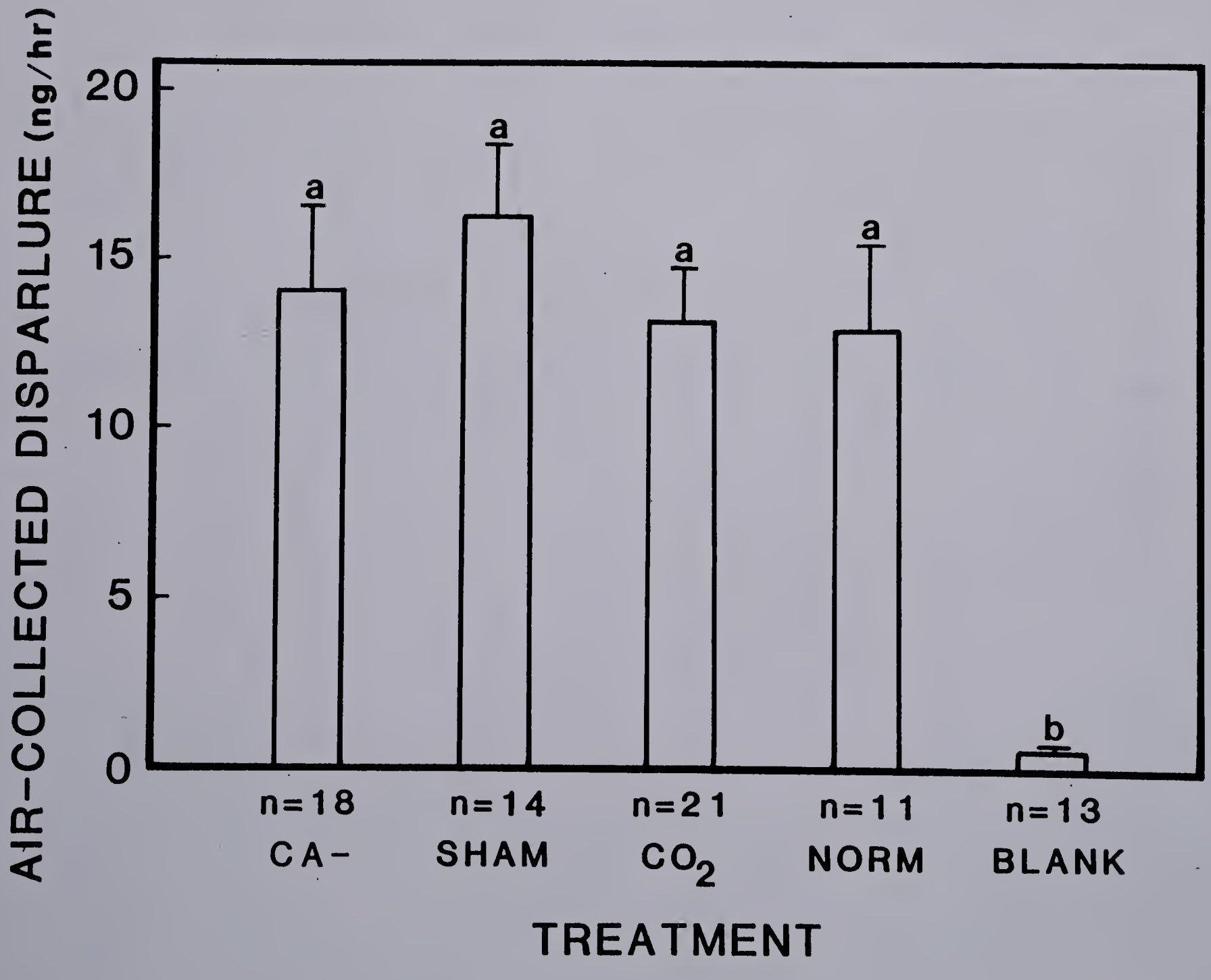
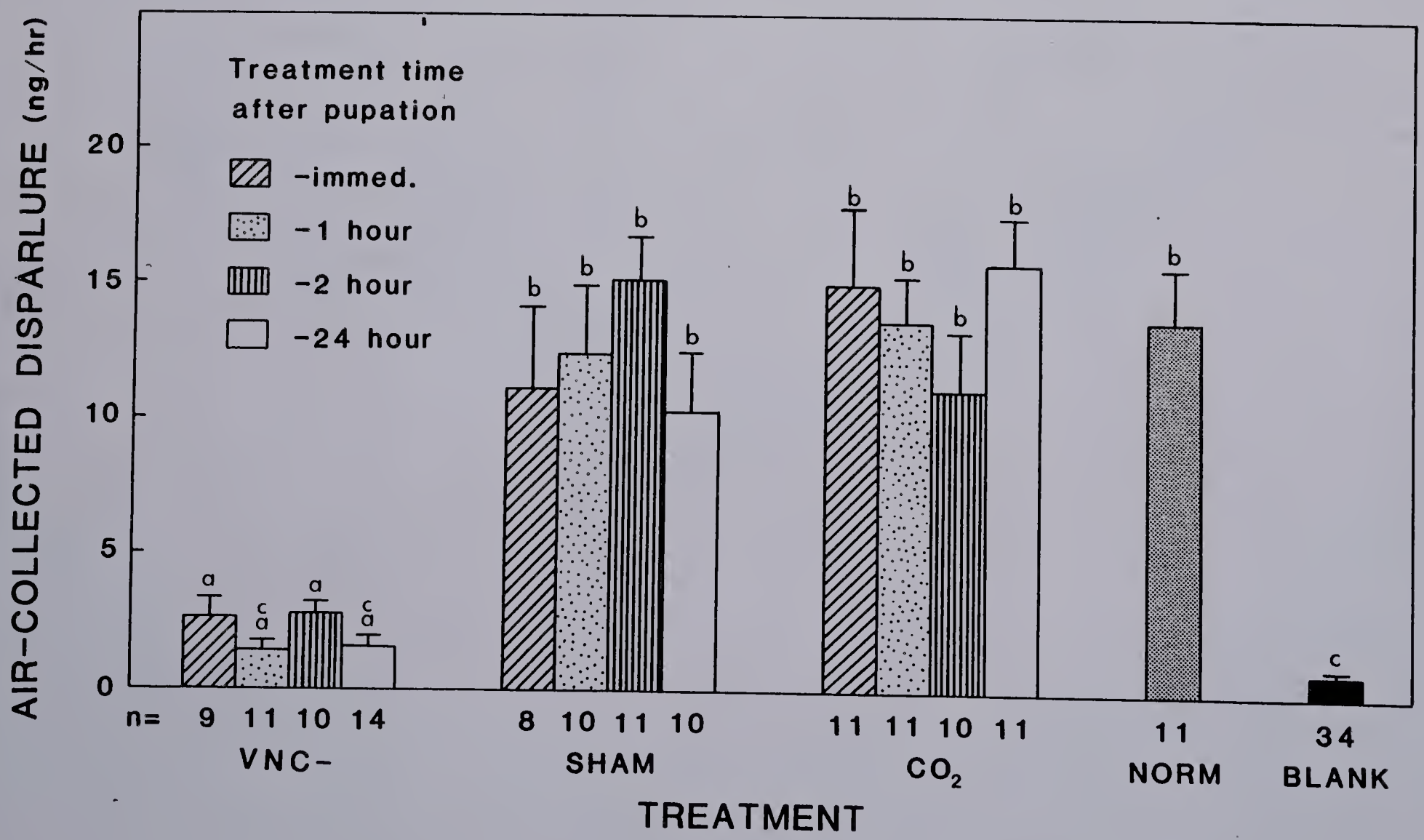

AIR-COLLECTED DISPARLURE (ng/hr)
20
15
10
5
0
a
a
a
a
b
n=18
n=14
n=21
n=11
n=13
CA-
SHAM
CO_2
NORM
BLANK
TREATMENT

Figure 3

Influence of VNC transection anterior to the TAG on pheromone emission rates of 2-day-old females as compared with control moths and blank air collections. Logarithmic transformation of the data was necessary to satisfy requirements of homogeneity of variance. Means followed by the same letter were not significantly different (P> 0.05) by the Student-Newman-Keuls multiple range test. Vertical bars denote standard errors of the mean.

