INSECT PHEROMONE

Insect Pheromone Biochemistry and Molecular Biology

The biosynthesis and detection of pheromones and plant volatiles

This book provides an up-to-date and in-depth coverage of how insects produce pheromones and how they then detect both pheromones and plant volatiles. Well over half the species on the planet are insects - more than 800,000 in all. Their perception of each other and their world is achieved through the production and reception of chemical odors that provide essential information for the location of prospective mates and food supplies. These chemical messages are unique for each species and thus represent a vast landscape in which we may explore the evolution of behavior and communication.

Many insects such as moths, beetles, aphids and grasshoppers are also pests of crops. Attracted by the specific smells released by these plants, they not only find food, but also each other, aided further by the odorous pheromones that they synthesize and release. Feeding and breeding are thus equally served by their extraordinary sense of smell. Understanding the underlying mechanisms of odor detection and pheromone biosynthesis offers us the means to disrupt their predations and populations without the use of harmful and poisonous pesticides. In the realm of disease transmission, insects such as mosquitoes, ticks and fleas feed on the blood of humans and other animals and in so doing transfer the dangerous pathogens which cause illnesses such as malaria, lime disease and plague. As with the plant feeders, these insects find their hosts by smell. Again, understanding the underlying mechanism of odor detection can help us to combat the process and contribute to improving human health. Continuing research into insect olfaction, founded on the study of insect pheromones, thus provides tremendous scope for mitigating the profound socio-economic impact of insects.

Gary Blomquist and **Richard Vogt** have been leaders in pheromone production and reception, respectively, for over 20 years. Blomquist was co-editor of the very successful 1987 book "Pheromone Biochemistry", and has organized and been part of numerous symposia on the biosynthesis and endocrine regulation of pheromone production. Vogt was involved in the pioneering work on the biochemistry and molecular biology of pheromone reception in insects and has remained a leader in this area. He has organized and participated in numerous symposia and written several reviews ion the subject.

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The biosynthesis and detection of pheromones and plant volatiles

Gary J. Blomquist
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NEW BOOK

"Insect pheromone Biochemistry and Molecular Biology: the biosynthesis and detection of insect pheromones and plant volatiles."

Edited by Gary J. Blomquist and Richard G. Vogt
Department of Biochemistry, University of Nevada
Department of Biological Sciences, University of South Carolina

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The 1987 book "Pheromone Biochemistry" summarized what was then known about the production and reception of insect pheromones. Remarkable advances in our understanding of pheromone production have occurred in the last one and a half decades, which is mirrored by similar advances in our understanding of pheromone reception. This progress is detailed herein by selected authors who are the leaders in the field. We have assembled contributed chapters from experts who are at the frontiers of pheromone chemistry, neurobiology, chemical ecology, molecular biology and biochemistry.

10

Regulation of pheromone biosynthesis, transport, and emission in cockroaches

Coby Schal, Yongliang Fan, and Gary J. Blomquist

10.1 Introduction

Chemical signaling is fundamental for the survival and reproduction of most animals. Chemical cues allow animals to appraise their environment, to detect food, toxins, prey, predators and pathogens, to identify kin, and to evaluate and bias mate-choice decisions. Many insects depend on sex pheromones for the identification of reproductive partners and the onset of reproductive physiology and mating behavior. However, the prevalence and importance of chemical signaling in various insect groups depends on the natural history of the insects. Cockroaches are the quintessential chemical communicators. In contrast to the closely related grasshoppers and crickets (Orthoptera), which rely on sound as their primary modality in communication, cockroaches use olfactory and tactile signals in their social behavior. Most of the 4000+ described species of cockroaches are nocturnal. Many use long-range volatile pheromones in mate-finding and cuticular contact pheromones in the final recognition process. Short-range volatile pheromones emitted by the male, coupled with nuptial tergal secretions, facilitate proper alignment of the pair prior to copulation. Pheromones also mediate intrasexual conflicts, especially when males establish dominance hierarchies and territories, in parent–offspring communication, stage and population recognition, trail-following behavior, and as epidiectic pheromones that mediate dispersion behavior. In this chapter we discuss only sex pheromones that are

used in mate-finding and recognition. First, we describe reproductive modes in cockroaches as they relate to pheromone production. We then present a conceptual framework for neuroendocrine regulation of cockroach reproductive biology. Last, we discuss pheromone production in several of the best studied species, reviewing for each what is known about the tissues, biochemical pathways, transport routes, and neuroendocrine regulation of pheromone production. Cockroach sex pheromones in general were last reviewed by Gemeno and Schal (2003) and regulation of pheromone production in cockroaches was last reviewed by Schal and Smith (1990), Tillman *et al.* (1999) and Blomquist *et al.* (2003).

