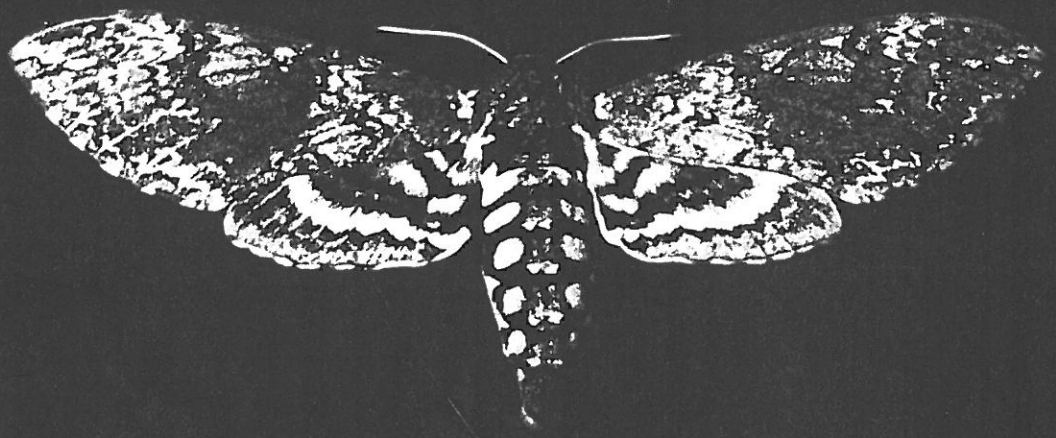
INSECT PHEROMONE RESEARCH

NEW DIRECTIONS



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Neural and Endocrine Control of Pheromone Production and Release in Cockroaches

Coby Schal, Dangsheng Liang, and Gary J. Blomquist

1. Regulatory Mechanisms of Pheromone Production and Release

Integration of physiological and behavioral events is required to produce and emit sex pheromones in coordination with other reproductive events. Thus, insects usually produce and emit sex pheromones, and exhibit specific behaviors associated with sexual receptivity, only when they are reproductively competent. While the production of pheromones is regulated biochemically or developmentally, emission of pheromones, particularly of volatile attractants, can also be regulated behaviorally, involving specific central nervous (CNS)-generated motor patterns. Insects thus employ a variety of mechanisms in the production and emission of sex pheromones:

- Pheromone production in some insects depends upon availability of biosynthetic precursors from host plants. Some danaid butterflies and arctiid moths modify plant pyrrolizidine alkaloids into volatile derivatives that are used as male pheromones (e.g., Schneider et al. 1975). It has become accepted that bark beetles detoxify tree monoterpenes through allylic oxidation into corresponding alcohols, which are then used as pheromones (review: Vanderwel 1994). However, recent data have demonstrated that *Ips paraconfusus* and *I. pini* synthesize the monoterpene mercene and the hydroxylated pheromone products ipsenol and ipsdienol (*I. pini* synthesizes only ipsdienol) from labeled acetate injected into the insects (Seybold et al. unpublished results). This raises the question of the relative importance of the *de novo* pathway *versus* the use of plant derived precursors to form the hydroxylated pheromone components in this group of insects.
- Neural mechanisms are involved in regulating both production and emission of pheromone in the gypsy moth, *Lymantria dispar*; neural messages

from the anterior portion of the CNS descend to the terminal abdominal ganglion (TAG) through the ventral nerve cord (VNC) (Tang et al. 1987). In this, as well as in other insects, neural signals originating from the abdomen of mated females ascend the VNC and inactivate sex pheromone production and/or release (e.g., Foster and Roelofs 1994).

- The bulk of recent work on regulation of pheromone production in insects, as well as emphasis in the present volume, is on humoral pheromonotropins, particularly the pheromone biosynthesis activating neuropeptides (PBAN) and related myotropic peptides with homologous carboxyl-terminal amino acids (reviews: Raina 1993; Nagasawa et al. 1994). In some insects, PBAN may stimulate release of other factors, such as a bursa copulatrix factor in the redbanded leafroller moth, *Argyrotaenia velutinana*, which in turn stimulates pheromone production (Jurenka and Roelofs 1993).
- Work with the housefly, *Musca domestica*, has shown that ecdysteroids, produced by the vitellogenic ovary, stimulate synthesis of the sex pheromone Z-9-tricosene (review: Blomquist et al. 1993). On the other hand, evidence from the moth *Heliothis virescens* suggests the involvement of 20-hydroxyecdysone in suppression of pheromone production after mating (Ramaswamy and Cohen 1992).
- Juvenile hormone (JH), a product of the corpora allata (CA), was shown to induce pheromone production and release in several coleopterans, cockroaches, and some lepidopterans (reviews: Cusson et al. 1994a; Schal and Smith 1990; Vanderwel 1994). In some insects, however, indirect evidence suggests that JH may also exert pheromonostatic effects (Webster and Cardé 1984). Although evidence for a direct action of JH on the site of pheromone production is lacking in the majority of cases, recent evidence from the boll weevil suggests that JH III enhances the production of sex pheromone by isolated male fat body *in vitro* (Wiygul et al. 1990).
- Developmental regulation of pheromone-producing glands occurs in all insects in which a sex-specific gland appears in the adult. In some lepidopterans, functional competency of the gland may be regulated by changing ecdysteroid titers during organogenesis (e.g., Tang et al. 1991). In cockroaches, cyclic maturational changes in the gland coincide with the ovarian cycle (Liang and Schal 1993c).
- Multiple mechanisms probably occur in most insects. In cockroaches and lepidopterans that respond to JH, it is likely that peptides are released in response to elevated titers of JH. For example, while either JH or PBAN can stimulate sex pheromone production in allatectomized *Agrotis ipsilon* (black cutworm moth), JH alone fails to induce pheromone production in decapitated females; it is therefore suggested that JH induces

release of pheromonotropic factors from the CNS (Picombon et al. 1995). Also, neural inactivation of pheromone production in many lepidopterans conceivably operates through suppression of pheromonotropic peptide release.