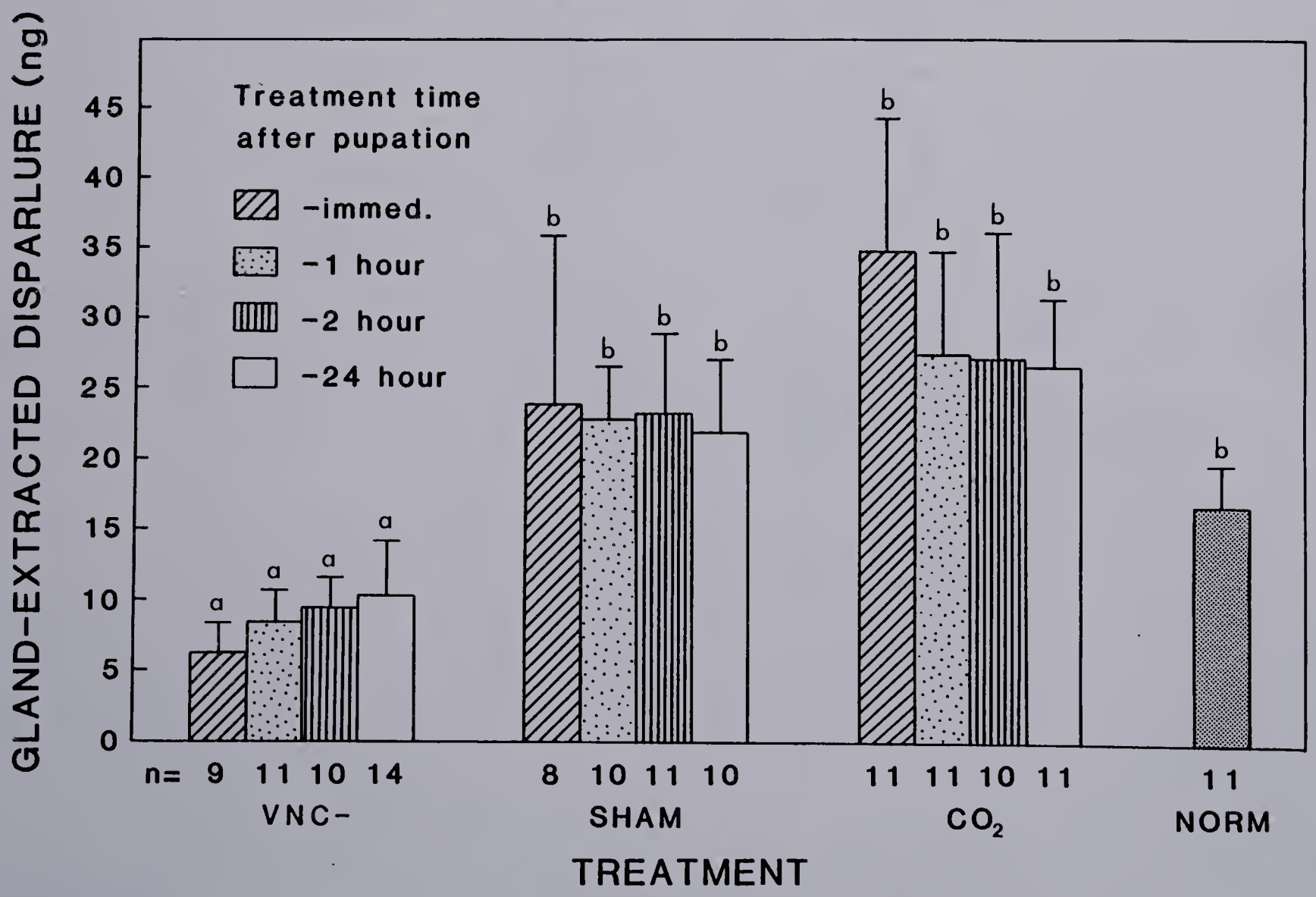

AIR-COLLECTED DISPARLURE (ng/hr)
Treatment time after pupation
-immed.
-1 hour
-2 hour
-24 hour
20
15
10
5
0
n= 9 11 10 14 8 10 11 10 11 11 10 11 11 34
VNC-
SHAM
CO2
NORM
BLANK
TREATMENT

pheromone release rates were 2.6 ± 0.7 SE ng/hr, 1.4 ± 0.3 SE ng/hr, 2.7 ± 0.5 SE ng/hr, and 1.5 ± 0.4 SE ng/hr, respectively. Mean pheromone release rates from females receiving sham-operations 0, 1, 2, and 24 hrs following pupation were 11.1 ± 3.0 SE ng/hr, 12.4 ± 2.5 SE ng/hr, 15.2 ± 1.7 SE ng/hr, and 10.0 ± 2.1 SE ng/hr, respectively. Mean pheromone release rates from anesthetized females ranged between 11.1 ± 2.1 SE ng/hr and 15.8 ± 1.7 SE ng/hr and untreated females averaged 13.7 ± 2.0 SE ng/hr. Although the treatment times, 0, 1, 2, and 24 hrs after pupation, were originally chosen to encompass the operation times of Hollander and Yin (1982), the data in Fig. 3 indicate that treatment effects on pheromone emission rates were independent of operation time. In addition, no significant differences in release rates were found among the 3 control groups, sham-operated, anesthetized, or untreated individuals. Therefore, VNC transection 0, 1, 2, or 24 hrs after pupation effected a dramatic reduction in pheromone emission rate of the adult compared to either control treatments performed at similar times after pupation or to untreated controls.

Nerve transection also resulted in significant reductions of extractable glandular pheromone (Fig. 4). From females operated at 0, 1, 2, and 24 hrs after pupation extractions yielded 6.3 ± 2.2 SE ng, 8.4 ± 2.4 SE ng, 9.5 ± 2.1 SE ng, and 10.3 ± 2.9 SE ng, respectively. Mean amounts of pheromone extracted from females receiving sham-operations at the same time periods were 24.0 ± 12.1 SE ng, 22.9 ± 3.9 SE ng, 23.2 ± 5.9 SE ng, and 21.9 ± 5.2 SE ng, respectively.