10.2 Reproduction and pheromones in cockroaches

Blattodea (Order Dictyoptera) is divided into five families, three of which – Blattidae, Blattellidae, and Blaberidae – contain most of the cockroach species (McKittrick, 1964) and all the species for which sex pheromones have been identified to date. The other two families, Cryptocercidae and Polyphagidae, are poorly known. This highly diverse group of insects exhibits a variety of reproductive strategies, including obligatory and facultative parthenogenesis, oviparity, ovoviviparity, and viviparity (Roth, 1970). Their social organization ranges from solitary individuals to genetically related families with monogamous parents. They live in temperate as well as tropical habitats, deserts, caves and hollow trees, bromeliad pools, in the nests of birds and social insects, and in association with human-built structures, sewers, and dump sites (Schal *et al.*, 1984).

Oviparous females oviposit their eggs into a protective egg case (ootheca), which is either deposited soon after being produced or carried by the mother until the nymphs hatch, depending on the species. In ovoviviparous and viviparous species the egg case is reduced, the embryos are incubated within a brood sac or uterus, and live nymphs hatch from the mother (parturition). The reproductive cycle in cockroaches is regulated by several lipid and peptide hormones, but juvenile hormone III (JH III), a C_{16} sesquiterpenoid, is a critical adult gonadotropic hormone. JH III stimulates the fat body to produce vitellogenin – a yolk protein precursor – and the oocytes to endocytose vitellogenin, it stimulates the production of oothecal proteins by accessory glands, and it elevates food intake in vitellogenic females. As will become apparent in this chapter, behavioral and physiological events related to mate-finding and sexual receptivity are also regulated in a coordinated manner by this vital hormone. Generally, the female cockroach undergoes sexual maturation for 1–3 days after the imaginal molt, she feeds and drinks extensively as her corpora allata (CA) synthesize and secrete JH III, and the fat body responds by synthesizing massive amounts of vitellogenin. In response to JH III the virgin female also produces and releases sex pheromones, and mates. Her basal oocytes continue to synchronously take up yolk protein precursors

and grow, and as hemolymph ecdysteroids peak the JH III titer dramatically declines, the oocytes become chorionated, they are ovulated, fertilized, and the eggs packaged into an ootheca. Most oviparous cockroaches carry the ootheca for just one to a few days, but ovoviviparous species retract it into the uterus and incubate the embryos for several weeks or months. During this gestation period, the JH III titer in the hemolymph is low, the gravid female feeds little and only sporadically, and she remains sexually unreceptive. As the neonates synchronously hatch from the egg case, a second gonotrophic cycle ensues. Depending on the species, an adult female can undergo ~6–30 such cycles. In connection with pheromone production, sexual receptivity may be permanently suppressed after mating, or it may reappear during the vitellogenic stage of each successive reproductive cycle.

Cockroaches have long served as a model system for studies of invertebrate biochemistry, endocrinology, and neurobiology (Tobe and Stay, 1985; Scharrer, 1987; Huber *et al.*, 1990). Therefore, a wealth of information is available about endocrine regulation of reproduction, which provides a useful foundation for studies of the neural and endocrine bases of pheromone production and emission. The cockroach system has other advantages. First, both volatile and contact pheromones have been characterized chemically and behaviorally in some species. Second, their large size facilitates biochemical and physiological investigations. And finally, cockroaches are major urban pests and cockroach infestations spread disease and serve as a major source of allergens that can cause asthma. It is therefore conceivable that understanding when, where, and how sex pheromones are produced will help shape new approaches on environmentally compatible pest control.

10.3 Regulatory mechanisms of pheromone production and release

Regulation of sex pheromone production and emission can occur through several major regulatory mechanisms, but it is likely that several of these mechanisms operate in concert in most insects:

1 Organogenesis of sex pheromone-producing glands occurs during pre-imaginal development. In the adult stage, a period of glandular differentiation is followed by alternating phases of sexual receptivity followed by sexual inactivity, generally during gestation. In some insects, pheromone secretory cells hypertrophy and gain competence for the production of pheromone during periods of sexual receptivity and regress during stages of sexual inactivity.
2 Availability of biosynthetic precursors from food, as in some danaid butterflies and arctiid moths, which modify plant pyrrolizidine alkaloids into volatile derivatives that are used as male pheromones (e.g. Schneider *et al.*, 1975). In

insects that produce large amounts of cuticular lipids, food availability (e.g. malonyl-CoA) may control the flux of carbon into the pheromone biosynthetic pathway. A deficiency of specific methyl branch donors, for example, may impede production of methyl-branched hydrocarbons.