2. Juvenile Hormone: A Master Regulator in Cockroach Reproductive Biology

Cockroaches are a diverse group. Some exhibit parthenogenesis, whereas most reproduce sexually with reproductive modes including oviparity, ovoviviparity, and viviparity (Roth 1970). They therefore offer excellent models for comparative studies of the mechanisms that regulate female reproductive physiology and behavior, including the synthesis and emission of pheromones. *Blattella germanica* (German cockroach) and *Supella longipalpa* (brown-banded cockroach) are both oviparous blattellids. Females of both species emerge as sexually unreceptive adults, undergo several days of sexual maturation (which can be extended indefinitely if inappropriate conditions prevail), become sexually receptive, recruit conspecific males with sex pheromones, mate, and oviposit their vitellogenin-laden basal oocytes into an ootheca. Females of the brown-banded cockroach deposit their oothecae within 36 hours of oviposition and immediately enter a new vitellogenic wave. In contrast, under identical rearing conditions in our laboratory, *B. germanica* females carry the ootheca externally for about 21 days until the young hatch. During this time, oocyte development is inhibited, as in ovoviviparous and viviparous cockroaches in which the ootheca is retracted for embryonic development within the mother. Thus, while *S. longipalpa* females exhibit relatively uninterrupted cycles of oocyte development, *B. germanica* females experience discrete patterns of oocyte development which are interrupted by long pregnancies. These complex adult reproductive life histories contrast with most moth species, which mate soon after emergence, do not feed, and have short adult lives.

In *Blattella* and *Supella*, as in all cockroaches studied to date, vitellogenesis and cyclic maturation of oocytes parallels and is dependent upon JH III synthesis by the CA (Feyereisen 1985; Tobe and Stay 1985; Scharrer 1987). Thus, in females of both species the size of basal oocytes serves as a reliable predictor of relative CA activity (Bellés et al. 1987; Gadot et al 1989b; Smith et al. 1989). Likewise, synthesis of oothecal proteins is JH-regulated, and in the absence of JH (e.g., surgical allatectomy) both oocyte maturation and colleterial (accessory) gland growth are arrested (Zalokar 1968; Burns et al. 1991). JH production increases as the oocytes grow after the imaginal molt; and it declines just before ovulation, as the oocytes become chorionated, and remains low during pregnancy, while oocyte development is arrested. The JH biosynthetic activity of the CA in adult female cockroaches is dependent upon and modulated by (a) intrinsic signals

originating from the brain, ovary, mating, and nutrients and (b) extrinsic signals including temperature, pheromones, tactile cues, and social conditions, such as isolation and crowding, that act through sensory pathways (Engelmann 1970). The degree of dependence of allatal activity on any one of these inhibitory or stimulatory signals varies widely and appears to be species-specific. In *B. germanica*, activity of the CA is potentiated more by signals from grouping than by those from mating (Gadot et al. 1989a). Conversely, isolated and grouped adult *S. longipalpa* females exhibit similar patterns of oocyte development, and mating stimulates JH synthesis (Chon et al. 1990). In *Blattella*, diet quantity and quality also influence activity of the CA (Schal et al. 1993).

In all cockroaches studied to date, the CA have been shown to be restrained to varying degrees by neural signals from the brain (see Feyereisen 1985; Tobe and Stay 1985; Khan 1988). Recent evidence supports the idea that allatostatic peptides are in large measure responsible for this inhibition (see Woodhead et al. 1994 and references there). Specific relevant cues, such as those from mating in *Supella* or social interactions and food quality in *Blattella*, can, together or independently, lift the brain inhibition on the CA. Conversely, in gravid females, especially in ovoviviparous and viviparous females and in oviparous females with nonoverlapping growth of basal and penultimate oocytes, low JH titers are necessary to sustain pregnancies and to avoid premature expulsion of oothecae. This is accomplished through ascending neural signals from the uterus or vestibulum that suppress CA activity until "the right time." The brain integrates the multiplicity of stimulatory and inhibitory signals; and through a graded and gradual lifting of neural inhibition of the CA, it effects a cycle of JH biosynthesis. Therefore, transection of the nervous connections between the brain and the CA, which removes this brain inhibition and facilitates JH synthesis, has been used as an effective tool in studies of JH regulation of reproduction and, in turn, pheromone production.

Females of both cockroach species may mate once or multiple times, depending on the amount of sperm in their spermathecal reservoirs. Because adult cockroaches are long-lived and most of their gonadal maturation cycles do not require reinsemination of the female, it is expected that they would evolve mechanisms to effect pheromone release and associated behaviors only during specific gonotrophic stages. At other times, suppression of sexual receptivity would conserve energy and reduce exposure to predators and parasites. In this review we compare and contrast the hormonal and neural mechanisms that regulate sexual receptivity in *Blattella* and *Supella*. Since both share JH III as a common regulator of reproduction, we are particularly interested in examining the effects of modulators of CA activity upon synthesis and emission of sex pheromones. Our pivotal question is whether cockroaches that exhibit different patterns of CA activity (long *versus* short pregnancy) share common underlying regulatory mechanisms of pheromone production. Moreover, what mechanisms are involved in suppression of mate-recruitment during pregnancy? And lastly, we review the biosynthe-

sis, regulation, and internal transport of a contact sex pheromone in the German cockroach.

Pheromones, Glands, and Calling Behavior in *Blattella* and *Supella*

In spite of the vast economic importance of pest cockroaches, to date, only two major attractant sex pheromones have been identified. In the American cockroach, *Periplaneta americana*, periplanone-B, a 10-membered germacranoid structure, was identified and confirmed by synthesis (Persoons et al. 1976; Still 1979). Recent work with related blattids has elucidated related compounds that serve in species-specific blends and thus act in species recognition and isolation.