Figure 4

Effect of VNC transection anterior to the TAG on glandular pheromone titer of 3-day-old females as compared with control moths. Logarithmic transformation of the data was necessary to satisfy requirements of homogeneity of variance. Means followed by the same letter were not significantly different ($P > 0.05$) by the Student-Newman-Keuls multiple range test. Vertical bars denote standard errors of the mean.


GLAND-EXTRACTED DISPARLURE (ng)
Treatment time after pupation
-immed.
-1 hour
-2 hour
-24 hour
0
5
10
15
20
25
30
35
40
45
a
a
a
a
b
b
b
b
b
b
b
b
b
n= 9 11 10 14
8 10 11 10
11 11 10 11
11
VNC-
SHAM
CO2
NORM
TREATMENT

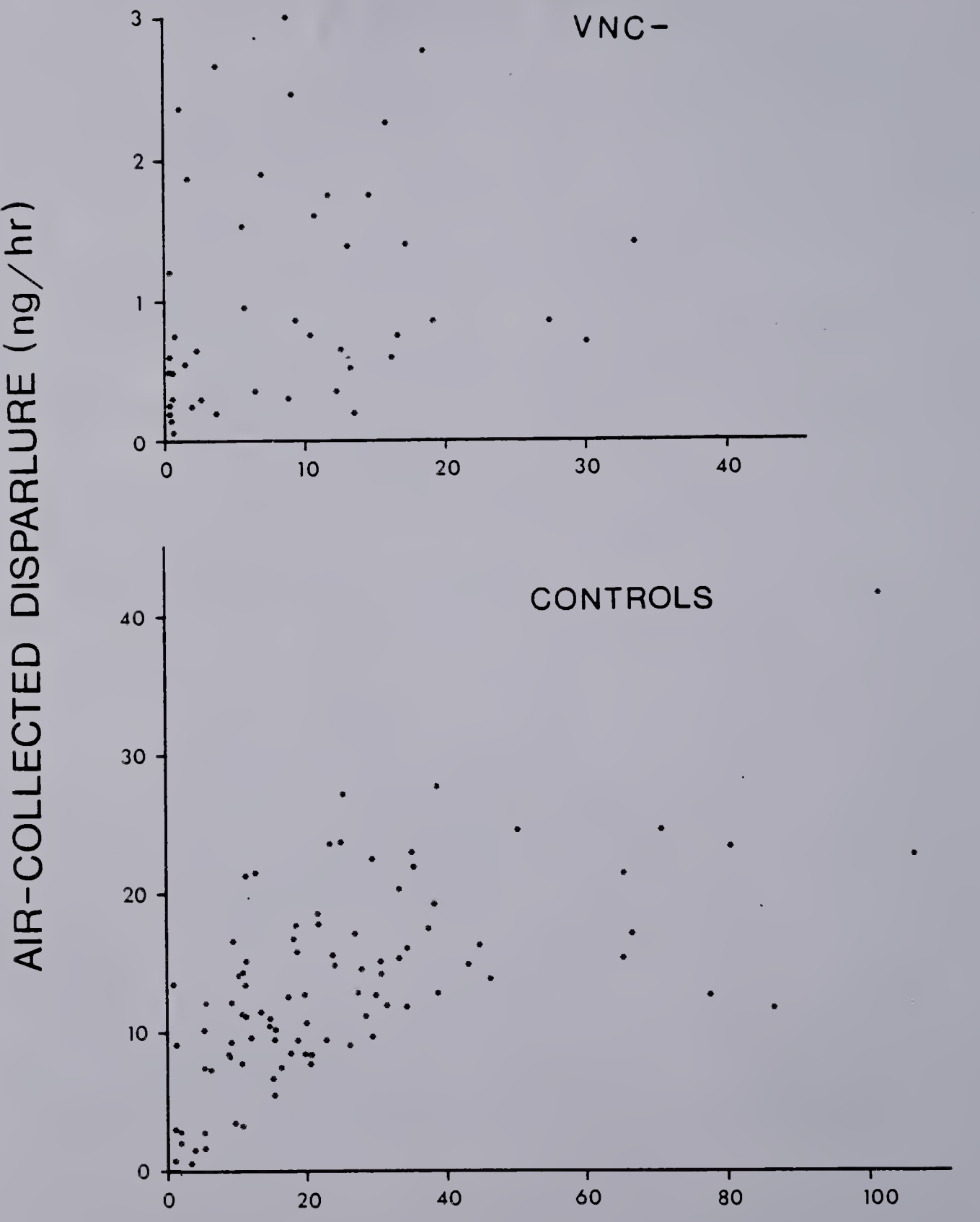
Extractable glandular pheromone from anesthetized females ranged between 27.2 ± 9.0 SE ng and 34.9 ± 9.4 SE ng and untreated females yielded 16.8 ± 2.8 SE ng. In accord with the pheromone emission rate results, the time of the treatment did not influence the amount of gland-extractable pheromone, and no significant differences were found among sham-operated, anesthetized, or untreated controls. Thus, the reduction in extractable pheromone from operated females compared to controls indicated that VNC transection also affected pheromone production by the gland.

It should be noted that the structure of disparlure was identified by epoxidation of the olefin precursor found in an extract of 78,000 tips since quantities of the natural attractant, although detectable by male response bioassay, were considered insufficient for characterization (Bierl et al., 1970). Analysis of our extractions, however, showed that the amounts of olefin per gland was insignificant compared to the amounts of disparlure. Differences between results presented here and those of Bierl et al. (1970) may be attributed to the type of extraction procedure performed and to the levels of impurities found in the extracts.

Figure 5 depicts plots of female pheromone emission rate versus pheromone titer extracted from the gland for each female used in the VNC transection experiment. The overall significant reductions exhibited by operated females compared to controls can be seen from data obtained from the individual females. Furthermore, data from control females indicate that although pheromone emission rates tended

Figure 5

Plots of pheromone emission rate versus gland-extracted pheromone from each operated and control female used in the VNC transection experiment. Measurements of pheromone emission rate were taken from 2-day-old females and gland-extractable quantities from 3-day-old females.


VNC-
CONTROLS
AIR-COLLECTED DISPARLURE (ng/hr)
GLAND-EXTRACTED DISPARLURE (ng)
0
1
2
3
0
10
20
30
40
0
10
20
30
40
0
20
40
60
80
100

to increase as gland-extracted quantities increased, emission began to level off at ca. 20 ng/hr despite higher gland-extracted quantities. Our approximation of the data curve which stabilized the variance due to heteroscedasticity was found following log transformations of both pheromone emission rate and gland-extracted quantities ($r^2 = 0.44$).

Calling Behavior

A description of the calling behavior for VNC-transected females and unoperated controls is shown in Table 2. No significant differences were found among the 3 control treatments with respect to pulse frequency, duration of protraction, duration of retraction, or peristaltic wave frequency. While calling, control females displayed ca. 4 extrusions/min, protrusion of the ovipositor endured ca. 16 sec, periods of retraction were brief, about 1.3 sec, and 7 or 8 peristaltic waves/min were visible. The direction of ovipositor extrusion and retraction paralleled the longitudinal axis of the female's body. If a female was disturbed while calling, the ovipositor was immediately retracted. Calling recommenced, however, within a few minutes after the disturbance, as similarly noted by Doane (1968).

Females receiving transections of the VNC exhibited two modes of behavior, both unlike the typical calling behavior of control insects. One of the behaviors (referred to as "inactive" in Table 2) displayed no pulsing activity or retraction and the ovipositor was maintained in either a fully or partially protruded position for periods longer than 300 sec. The degree of protrusion also reflected the degree of

Table 2. Effect of ventral nerve cord transection anterior to the TAG on parameters of calling in 2-day-old females.

treatment*	n	pulses per min x(SE)	protraction sec x(SE)	retraction sec x(SE)	waves per min x(SE)
operated ("inactive" mode)	10	0a	300a	0a	7.5a (0.9)
operated ("erratic" mode)	9	12.5c (1.9)	1.9c (0.2)	3.9b (0.8)	ND
sham-operated	10	4.3b (0.7)	17.0b (3.5)	1.2b (0.1)	6.9a (0.7)
anesthetized-only	10	3.5b (0.3)	16.9b (1.6)	1.3b (0.1)	8.1a (0.4)
untreated	10	4.3b (0.4)	15.3b (3.2)	1.3b (0.1)	7.1a (0.8)

Data were based on 5-min sequences except in the case of the erratic mode exhibited by operated females which only lasted between 1 and 3 min before a transition back to the inactive mode occurred. Means in the same column having no letters in common were significantly different according to the Student-Newman-Keuls multiple range test. Logarithmic transformation of the data was necessary to satisfy requirements for homogeneity of variance.

* The 2 modes of ovipositor activity exhibited by operated moths were distinctly different. Therefore, each was treated separately in the analysis. Under normal circumstances, the ovipositor of operated females typically showed no movements and was continuously held in a full or partially protruded state (inactive mode). In 9 of 19 operated females videotaped, erratic ovipositor activity was triggered when the light from the fiber optics was illuminated. The behavior was termed erratic because ovipositor movements were rapid and often directed at angles not normally associated with calling.

ND - Due to the rapid movement of the ovipositor, peristaltic waves were not discernible.

exposure of pheromone gland regions. The frequency of peristaltic waves seen during the inactive phase appeared to be unaffected by the operation. During the 1-hr air-borne pheromone collections, females were only observed in this inactive mode of behavior.

The second type of behavior (referred to as "erratic" in Table 2) was never observed occurring spontaneously but was provoked in 9 of the 19 VNC-transected females videotaped after the light from the fiber optics was turned on. (This behavior was also sometimes seen immediately after operated females were moved to the balsa wood substrate.) The mean duration of the erratic behavior was ca. 3 min, after which ensued a gradual transition over the next few minutes back to the inactive phase. Separate VNC transected females were used to describe the inactive and erratic behavior modes. In addition, analysis of the erratic mode was terminated when the transition became obvious.

Characterization of the erratic behavior showed that compared to control treatments, pulsing activity was significantly higher (12.5 extrusions/min), resulting in a significant decrease in the time spent in ovipositor protraction, and a slight but not significant increase in retraction duration. Moreover, the direction of ovipositor protrusion and retraction was highly irregular, exihibiting frequent angled and rotational movements.

Thus, females receiving VNC transection did not call normally. Operated females displayed an inactive mode characterized by continuous ovipositor protraction. If disturbed, some operated females abrubtly

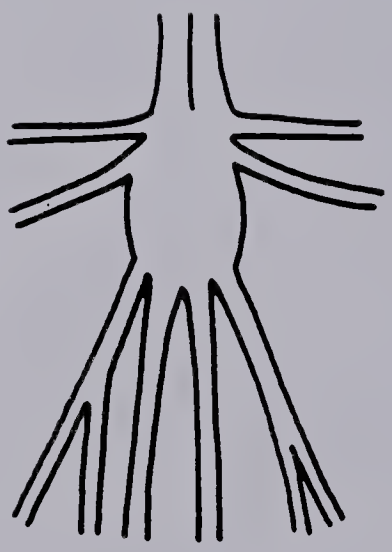
began to pulse in a rapid and erratic fashion. Control females, on the other hand, protruded the ovipositor for ca. 17 sec before a brief period of retraction. If disturbed, the ovipositor of control females was immediately retracted. Calling recommenced, however, within a few minutes.

TAG Morphology

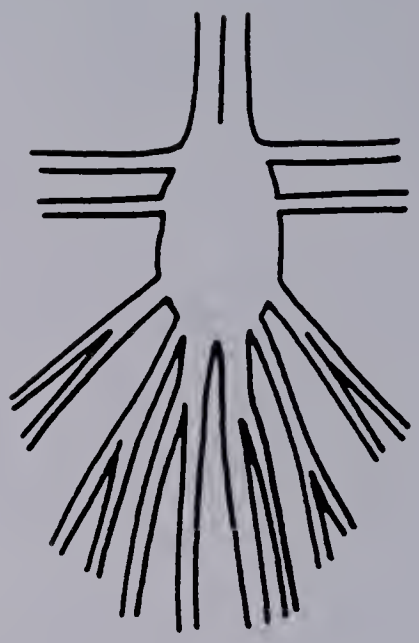
Post-operative inspection of adult females receiving nerve transections as pupae (regardless of the time of the treatment) revealed morphological differences of the TAG compared to controls. Figure 6 shows diagrammatic representations of the TAG from pupae (n=10) and from adults (n=10 for each treatment). At the time of the treatment, the pupal TAG had 4 major pairs of nerve branches in addition to the anteriorly located paired VNC. The adult TAG from all control treatments had 5 major pairs of nerve branches in addition to the paired VNC. The adult TAG from operated females showed only 4 pairs of major nerve branches and the severed ends of the paired VNC were completely degenerated by the time females were dissected as adults. Transection of the VNC of pupae apparently affected the normal development of the TAG during metamorphosis.

Figure 6

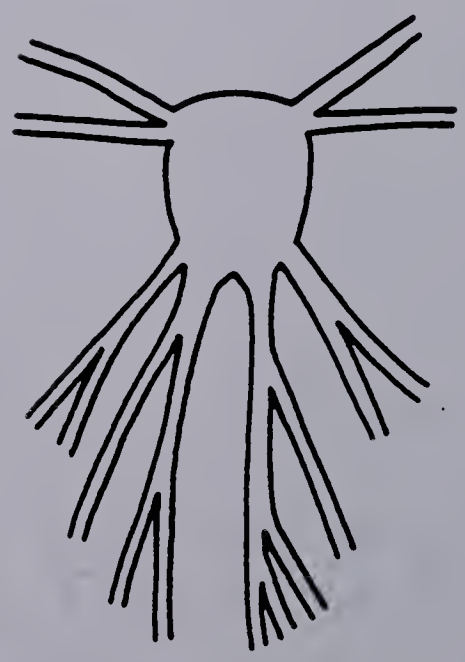
Schematic diagram of representative TAG from (A) pupae 0-24 hrs old (n=10), (B) 3-day-old females which were nerve-transected between 0 and 24 hrs after pupation (n=10), and (C) 3-day old females from the 3 control groups: sham-operated between 0-24 hrs (n=10), anesthetized-only between 0 and 24 hrs (n=10), and untreated (n=10).



pupal



adult controls



adult operated
VNC-

Discussion

Data presented here which quantifies the pheromone emission rate from allatectomized females showed that the CA did not influence pheromone release in L. dispar. In a previous study of the gypsy moth, Hollander and Yin (1985) drew similar conclusions based on male response (wing-fanning) to allatectomized and control females. Although their bioassay indicated that allatectomized females emitted pheromone, however, Hollander and Yin (1985) did not use their bioassay to measure differences in pheromone emission rates, e.g. by measuring latency of the male wing-fanning response (Cardé and Hagaman, 1979; Hagaman and Cardé, 1984). Similarly, in G. mellonella (Röller et. al., 1963) and A. pernyi (Barth, 1965), male attraction and subsequent copulation in the field indicated that allatectomized females released pheromone, but differences in release rate were not determined. Due to the low response threshold of males to pheromone (Cardé and Charlton, 1984), significant reductions in pheromone release among the different treatments could have escaped detection. Therefore, our results show conclusively that allatectomized L. dispar females emit similar amounts of pheromone ($\bar{x}$ = 14.1 ng/hr) as unoperated controls (means ranged between 13.1 ng/hr and 16.4 ng/hr).