In the scotophase, *S. longipalpa* females engage in calling behavior, characterized by elevated tegmina and wings, a recurved abdomen, and occasional expansion of the genital atrium (Hales and Breed 1983). Calling is expressed only by receptive females at least 5 days old, is under circadian control, and is associated with the release of a volatile sex pheromone that elicits anemotactic responses in males (Smith and Schal 1990a, 1991). Behavioral assays of males and electroantennographic (EAG) assays with hexane extracts of various female body parts identified the fourth and fifth tergites as the major sites of sex pheromone production (Schal et al. 1992). We extracted the pheromone from integumental secretory units, each consisting of a secretory cell that discharges into a cuticular pore via a long subcutaneous duct supported by a duct cell (Liang and Schal unpublished). These cuticular pores are distributed throughout all tergites, but their density is highest on the lateral margins of the fourth and fifth tergites (Schal et al. 1992). The morphological evidence, together with behavioral and electrophysiological data, strongly implicate these structures as the site of pheromone production and release while the female engages in calling behavior.

An arduous rearing, extraction (from the tergites of some 20,000 virgin females and from air blown over calling females), and purification effort recently yielded supellapyrone (Fig. 1.1A) as the *S. longipalpa* sex pheromone (Charlton et al. 1993b). A synthetic racemic blend of supellapyrone [5-(2,4-dimethylheptanyl)-3-methyl-2*H*-pyran-2-one] elicits strong behavioral (long-distance anemotaxis) and electrophysiological (EAG) responses in males, as does the natural pheromone. The stereochemistry of supellapyrone was shown to be (2′*R*,4′*R*) by dual electroantenographic and flame ionization detection coupled to chiral capillary gas chromatography (Leal et al. 1995). Elucidation of the chemical structure of this pheromone and availability of synthetic supellapyrone will now facilitate *in vivo* and *in vitro* studies of endocrine regulation of its biosynthesis.

Virgin adult *B. germanica* females also exhibit a characteristic calling behavior; but in this species, calling peaks just before the end of the scotophase and extends well into the photophase; the diel pattern of calling coincides with the periodicity of mating (Liang and Schal 1993b). During calling, the female emits a volatile

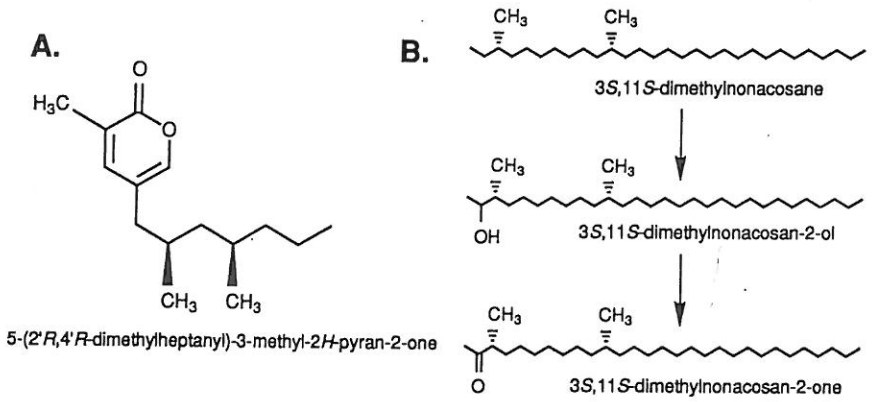

Figure 1.1. **(A)** Structure of supellapyrone, the sex pheromone of *Supella longipalpa*. **(B)** Biosynthesis of the contact sex pheromone of *Blattella germanica* showing conversion of the dimethylalkane to methyl ketone.

sex pheromone that serves to attract males from a distance. Olfactometer assays showed that the attractant is produced exclusively by adult females and that it elicits behavioral responses only in adult males. Behavioral and EAG assays localized the site of pheromone production in the tenth abdominal tergite, at the anterior of which an adult female-specific gland is found (Liang and Schal 1993a). This gland, too, is made of aggregates of integumental secretory cells that open via long ducts into orifices within deep depressions on the cuticular surface. The secretory cells of active glands (virgin day-6 females) are characterized by a large number of electron-lucid secretory vesicles, abundant rough and smooth endoplasmic reticulum (RER and SER), a large nucleus, and a long, convoluted end-apparatus which is surrounded by numerous long microvilli (Fig. 1.2) (Liang and Schal 1993c). The contents of the secretory vesicles are exocytosed into extracellular reservoirs at the base of microvilli and then transported to the cuticular surface through the long ducts. The chemical identity of the attractant sex pheromone of *B. germanica* remains unknown.

Figure 1.2. Schematic diagram showing maturation and developmental changes in the pheromone gland of *Blattella germanica* females in relation to the gonadal maturation cycle, juvenile hormone synthesis, and pheromone production. The immature secretory cell (SC) of a day-0 pheromone gland enlarges by approximately threefold by day 6, in relation to increasing JH production, basal oocyte size, and pheromone content. Active secretory cells contain numerous secretory vesicles (Sv), many of which discharge through large microvilli into an end-apparatus (EA). After oviposition, and throughout pregnancy, secretory cells regress, as do JH biosynthesis and pheromone content of the gland. Secretory cells enlarge again in the second gonotrophic cycle, coincident with an increase in JH