After transection of the VNC anterior to the TAG, operated females exhibited significant reductions in pheromone release rate, extractable glandular pheromone, and did not call normally. If left undisturbed, the ovipositor of operated females was maintained

continuously in a full or partially protruded state. If physically disturbed, however, a brief interlude of erratic ovipositor protrusion and retraction ensued. This behavior was characterized by rapid pulsing, short protraction duration, and frequent rotational movements of the ovipositor. Since neither of these behaviors resembled the calling behavior of control females, nervous input from higher centers appears essential for normal calling to occur. The influence on moth calling of exogenous factors such as temperature, photoperiod, and wind velocity (Sower et al., 1971; Kaae and Shorey, 1972; Cardé and Roelofs, 1973; Fatzinger, 1973; Nordlund and Brady, 1974; Baker and Cardé, 1979; Alford and Hammond, 1982; Webster and Cardé, 1982; Conner et al., 1985; Webster, 1986) further suggests that the brain is the control center which can initiate, maintain, and inhibit calling.

Neural control of calling has also been demonstrated in other species of Lepidoptera. In H. cecropia and A. polyphemus, Sasaki et al. (1983) showed that removal of the first abdominal ganglion eliminated calling. In M. sexta and U. ornatrix, Itagaki (1984) performed transection of the VNC at different levels of the abdominal nervous system, and in all cases, females were unable to call.

Evidence against control of calling by higher centers of the nervous system has been presented by Hollander and Yin (1982) in L. dispar. They found that females receiving VNC transection immediately anterior to the TAG called. Differences between results presented here and those of Hollander and Yin (1982) may be based on the definitions of calling used. In their study, they defined calling in the broad

sense, i.e. if ovipositor protrusion and retraction occurred. Our definition, on the other hand, characterized calling in more detail based on pulse frequency, and protraction and retraction durations.

According to their definition, the erratic mode of ovipositor activity which we observed when operated females were physically disturbed would be interpreted as calling behavior. In their experiment, evaluation of calling behavior was determined while the female was being tested for pheromone release in their male wing-fanning bioassay. Since a female had to be moved to the upwind end of the bioassay tube, it is possible that the physical disturbance triggered expression of the erratic behavior which was interpreted as calling. Environmental and procedural differences may further explain why they observed ovipositor protrusion and retraction in 81% of the operated females, whereas we observed expression of the erratic mode when the light from the fiber optics was turned on in 47% of the operated females.

Although input from higher centers of the nervous system appears essential for normal calling to occur, expression of erratic ovipositor movements in operated females after the fiber optics light was turned on indicates that the TAG is capable of some endogenous activity. Hollander and Yin (1982) postulated the existence of a CPG in the TAG for calling. Our results, on the contrary, showed that the erratic behavior of operated females displayed fundamental dissimilarities with calling female controls. Before any functional role can be ascribed to this endogenous activity, electrophysiological characterization of the

TAG is required since behavioral observations of abdominal tip activity (as presented here) or scoring (+) (-) for calling (Hollander and Yin, 1982) are simply not adequate approaches to prove the existence of a CPG.

In U. ornatrix, calling females exhibit very rapid and rhythmic extrusions of the ovipositor (1.57 ± 0.06 SD extrusions/sec, Conner et al., 1980). Although VNC transection resulted in the cessation of calling behavior in U. ornatrix, electrical stimulation of the TAG did elicit a sequence of ovipositor protrusions and retractions which was indistinguishable from the calling behavior of normal females (Itagaki, 1984). Thus, in U. ornatrix, patterned output from the TAG for calling is likely, but nervous input from higher centers still appears essential for the initiation and continued expression of calling.

Despite previous evidence for neuroendocrine control of pheromone production (Raina and Klun, 1984), our results on VNC transection in the gypsy moth indicate that the presence of an intact nervous system strongly influences pheromone production by the gland. Females which received VNC transection anterior to the TAG but did not receive surgical alteration of the brain or CC still exhibited significant reduction in the amount of gland-extractable pheromone. Direct nervous control of pheromone production would seem unlikely since no nervous innervation of the intersegmental membrane bearing the pheromone gland regions was found in L. dispar (Tang, unpublished) or other species (Eaton, 1985).

Indirect nervous control of pheromone production, on the other

hand, may be achieved through possible neural/neuroendocrine interactions. Raina and Klun (1984) reported that elevated quantities of pheromone from gland extractions of neck-ligated or decapitated H. zea females occurred following injections of H. zea brain homogenates or from brain homogenates from 6 other species of Lepidoptera, including L. dispar. If a neuroendocrine factor is involved in pheromone production in L. dispar, its release from neurosecretory cells may be modulated by communication with those neurons which control calling.

In addition to the significant reduction in gland-extractable pheromone, females receiving transection of the VNC also exhibited a significant reduction in pheromone emission rate compared to controls. Hollander and Yin (1982) concluded that the lack of pheromone release (as detected by their bioassay) in VNC-transected females could be explained by direct nervous control of pheromone release by the CNS. Our results indicate, however, that the reduction in emission rate probably arose from the concurrent reduction in gland-extractable pheromone and from the elimination of normal calling.

Based on ultrastructural studies of the gland cells in Trichoplusia ni, Percy (1979) proposed a model outlining steps whereby pheromone is transported from within the cell to the cuticle surface. Cellular control of pheromone release from the cells may in turn rely on the control mechanisms which regulate pheromone production.

Although calling, pheromone production, and pheromone release can be considered separate phenomena with separate diel periodicities, it

appears that the underlying control mechanisms for at least the first two are closely linked. Future investigations will hopefully reveal in more detail how control for each is achieved as well as what interactions exist to ensure the appropriate timing of their respective diel periodicities.

C H A P T E R I I I

EFFECTS OF AGE AND MATING ON THE DIEL PERIODICITY OF SEX PHEROMONE TITER IN *LYMANTRIA DISPAR*

Abstract

The diel periodicity of pheromone titer of female *Lymantria dispar* was described. Individual abdominal tips were extracted in hexane every 4 hrs, 6 times a day, from females 0-, 1-, 2- and 3-days-old. Quantification of sex pheromone was measured using GLC analysis. The diel periodicity of pheromone titer became apparent the day following eclosion after which maximal amounts (means ranged from 19.0 to 26.0 ng) occurred 0-4 hrs before lights-off and minimal amounts (means ranged from 5.1 to 7.4 ng) 0-4 hrs after lights-on. Periods of minimal titer were associated with lowest percent female calling and periods of maximal titer were always observed within periods of maximal calling. Day-0 females exhibited significantly less average daily titer (4.5 ng) than day-1 (12.4 ng), day-2 (15.4 ng), and day-3 (13.5 ng) females. Mated females exhibited a rapid reduction in titer ($\bar{x}$ = 2.0 ng for females extracted 0 to 60 min after pair separation) compared to virgin females ($\bar{x}$ = 19.6 ng) of similar age.

Introduction

Investigations of sex pheromone titer within the gland of several species of moths have revealed that characteristic patterns exist describing the amount of pheromone in the gland at different times of the day. In Heliothis zea (Raina and Klun, 1986), Amyelois transitella (Coffelt et al., 1979); and Plodia interpunctella and Ephestia cautella (Coffelt et al., 1978), comparison of the diel periodicity curve of pheromone titer with that of calling has shown that the periodicity curves for pheromone titer followed the same pattern as periodicity curves for calling; periods of maximal and minimal titers coincided with periods of maximal and minimal calling.

The effect of female age on pheromone titer is variable. In Trichoplusia ni (Shorey and Gaston, 1965), other noctuids (Shorey et al., 1968), P. interpunctella (Brady and Smithwick, 1968), and Choristoneura fumiferana and C. occidentalis (Grant et al., 1982), the pheromone titer either was not affected by age or rapidly increased during the first few hours following eclosion and then remained constant over subsequent days (for a given time of day). In Platynota stultana (Webster and Cardé, 1982) and H. zea (Raina and Klun, 1986) increasing titers were found as females aged, then after the second and third scotophase, respectively, titer began to decrease with age.

It has been generally assumed that the effect of mating on titer depends on the number of times females typically mate i.e. female attractiveness and sexual receptivity would cease after mating in

monogamous females. Thus far, P. stultana represents the sole species for which the titer of monogamous females has been examined after mating (Webster and Cardé, 1984). In accord with the hypothesis, titer eventually decreased until pheromone fell below the limits of detection (<0.1 ng) within 14 hrs. In polygamous female species, titer remained unaffected in T. ni (Shorey and Gaston, 1965), exhibited a decrease 24 hrs later in A. transitella (Coffelt et al., 1979), or in H. zea revealed only a temporary but rapid decrease (after 2 hrs) which increased over subsequent nights to levels comparable to virgin females (Raina and Klun, 1986). Data for P. interpunctella are conflicting; Brady and Smithwick (1968) observed no effect of mating on titer, whereas Lum and Brady (1973) found a 100-fold decrease in titer.

For the female gypsy moth, Lymantria dispar, which is typically monogamous (Doane, 1968) and short-lived as an adult, profiles of calling and pheromone emission periodicities have been described (Charlton and Cardé, 1982). In addition, physiological investigations have shown that the control mechanisms of calling and pheromone production are linked (see Chapter II). Therefore, to further delineate relationships which may exist among calling, pheromone release, and pheromone production, we describe the diel periodicity of pheromone titer in the gypsy moth and report on the effects of mating and age on titer.

Materials and Methods

Moths

Egg masses were supplied by the USDA Gypsy Moth Methods Laboratory, Otis AFB, MA. Larvae were reared on a modified wheat germ diet (Bell et al., 1981) in an environmental chamber held at 24 ± 2°C, 50% r.h., and 16h:8h light:dark cycle. Under these conditions, development to adult eclosion required approximately 6.5 wks. Eclosion was monitored every 4 hrs after lights-on. Eclosion was strictly diurnal with 24.8% of the females eclosing between 0-4 hrs, 50.4% between 4-8 hrs, 23.6% between 8-12 hrs, and 1.2% between 12-16 hrs of photophase. This profile of eclosion periodicity concurs with previous reports (ODell, 1978; Ma et al., 1982). Unless specified otherwise, the age in days was based on the age in hours from the time of eclosion. Day-0 refers to females 0-23 hrs old, day-1, 24-47 hrs old, day-2, 48-71 hrs old, and day-3, 72-95 hrs old.

Gland-extractions

The sex pheromone of the gypsy moth, commonly known as disparlure, has been identified as cis-7,8-epoxy-2-methyloctadecane (Bierl et al., 1970). The pheromone gland of the gypsy moth (Hollander et al., 1982) like other female Lepidoptera (Percy and Weatherston, 1971) lies on the intersegmental membrane between abdominal segments VIII and IX. The abdominal tip which included sclerite IX, the pheromone gland tissue and part of sclerite VIII was excised and

extracted in 150 ul hexane for 1-hr. The internal standard (25 ng), cis-9,10-epoxy-eicosane, was added to the hexane immediately before the gland was extracted. Samples were stored in vials with Teflon-lined caps at -15°C until analysis.

Efficiency of extraction was determined by extracting the same gland (n=5) sequentially for 1-min, 1-hr, and 24-hrs. One-min, 1-hr, and 24-hr extractions yielded, respectively, 83.2% (± 2.6 SD), 16.8% (± 2.6 SD) and less than 0.1 ng of the available pheromone. Moreover, 24-hr extractions also contained extraneous compounds which had similar retention times as the internal standard. Therefore, glands were extracted for 1-hr.

Extractions and observations of female calling behavior were performed every 4 hrs, 6 times a day, beginning at lights-on for day-0, -1, -2, and day-3 females. All extractions and calling behavior observations were made under the same light and temperature conditions as the rearing regime. Hence, during scotophase, a light covered with a Kodak Wratten filter no. 70, which effectively eliminates all light less than 650 nm, was used.

To determine the effects of mating on pheromone titer, males were placed with females (48-56 hrs old) 8 hrs after lights-on. Mating ensued within a few minutes and pairs remained *in copula* for approximately 1 hr. After uncoupling, females were used for gland-extractions. Mean elapsed time between uncoupling and extraction was 33 min (ranged from 0 to 60 min). The titer from mated females was compared with the titer from virgin females of similar age extracted at

8 and 12 hrs after lights-on.