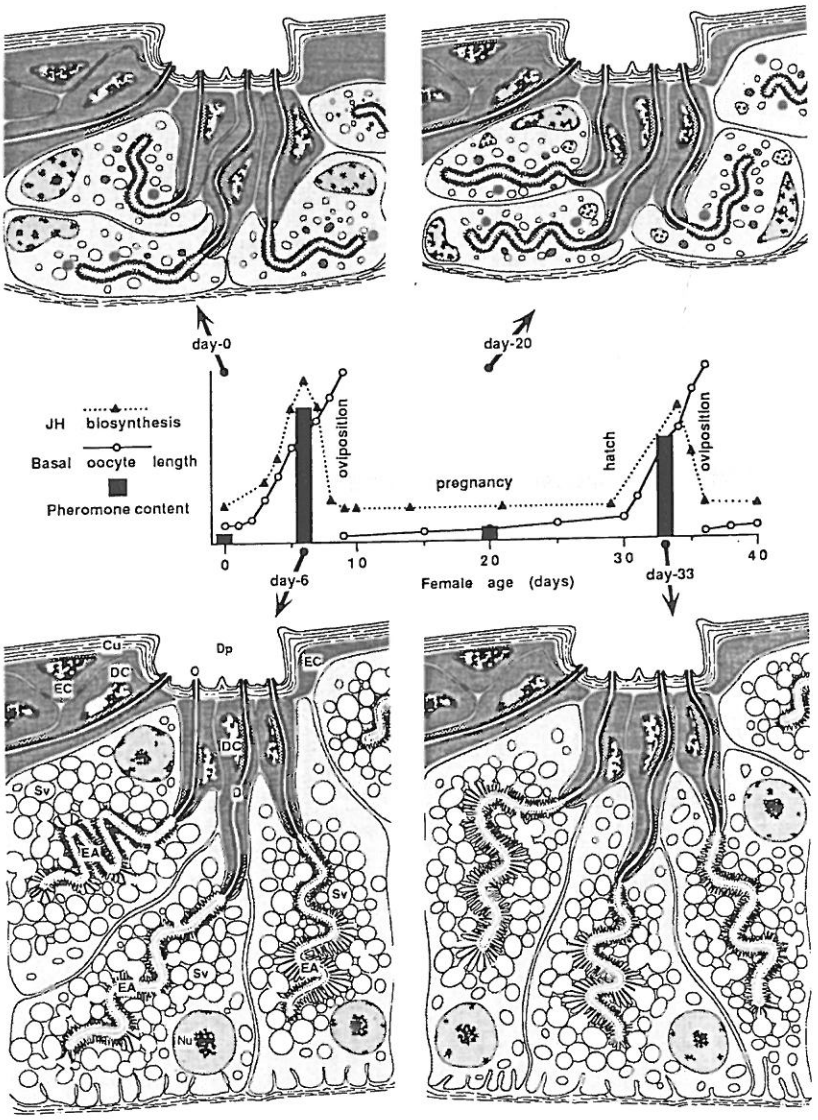

Figure 1.2. (Con't.) production, pheromone production, and oocyte growth. Rates of JH biosynthesis were determined with an *in vitro* radiochemical assay and were redrawn from Chiang et al. (1991b); data for basal oocyte length were redrawn from Chiang and Schal (1994). Pheromone content of the gland was determined by electroantennograms of gland extracts, as described in Liang and Schal (1993c). Schematics are based in part on descriptions in Liang and Schal (1993c). Schematics are based in part on descriptions in Liang and Schal (1993c). Cu, cuticle; D, duct; DC, duct cell; Dp cuticular depression; EC, epidermal cell; M, mitochondria; Nu nucleus; O, duct orifice.

4. Juvenile Hormone Induction of Sexual Receptivity and Pheromone Production and Emission in *Blattella* and *Supella*

JH involvement in the control of sexual behavior has been shown in several insects. Engelmann (1960) provided early evidence for the regulation of mating behavior by the endocrine system: Only 30% of 1-day-old *Leucophaea maderae* female cockroaches whose CA were removed mated during the next month. Subsequent work by Roth, Barth, and Bell (review: Barth and Lester 1973) showed that pheromone production in some cockroaches is under endocrine control. A major conclusion from several comparative studies of cockroaches (see reviews: Barth and Lester 1973; Truman and Riddiford 1974; Schal and Smith 1990) is that regulation of pheromone production, its release, and copulatory readiness by JH is species-specific. Thus, in *Byrsotria fumigata*, JH controls pheromone production but not mating readiness (Roth and Barth 1964); in some species (e.g., *Diploptera punctata*), newly eclosed females mate immediately, while still teneral, before the CA become active (Stay and Roth 1958; Stay and Tobe 1977), and yet in others (e.g., *B. germanica*) JH controls all three events (see below). Similarly, in a cricket, JH is responsible for the directed phonotactic responses of females to males, presumably by reducing the phonotactic threshold of an auditory neuron in the prothoracic ganglion (Stout et al. 1992; but see Loher et al. 1992). The regulation of copulatory readiness is much less clearly understood in this insect, since females with extripated CA mate when placed in contact with males (Koudele et al. 1987).

4.1. Sexual Receptivity

The close relationship between JH biosynthesis and the onset of pheromone production, release, and oocyte maturation in *Supella* and *Blattella* (Smith et al. 1989; Smith and Schal 1990a; Liang and Schal 1993b) suggests that the CA might be required for production of sexual signals and development or expression of female receptivity. Females emit these signals and mate only when JH titers reach a certain threshold level. Thus, teneral adult *B. germanica* females are sexually unreceptive; they routinely mount courting males, but by extending their legs, they move the abdomen out of the reach of males, or by mounting the male from the side, they avoid copulation. There is a clear relationship between copulatory readiness and size of the basal oocytes in virgin females with daily access to males: In a group of females of the same age, females that mate early have larger oocytes (Liang and Schal 1993b). Unobtrusive time-lapse video records showed that females mated on average 5.7 ± 0.13 (SEM) days after the imaginal molt (Schal and Chiang 1995), when their basal oocytes averaged 1.36 ± 0.03 mm, 54% of their maximal length at ovulation (see Fig. 1.2). Similarly, after virgin females abort their second infertile ootheca, they refuse to mate until their oocytes reach 1.28 ± 0.03 mm. This suggested a relationship between oocyte

maturation, or factors controlling oocyte growth (i.e., JH), and sexual receptivity. We therefore exposed females to conditions that would uncouple the usually tightly linked factors of female age and physiological (endocrine) state. An association between sexual receptivity and JH, independent of age, would provide support for the hypothesis that JH affects sexual receptivity. Various experimental manipulations, including ablation of the CA, therapy with JH analogs, CA denervation, ovariectomy, and changing nutrient quality, coupled with time-lapse infrared video recording, support the hypothesis that JH controls readiness of the female German cockroach to copulate (Schal and Chiang 1995).