Sample Analysis

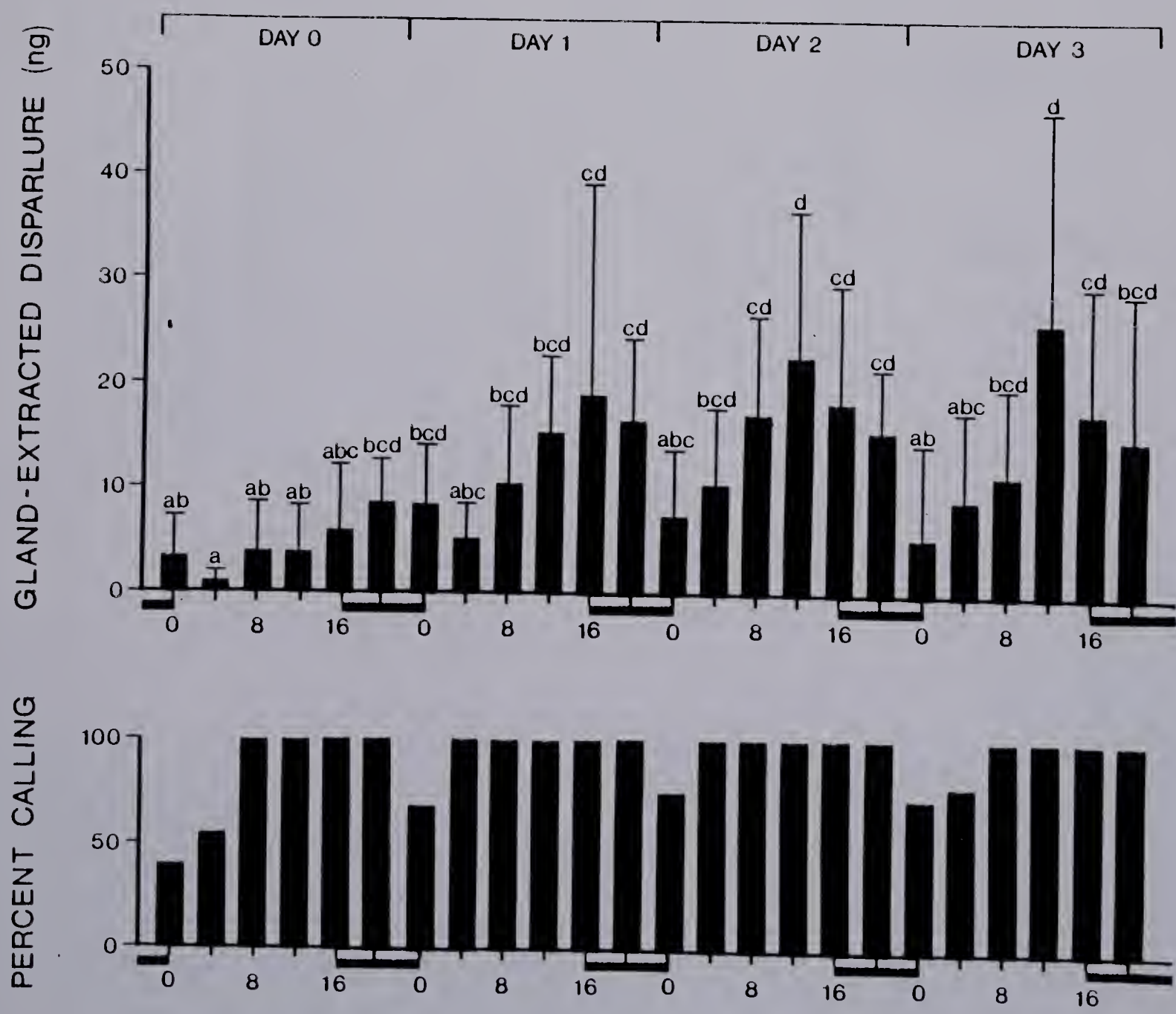
Samples were concentrated to ca. 0.8 ul under filtered N_2 and injected onto a 2 m X 2 mm ID glass column packed with SP-2100 (3% loading on 100/120 Supelcoport; N_2 carrier, 30 ml/min). Quantification of FID peak areas was provided by an HP 3390 integrator. Under isothermal operation at 185^{0}C, disparlure and the internal standard eluted at 6.5 and 10.7 min respectively. The lower analytical limit of the system was ca. 0.1 ng.

Results

The effect of the time of day on pheromone titer is shown in Figure 7. For day-0 females, the pheromone extracted during photophase ranged between 1.0 and 4.0 ng with no significant differences evident between different extraction times. The titer of day-0 females slowly increased during early scotophase and peaked (8.4 ng) 4 hrs after lights-off. On day-1 the lowest pheromone titer (5.1 ng) was found 4 hrs after the onset of photophase and was not significantly different from the peak titer (19.0 ng) observed at lights-off. For day-2 and day-3 females the lowest titer occurred at lights-on (7.4 ng and 5.3 ng, respectively) and was significantly different from the peak exhibited 4 hrs before lights-off (22.7 ng and 26.0 ng, respectively). Therefore, the diel periodicity of pheromone titer became apparent in

Figure 7

Diel periodicity of pheromone titer from individual day-0 through day-3 females (upper graph) and the observed percent female calling immediately before each extraction period (lower graph). Values for each extraction period were obtained from 8 to 12 females. Scotophase began 16 hrs after lights on. Means sharing letters in common were not significantly different ($P > 0.05$) after square root transformation of the data by the Student-Newman-Keuls multiple range test. Vertical bars denote standard deviations of the mean.


DAY 0
DAY 1
DAY 2
DAY 3
GLAND-EXTRACTED DISPARLURE (ng)
50
40
30
20
10
0
ab a ab ab abc bcd bcd abc bcd cd cd abc bcd cd d cd ab abc bcd cd bcd d
0 8 16 0 8 16 0 8 16 0 8 16
PERCENT CALLING
100
50
0
0 8 16 0 8 16 0 8 16 0 8 16
TIME DURING 16L:8D PHOTOPERIOD (hr)

females older than day-0. Peak titers were observed 0-4 hrs before onset of scotophase and minimum titers were found 0-4 hrs after onset of photophase.

Figure 7 also describes the percent females calling at each extraction time. The percentage of females calling was lowest 0-4 hrs after lights-on. For all other times of the day, 100% of the females called. These results concur with the previous description of the diel periodicity of calling reported by Charlton and Cardé (1982).

Comparison of the titer and periodicity curves revealed that periods of maximal titer always fell within periods of maximal calling activity, and minimal titer was generally associated with minimal calling. In addition, maximal calling was often associated with pheromone titers less than the peak titer. There appeared to be a delay (4 to 16 hrs) between the time when maximal calling was initiated and the time when the peak titer was exhibited. Also, during the declining phase of the titer periodicity curve, maximal calling continued.

The influence of age on the average pheromone titer found for each day is shown in Table 3. Day-0 females had significantly less pheromone in their glands (4.5 ng) compared to day-1 (12.4 ng) , day-2 (15.4 ng), and day-3 females (13.5 ng). No significant differences, however, were found among day-1, -2, and day-3 females. Thus, pheromone titer increased with age only between day-0 and day-1 females, after which, the average amount of pheromone found in the gland was independent of age.

Table 3. Effect of female age on the average daily pheromone titer.*

female age	n	pheromone (ng) x (SD)
day-0	60	4.5a (4.7)
day-1	59	12.4b (11.1)
day-2	60	15.4b (10.7)
day-3	60	13.5b (13.3)

* The average daily pheromone titer was calculated from the 6 extraction periods beginning at lights-on. Means followed by the same letter were not significantly different (P> 0.05) after square root transformation of the data by the Student-Newman-Keuls multiple range test.

Table 4 describes the pheromone titer in virgin and mated females of similar age (ca. 52 hrs old). After mating, females ceased calling. and exhibited a significant decrease in the amount of extractable pheromone (2.0 ng) compared to virgin females extracted during the same time period (19.6 ng). Furthermore, the decrease in titer in mated females occurred relatively quickly since females extracted immediately after pair separation contained similar amounts of pheromone as females extracted 1 hr later.

It should be noted that the structure of disparlure was identified by epoxidation of the olefin precursor found in an extract of 78,000 tips since quantities of the natural attractant, although detectable by male response bioassay, were considered insufficient for characterization (Bierl et al, 1970). Analysis of our extractions, however, showed that the amounts of olefin per gland was insignificant compared to the amounts of disparlure. Differences between the results presented here and those of Bierl et al. (1970) may be attributed to the type of extraction procedure performed and to the levels of impurities found in the extracts.

Discussion

Female gypsy moths possessed a daily rhythm of pheromone titer, which became apparent following the day of eclosion. After which, maximal titer occurred 0-4 hrs before lights-off and miminal titer 0-4 hrs after lights-on. Comparison of the titer and calling periodicity

Table 4. Effect of mating on pheromone titer.*

treatment	n	female age hr x (SD)	pheromone ng x (SD)
virgin	15	52.3a (2.2)	19.6a (11.0)
mated	11	52.0a (2.4)	2.0a (1.6)

* Females were mated on day-2 beginning 8 hrs after lights on. All mated females ceased calling. Gland extractions were performed an average of 30 min after pair separation. Titer from mated females was compared with the titer from virgin females 8 to 12 hrs after lights on. Column means are not significantly different ($P > 0.05$) by the Mann-Whitney test.

curves showed that the peak titer was found within the period of maximal calling and minimum titer generally coincided with minimal calling. Day-1 females had significantly more pheromone in the gland than day-0 females. Subsequent increases in age, however, had no effect on the average daily pheromone titer.

The pheromone titer periodicity in the gypsy moth can also be compared with the emission rate periodicity. In lab-reared moths (24^{o}C, 16L:8D), emissions in day-0 and day-1 females exhibited maxima (ca. 7.5 ng/hr and 14 ng/hr, respectively) 2 and 5 hrs before onset of scotophase and minima were observed shortly after lights-on (Charlton and Cardé, 1982). Thus, titer and emissions show similar times for both peaks and minima. A good correlation (r^2 = 0.44 following log transformation of the data) between gland-extracted and emitted quantities from individual females also exists (see Chapter II). Consequently, there does not appear to be appreciable amounts of pheromone stored in the gland during periods of reduced emission.

Similar comparisons can be drawn in H. zea. Pope et al. (1984) observed peak emission of the major sex pheromone volatile 2 hrs after the onset of scotophase, whereas Raina and Klun (1986) observed the peak titer of the same compound 4 hrs after lights-off. During photophase females did not call, had significantly lower amounts of pheromone in the gland (Raina and Klun, 1986), and released significantly lower amounts (Pope et al., 1984). Again, these data indicate that appreciable storage of pheromone probably does not occur.

In P. stultana, on the other hand, evidence indicates that

storage of pheromone does occur (Webster and Cardé, 1982). Maximal amounts of pheromone were found in the gland close to the onset of calling. Once maximal calling began, titer immediately started to decline rapidly suggesting that the rate of release was much higher than the rate of production. Investigation of the actual pheromone emission periodicity in P. stultana should further substantiate this hypothesis. Bjostad et al. (1980) observed a high emission rate at the onset of calling in T. ni which then exhibited an exponential drop as the time spent calling increased. These results suggested that release occurred more rapidly than production. The expected decrease in titer during maximal calling periods, however, was not observed by Shorey and Gaston (1965) who found no diel periodicity of titer in T. ni.

The chronological relationships which exist between the calling and titer periodicity curves in L. dispar suggest that there are links between their respective control mechanisms. Further evidence for related control was found after transection of the ventral nerve cord which interfered with calling, pheromone production, and pheromone release (see Chapter II). Raina and Klun (1984) demonstrated a factor in saline-extracted brain homogenates of of H. zea and 6 other species of moths, including L. dispar, which induced significant increases in pheromone titer when injected into neck-ligated or decapitated H. zea females compared to un-injected controls. Possibly, neural control of calling in the gypsy moth also mediates release of a neuroendocrine factor for increased pheromone production. This linked relationship between calling and pheromone production may account for the delay

observed between the onset of maximal calling and the peak in pheromone titer. Since calling remains maximal after the peak pheromone titer, a different factor may mediate the decline in titer.

The effect of age on pheromone titer in the gypsy moth indicates that females exhibit an increase in titer between the day of eclosion and day-1, and do not exhibit the periodicity of titer characteristically found in older females. The slow increase in pheromone on the day of eclosion and the inapparency of titer periodicity may be due to inertia of the pheromone biosynthesis "machinery" and/or expression of periodicity requires at least one lights-on/off exposure. Nevertheless, in day-1, -2, and day-3 females, the diel periodicity of titer is established and remains relatively constant.

In the gypsy moth, the rapid decline in titer observed after mating and the termination of calling would decrease the likelihood that a female would attract additional males before oviposition. Since virgin female calling behavior is neurally controlled (Hollander and Yin, 1982; see Chapter II), neural inhibition (possibly induced by sensory feedback information from reproductive structures) of calling may be implicated in the behavioral change following copulation. In addition, combined factors may arrest pheromone biosynthesis, prevent further pheromone release from glandular epithelia, and remove pheromone from surrounding cuticle (possibly through enzymatic degredation). Evidence shows that females receiving transection of the ventral nerve cord anterior to the terminal abdominal ganglion did not

call normally, exhibited a significant reduction in pheromone release rate, and contained significantly less pheromone in the gland compared to controls (see Chapter II). Although pheromone production does not appear to be under direct nervous control in the gypsy moth, indirect control may be achieved via release of neuroendocrine substances (see Chapter II).

In P. stultana, the transition from virgin to mated behavior may involve several factors. Virgin females which were decapitated or received topical applications of juvenile hormone (JH) ceased calling and exhibited a significant decline in glandular pheromone titer comparable to mated females (Webster and Cardé, 1984). Although decapitation also elimated oviposition, topical JH application induced egg deposition in a pattern similar to mated females. Apparently, interaction of both neural and endocrine factors may be important during the switch from virgin to mated behavior.

Female gypsy moths are typically monogamous obtaining sperm sufficient for over 95% fertilization from one mating (Forbush and Fernald, 1896; Doane, 1968). Multiple matings have been observed, however, particularly in dense populations (Cardé and Hagaman, 1984). Although sperm transfer for successful fertilization occurs during the first 5 to 7 min of copulation, males remain in copula with females for on average 1 hr (Doane, 1968). Extended copulation may ensure that the physiological transition of the female from a sexually receptive state to a non-receptive state is initiated.

Alternatively, in the Lepidoptera sperm precedence has been shown

in several instances and extended copulation appears to be a form of mate-guarding (see Drummond, 1984 for a review). A male monopolizes the female long enough to ensure establishment of sperm in the ductus seminalis, thereby decreasing the probability of sperm displacement by subsequent matings. The occurrence of sperm precedence in the gypsy moth, however, is unknown.

We have described the diel periodicity of pheromone titer in the gland of gypsy moth females and examined the effects of age and mating on titer. These results along with results from other investigations bring up basic questions concerning the nature of and relationships among the control mechanisms for calling, pheromone release, and production. Although investigations of endogenous control mechanisms have recently received much more attention than a decade age, we still know very little of the details. Why is pheromone stored in the gland in some species and not in others? How is pheromone release from glandular cells controlled? How is sexual receptivity terminated after mating? Future work will hopefully provide more answers to these basic questions of control.

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