4.2. Onset of Pheromone Production and Calling

Because the chemical identity of pheromones of both species was not known, regulation of the onset of pheromone production was studied by employing behavioral and EAG assays of whole-body or gland extracts. Thus, the relative amount of pheromone was estimated, not *de novo* biosynthesis. In both *B. germanica* and *S. longipalpa*, JH is required for production of sex pheromones and the expression of calling. Virgin *Supella* females initiated pheromone production at 4 days old and were calling at 6.15 + 0.91 days (Smith and Schal 1990a), in relation to increasing JH biosynthesis by the CA *in vitro* (Smith et al. 1989). Likewise, in *Blattella*, onset of calling and pheromone production in relation to increasing rates of JH synthesis suggested involvement of the CA (Liang and Schal 1993c, 1994). In both species, denervation of the CA from the brain of teneral females through nervi corporis allati I (NCA-I) or nervi corporis cardiaci I and II (NCC-I and II) transection significantly accelerates the onset age of calling (Fig. 1.3) (Smith and Schal 1990a; Liang and Schal 1994). Since this operation accelerates the rate of JH synthesis by the CA (Gadot et al. 1989b; Schal et al. 1993), these results lend further support that JH is a regulator of calling in both species. Extirpation of the CA (allatectomy) of newly emerged adult females prevents pheromone production and calling in both species, and both events can be restored by reimplantation of active CA or by treatment with JH or JH analogs. Although growth of the basal vitellogenic oocytes is controlled by and highly correlated with JH biosynthetic rates, direct or even intermediary involvement of the ovaries in regulating calling and pheromone production was excluded by ovariectomies in both species (unpublished; Smith and Schal 1990a).

Thus, in both species, females synthesize pheromones and initiate calling when JH reaches a certain threshold titer. In the absence of JH, both events are not expressed. It remains to be determined whether JH exerts its actions directly on secretory cells of the pheromone gland, or whether it acts indirectly by inducing the synthesis and/or release of pheromonotropic neuropeptides. The latter model would be similar to that of several noctuid moths in which JH induces pheromone production presumably by promoting the release of PBAN (Cusson et al. 1994a; Picimbon et al. 1995). The primary site of JH action in this scheme is likely to be the brain; and, if so, JH receptors should be found in the CNS.

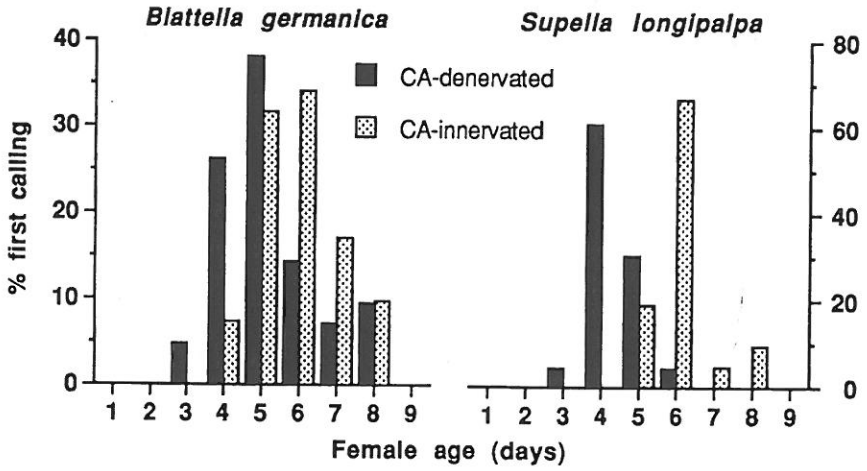

Figure 1.3. Effect of allatal nerve transection on calling behavior in *Blattella germanica* and *Supella longipalpa* females. Data were drawn from Liang and Schal (1994) and Smith and Schal (1990a), respectively.

5. Neural suppression of Pheromone Production and Calling in *Blattella* and *Supella*

Both pheromone production and calling are usually expressed only in receptive females seeking mates. In sexually immature adult females, absence of sexual signaling can be explained by low titers of JH since exogenous JH induces both calling and pheromone production in these females (see above). However, in older females, expression of both events (and sexual receptivity) is inhibited through specific signals to the CNS. Two important signals include cues associated with copulation and those from the ootheca. In spite of increasing rates of JH synthesis immediately after mating (Smith et al. 1989; Gadot et al. 1989a), the emission of sex pheromones is curtailed after copulation in both species. Termination of calling after copulation occurs through two successive events, with only minor differences between these two cockroach species. A first stage involves the physical insertion of a spermatophore into the bursa copulatrix, while a second stage involves presence of sperm in the spermathecae. In *Supella*, a brief and transient presence of a normal or spermless spermatophore (from a vasectomized male) in the female's genital atrium is sufficient to completely suppress calling in the first ovarian cycle; however, without migration of sperm into the spermathecae, females resume calling within several days, in relation to a new gonadal maturation cycle (Smith and Schal 1990b). While insertion of a spermatophore into the bursa copulatrix causes a temporary suppression of calling in *B. germanica*, sperm and/or associated secretions play a key role in the complete

suppression of calling in mated females even in the first ovarian cycle (Liang and Schal 1994).

Mechanical pressure from the spermatophore appears to be the relevant signal in cockroaches, since implantation of artificial spermatophores also terminated calling (Smith and Schal 1990b). Also, chemical factors from the male appear to not be involved, since injection of sperm, spermatophores, spermatophore extracts, or hemolymph from mated females did not suppress calling in virgin females. Since females that mate with castrated males resume calling, sperm appear to be involved in the second stage of matedness. In both cockroach species, an intact ventral nerve cord is required for the inhibitory signals from mating to be effective suppressers of calling behavior (Smith and Schal 1990b; Liang and Schal 1994) as in *Nauphoeta cinerea* females, where an intact VNC is required to turn off sexual receptivity after mating (Roth 1962). Thus, it appears that nervous signals resulting from mechanical distention of sexual organs in the female ascend the VNC and inhibit calling.

Virgin females of both *Supella* and *Blattella* do not exhibit calling behavior while carrying infertile oothecae (Smith and Schal 1990a; Liang and Schal 1993b). This is also due to signals that ascend the VNC, since transection of the VNC in gravid virgin or mated females released the expression of calling behavior. However, the ventral nerve cord also transmits signals that suppress JH synthesis in gravid females (Roth and Stay 1962; Chiang et al. 1991a; Gadot et al. 1991). Therefore, the occurrence of calling behavior in gravid *B. germanica* after VNC transection could also be explained by activation of the CA and a rise in the titer of JH in the absence of VNC-transmitted inhibitory signals. To distinguish between direct suppression of calling by neural directives and indirect suppression through inhibiting a rise in JH, we treated gravid females with a JH analog and either removed the ootheca or transected the VNC. Intact virgin gravid females did not exhibit calling behavior even when treated with exogenous JH; however, such females initiated calling immediately after the ootheca was removed or the VNC cut (Liang and Schal 1994). Therefore, the ootheca plays a dual function in the control of calling behavior: It inhibits calling directly, as well as indirectly by suppressing JH synthesis. Both signals ascend the VNC, and CA inhibition is effected by the brain (Gadot et al. 1991).

6. Developmental Changes Control Pheromone Gland Function in *Blattella*

Mature endocrine or exocrine glands, wherein all the cellular synthetic machinery is present and functional, can be regulated through precise and rapidly reversible quantitative changes in rate-limiting enzymes along the biosynthetic pathway. PBAN appears to operate in this manner in many, primarily short-lived, lepidopterans. For example, the pheromone gland of *Helicoverpa armigera* and *H.*

zea, taken from virgin or mated females of various ages and during various photoperiods, were capable of responding to PBAN *in vitro* (Rafaeli 1994). However, probably more universal among insects is a slow, developmental regulation of gland function through changes in the cellular machinery available for hormone or pheromone synthesis. Such regulation can occur in three phases:

1. Organogenesis of a sex-specific gland in the adult must be developmentally regulated during the imaginal molt. In short-lived insects that emerge as reproductively competent teneral adults, it is expected that functional competence of the gland to produce pheromone might be regulated by the same factors that control its organogenesis (e.g., ecdysteroids—see Tang et al. 1991 for example).
2. In insects whose gland acquires functional competence during an imaginal maturational period, developmental regulation might be involved at the target tissue level and manifested as ultrastructural changes. Examples include many insects that mature sexually for several days after eclosion, as well as adult insects that enter diapause or migrate before the onset of reproduction. Competence to produce pheromone may be maintained for the life of the insect.
3. In insects in which reproduction is interrupted by long periods of sexual inactivity, developmental regulation at the target tissue level results in the cyclic acquisition and loss of competence through periodic maturation and atrophy of glandular machinery.

Nothing is known about organogenesis of the sex pheromone gland in *B. germanica*. However, based on studies of adult male-specific glands (e.g., Sreng and Quennedey 1976), we infer that each secretory unit in the female pheromone gland (glandular cell, duct cell, duct) is formed during the imaginal molt from four progenitor cells in response to low JH titer throughout the last stadium and a rise in ecdysteroids late in the instar.

The imaginal gland exhibits a clear pattern of developmental maturation in relation to sexual maturation of the female (Liang and Schal 1993c) and subsequent development cycles that correspond to reproductive cycles. In correspondence to a low titer of extractable pheromone, the secretory cells of newly formed glands in teneral females are small (about 5–10 μm in diameter), the nucleus is elongated and conspicuous, occupying about one-third of the cell volume, and only a few small secretory vesicles are evident (Fig. 1.2). The microvilli surrounding the end-apparatus are extremely short (10–20% of active) and few in number. As pheromone production increases with age in virgin females, so does the size of pheromone-secreting cells: Mature day-6 glands are characterized by large secretory cells (about threefold larger than on day 0) containing a large number of spherical or oval electron-lucid secretory vesicles, an end-apparatus lined with numerous long microvilli, and a large spherical nucleus (Liang and Schal 1993c).

Shortly after mating, while JH titer is high, the secretory cell begins to decrease in size, the secretory vesicles begin to lyse, and the microvilli shorten in relation to a rapid decline in pheromone content. In gravid virgin and mated females the pheromone gland regresses to the appearance of a day-0 gland and its pheromone activity is barely detectable (Liang and Schal unpublished). The glandular layer in gravid females appears even thinner than in day-0 females, the nuclei decrease in volume and become elongated and branched, and densely packed residual bodies, probably containing degenerated organelles, appear in the cytoplasm. After the infertile ootheca is aborted in virgin females, the secretory cells of the pheromone gland become hypertrophied again, as in day-6 virgins, in relation to a reinstatement of sexual receptivity, pheromone production, and calling. This suggests that the sex pheromone gland in *B. germanica* females may be developmentally regulated during the reproductive cycles of the adult female as well as during organogenesis in the preimaginal insect. Further research will be needed to verify this point and to delineate whether the pheromone gland responds to JH and ecdysteroids.

It is reasonable to presume that, in order to conserve cell energy, this type of regulatory mechanism should occur in insects requiring long-term arrestment of pheromone synthesis because of sexual inactivity (long gestation period, diapause, migration). Given that, developmental regulation of pheromone biosynthesis should play a less significant role in *S. longipalpa*, which exhibits more continuous JH cycles with very short periods of CA inactivity. However, our morphometric studies of the CA indicate that cyclic growth and atrophy of CA cells play a major role in the regulation of JH biosynthesis during reproductive cycles in both *Blattella* and *Supella* females (Chiang et al. 1991a; Chiang and Schal 1994). Thus, it is plausible that developmental regulation of pheromone biosynthesis is not restricted to species with long periods of gestation. In *Supella*, developmental changes in the pheromone gland might be involved in the long-term suppression of pheromone production after mating, rather than in tracking JH titers at each ovarian cycle as in *Blattella* (see Smith and Schal 1990a; unpublished).

7. Courtship-Inducing Contact Pheromone in *Blattella*: Synthesis and Release

To elucidate which step(s) of pheromone biosynthesis is (are) endocrine-regulated, it is necessary to understand the biosynthetic pathway. The courtship-inducing contact sex pheromone of *B. germanica* has been extensively studied in this regard. It consists of oxygenated derivatives of cuticular hydrocarbons (HCs) and includes 3*S*, 11*S*-dimethylnonacosan-2-one (C_{29} methyl ketone) as a major component, derivatives containing an alcohol or an aldehyde group at the 29 position, and the C_{27} methyl ketone homolog, 3,11-dimethylheptacosan-2-one (Nishida and Fukami 1983; Schal et al. 1990b). Since the cuticular HCs of *B.*

germanica include 3,7-, 3-9-, and 3,11-dimethylnonacosane as major components (Augustynowicz et al. 1987), and only the 3,11- branching patterns are seen in the pheromone components, we proposed that the 3,11- HC isomer might give rise in the adult female to the methyl ketone pheromone with the same methyl branching pattern (Jurenka et al. 1989). Radiotracer and carbon-13 nuclear magnetic resonance (NMR) studies of incorporation of valine, isoleucine, and methionine, as well as succinate, into methyl-branched HCs revealed that the branched-chain amino acids serve as precursors to the methylmalonyl-CoA, which serves as the methyl-branch donor (Chase et al. 1990). Together with carbon-13 NMR studies of propionate incorporation, these studies clearly show that the methyl branches are inserted early in chain elongation. Presence of methyl-branched fatty acids, including *n*-3,11-dimethyl fatty acids with 16–20 carbons, in the integument of female *B. germanica* and incorporation of [methyl-^{14}C]methylmalonyl-CoA into methyl-branched fatty acids by a microsomal fraction provided evidence for a microsomal fatty acid synthetase (FAS) (Juarez et al. 1992). The methyl-branched fatty acids formed by microsomal FAS are then presumably elongated and converted to methyl-branched hydrocarbons. Comparative kinetic studies on the microsomal and soluble FAS have been reviewed by Blomquist et al. (1993).

During the first two ovarian cycles, biosynthesis of methyl ketone pheromone *in vivo* (assayed with [1-^{14}C]propionate incorporation into methyl branches) and its accumulation on the epicuticle correspond to the pattern of JH biosynthesis by the CA *in vitro* (assayed with L-[methyl-^{3}H]methionine as a methyl donor) and oocyte maturation (Schal et al. 1990a, 1991, 1994). This suggested that the CA and JH were involved in regulating pheromone production. Removal of the CA reduced the amount of C_{29} methyl ketone on the cuticle, whereas the JHA hydroprene significantly accelerated both oocyte development and pheromone production (Schal et al. 1990a). Since JH analogs also restore pheromone production in decapitated and head-ligated females, factors from the corpora cardiaca (CC), such as PBAN or related factors, appear to not be involved.

While pheromone production is JH-mediated, the synthesis of the parent methyl-branched HC is dependent to a large extent on food availability, not JH (Schal et al. 1994). Normal females synthesize methyl-branched HC rapidly during their vitellogenic feeding stage and HC synthesis declines well before any appreciable decrease in JH biosynthesis, but concomitantly with reduced food intake. Conversely, allatectomized females eat less and synthesize less HC during the same time. While gravid females feed only sporadically and concurrently synthesize little HC, rates of HC synthesis in allatectomized females (without oothecae) remain relatively high. An important site for deposition of HC in normal females is the basal oocytes, because the amount of HC deposited into oocytes equals the amount on the epicuticle (see below). In the absence of ovarian uptake of HC in alltectomized females, the hemolymph titer of HC increases well beyond normal levels (Schal et al. 1994). As internal HCs increase,

so do cuticular HCs and both internal (hemolymph) and cuticular methyl ketone pheromones. These patterns suggest that normally, stage-specific feeding regulates the amount of 3,11-dimethylnonacosane available for pheromone production. Thus, availability of *de novo* synthesized substrate is one mechanism by which *B. germanica* regulates contact pheromone production.

A second mechanism involves the stage-specific JH-mediated conversion of the HC to methyl ketone (Fig. 1.1B). Chase et al. (1992) concluded from radiolabeling studies that the pheromone arises from conversion of 3,11-dimethylnonacosane to 3,11-dimethylnonacosan-2-one via an alcohol intermediate, and they showed that JH analogs can stimulate the conversion of radiolabeled hydrocarbon to methyl ketone in females. Since [11,12-$^{3}H_2$]3,11-dimethylnonacosan-2-ol is readily metabolized to methyl ketone by both males and females, and only vitellogenic females efficiently hydroxylate the radiolabeled HC, it appears that formation of the 3,11-dimethyl alcohol occurs only in adult females and that this step might be under JH control (Chase et al. 1992).

To understand the mechanisms by which HCs and pheromones are compartmentalized among sites of synthesis, the hemolymph, the ovaries, and the cuticle, it is necessary to elucidate the sites of synthesis and dynamics of transport among target deposition sites. Both HC and contact sex pheromone in *B. germanica* females are synthesized primarily by abdominal integument, while incorporation of [^{14}C]propionate into HC and pheromone in other body parts (head, wings, legs, thorax, fat body, digestive tract, and vitellogenic ovaries) is negligible (Gu et al. 1995). Moreover, the sternites synthesize significantly more methyl-branched HC and methyl ketones than the tergites. These patterns strongly favor abdominal oenocytes or epidermal cells as the sites of synthesis of both HC and pheromone. However, occurrence of both lipids internally after extensive washing of the cuticle prompted us to examine the distribution of HC and pheromone in internal tissues. Most of the internal HCs and their methyl ketone derivatives can be recovered from ovaries and the integument. It appears that hemolymph carries and circulates HC and pheromone in the female.

In most insects the mechanisms of lipid transport involve lipophorin (reviews: Shapiro et al. 1988; Kanost et al. 1990; Van der Horst 1990; Law et al. 1992). Using *B. germanica* female hemolymph, we isolated and purified a high-density lipophorin (HDLp, 1.109 ± 0.002 g/ml) by KBr gradient ultracentrifugation and showed that it was the only hemolymph protein that carries HC and contact pheromone (Gu et al. 1995). Injected radiolabeled HC was recovered from HDLp, as was the radiolabeled pheromone to which it was converted. These results suggested the hypothesis that lipophorin loads newly synthesized HC and pheromone from the abdominal epidermis and transports them to various tissues, including the ovaries and epicuticle. A time course of incorporation of [^{14}C]propionate showed that newly synthesized methyl-branched HCs appear first in the epidermal fraction and hemolymph, and later on the epicuticle, supporting the Lp-mediated transport hypothesis. Experimental support for this idea was provided by

severing the veins to the forewings, thus blocking hemolymph transport to the wings. The amount of newly synthesized HC on the wings was significantly lower than on intact forewings, and the amount of topically applied radiolabeled HC that was recovered in the hemolymph was greater than on the surface of the wings (Gu et al. 1995). These results, and the abundance of epicuticular HCs on nonsynthesizing body parts (e.g., wings, legs), clearly show that transport of HCs and their derivative pheromones is mediated by a HDLp, which shuttles newly synthesized lipids from the abdominal oenocytes to epicuticular and internal deposition sites, including the ovaries.

8. Conclusions

Often, the reproductive state of individual insects can be inferred from the sexual signals that they produce; they exhibit specific behaviors associated with sexual receptivity only when they are reproductively competent. Receptive virgin individuals produce songs, displays, pheromones, and other signals that attract conspecifics or elicit courtship in members of the opposite sex, while immature virgins and mated individuals do not solicit mates. In females with reproductive cycles, such as cockroaches, these behaviors usually coincide with ovarian maturation, suggesting that these signals may ensure courtship by males only at appropriate stages in the female's gonadal maturation cycle. We propose that cyclic acquisition and loss of sexual receptivity and emission of mate-recruiting signals might be related to the ecology of the insect. In some species, especially in solitary short-lived insects, the female may remain sexually receptive throughout the reproductive cycle, including periods during which she does not produce or emit sexual signals, because she is unlikely to contact and be courted during such periods. In contrast, coordination of copulatory readiness with mate-solicitation should be more important in gregarious long-lived species. Mate attraction by one receptive female may cause nonsoliciting females within the group to respond to male courtship if their mating receptivity is not physiologically restrained. In such species, especially those with repeating gonadotrophic cycles (e.g., cockroaches), it is likely (but not necessary) that hormones that regulate sexual physiology will also coordinate the expression of specific sexual behavioral responses. Thus, in insects in which JH regulates reproductive physiology, it would be efficient for JH also to coordinate the production of sex recruitment signals and sexual receptivity.

Juvenile hormone exerts major pleiotropic effects on insect development and reproduction. It acts as a repressor in larval growth and development, and it regulates some major gene products in adult females, including vitellogenin, a yolk protein synthesized by the fat body, and oothecin, an egg case protein synthesized in the accessory sex glands. This may be why a close relationship has evolved in some cockroaches between gonadal maturation and sexual behavior, as

in many metazoans. Our results support the hypothesis that JH controls sexual receptivity and sexual signals emited by *Blattella* and *Supella* females, including sex pheromones. The activity of the CA in adult female cockroaches is dependent upon and modulated by both internal states (physiological, nutritional) and environmental stimuli (temperature, photoperiod, social interactions). Favorable stimuli result in a graded lifting of brain inhibition upon the CA, permitting the synthesis and release of JH. In addition to inducing protein synthesis in the female, in some species JH also stimulates the female cockroach to produce sexual signals, including both attractant and courtship-eliciting pheromones, and to become sexually receptive. Importantly, mated females also produce JH, but they neither emit sex pheromone nor regain sexual receptivity. It thus appears that while JH is required for the expression of copulatory readiness and pheromone production in *S. longipalpa* and *B. germanica*, signals associated with copulation (spermatophore, sperm, accessory secretions) can inhibit this behavioral state even when titers of JH are permissive for sexual receptivity.

Our results suggest that JH plays a "permissive" indirect role in the regulation of calling behavior. It is necessary for the expression of calling, but is not sufficient in the presence of ascending inhibitory signals from the terminal abdominal ganglion. These neural signals might inhibit the release of humoral signals from the brain, whose release is possible only when JH titers are high. Alternatively, neural signals that ascend the VNC might inhibit a JH-inducible motor program in the CNS. While signals associated with the ootheca are transient (up to 36 hours in *Supella*; 21 days in *Blattella*), inhibitory signals from mating are effective for the life of the female so long as enough viable sperm reside in the spermathecae. Clearly, the involvement of PBAN or PBAN-like peptides must be examined in production and emission of volatile pheromones in cockroaches. Conversely, JH appears to have direct effects on contact pheromone production since decapitated females treated with JH synthesize pheromone. The effect of JH needs to be confirmed *in vitro* using isolated epidermal tissues and oenocytes.

Developmental regulation of pheromone production and behavioral control of pheromone release are important mechanisms in *B. germanica*, and probably other insects that experience protracted periods of reproductive inactivity. Although we are aware of several reports on pheromone gland organogenesis during the imaginal molt, maturational phenomena, such as those we describe for the pheromone gland of *B. germanica* and for the CA of cockroaches, appear to be rare. This, we believe, is due to the scarcity of studies on long-lived insects with cyclic reproduction. It is likely that in such insects with long periods of reproductive inactivity (e.g., pregnancy, migration, diapause, nutrient deficiency), regression of pheromone glands will be found as a mechanism to conserve energy and ensure sexual inactivity. For example, our work with regulation of *B. germanica* CA reveals an early acquisition of competence to produce JH (CA maturation), followed by periodic volumetric regression of CA cells accompanied by loss of competence to biosynthesize JH, even in the presence of late precursors along

the biosynthetic pathway (Gadot et al. 1989b; Chiang et al. 1991a; Chiang and Schal 1994). These volumetric changes in CA cells are synchronized and paced by various cues, including ovarian factors, resulting in a precise correlation between JH cycles and oocyte development. Thus, in *B. germanica*, rates of JH synthesis during ovarian cycles are mainly regulated through cyclic maturation and regression of cellular machinery in the CA, and these rates can be modified rapidly by allatostatic and allototropic modulation of rate-limiting enzymes.

To our knowledge, *B. germanica* is the only species in which lipophorin transports a sex pheromone. It remains to be seen whether Lp-mediated transport of sex pheromone is unique to this species or a general principle for long-chain cuticular lipids. Indirect evidence from other species suggests that an internal transport pathway, possibly involving Lp, might also occur in species that utilize medium- and short-chain HCs as sex pheromones (Blomquist et al. 1993; Charlton and Roelofs 1991; Schal et al. unpublished).

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