3 Endocrine mediators, including specific pheromonotropins (e.g. pheromone biosynthesis activating neuropeptide [PBAN]) (reviewed in Raina, 1993; Nagasawa *et al.*, 1994; Rafaeli, 2002; Rafaeli and Jurenka, Chapter 5, in this volume), JH (reviewed in Tillman *et al.*, 1999), and ecdysteroids (reviewed in Blomquist *et al.*, 1993; Blomquist, Chapter 8, in this volume), can regulate pheromone biosynthesis in different insect species.

4 Neuronal signals that descend from the central nervous system (CNS) or ascend through the ventral nerve cord (VNC) can modulate sex pheromone production and/or its release.

5 Chemical signaling can be regulated at the level of calling – the emission of attractant sex pheromones during specific behaviors. In some insects, pheromone production continues uninterrupted in the adult stage, and mate-finding is regulated only through controlled release of the pheromone (e.g. Charlton and Roelofs, 1991; Schal *et al.*, 1998a).

10.4 Barth's hypothesis revisited

In 1965 Barth proposed a hypothesis relating neuroendocrine control of pheromone production to life history strategies of insects. Specifically, neuroendocrine control was predicted for insects with a long-lived adult stage and with multiple reproductive cycles interrupted by periods during which sexual receptivity and mating are not appropriate or not even possible anatomically. Conversely, in insects that eclose with mature oocytes, and live for only a few days as adults, Barth (1965) predicted that pheromone signaling would be part of the adult metamorphic process and not subject to neuroendocrine control. Early work on long-lived cockroaches and beetles and short-lived moths lent support to this hypothesis. However, the discovery of PBAN in a number of lepidopteran insects (see Rafaeli, 2002; Rafaeli and Jurenka, Chapter 5, in this volume) called for a reassessment of this hypothesis. Yet, two fundamental elements of Barth's hypothesis were overlooked in a rush to dismantle this rather useful conceptual framework. First, Barth clearly stated that "in those [Lepidoptera] species which do feed as adults, oocytes maturation requires the participation of JH and perhaps neuroendocrine factors as well. So far as we know the control of mating behavior in Lepidoptera which feed as adults has not been investigated but, . . . the existence of neuroendocrine regulating mechanisms for mating behavior would not be surprising. In fact we might venture to predict that some form of neuroendocrine regulation of mating behavior is likely in the majority of insects which feed as adults and

which depend on JH or neuroendocrine factors for oocytes maturation" (Barth and Lester, 1973). Second, the hypothesis relates the existence of neuroendocrine control of pheromone production to the type of reproductive cycle exhibited. It predicts that neuroendocrine control would also be found in short-lived moths that exhibit periods of sexual inactivity. Delaying reproductive activity until after migration is completed (reviewed in Dingle, 2002) or a suitable host plant is located (e.g. Raina *et al.*, 1997) are examples of such a strategy.

We propose a reconsideration of Barth's hypothesis, taking into account the coordination of reproductive developmental processes with mating-related events. In long-lived insects, such as cockroaches, pheromone production is regulated synchronously with other reproductive processes by the same hormone, usually JH. Cellular remodeling of the pheromone glands plays a prominent role in this group of insects, resulting in a slow stimulation of pheromone production. The cessation of pheromone production after mating is also slow, and precise control of pheromone signaling, therefore, is not at the level of pheromone production, but rather at the behavioral level through pheromone emission during calling. Conversely, in short-lived moths rapid modulation of rate-limiting enzymes in the pheromone biosynthetic pathway is much more prominent than developmental processes, and pheromone biosynthesis is turned on or off in coordination with activity cycles (day versus night) and sexual receptivity (virgin versus mated). Control of sexual signaling occurs at the level of pheromone production as well as emission, but these two events are usually regulated by different factors. Thus, both groups of insects exhibit neuroendocrine control of pheromone production. In cockroaches, pheromone production is coordinated with the gonotrophic cycle and the major gonadotropic hormone – JH – has been recruited to control both by acting at several target tissues. In most moths, on the other hand, reproduction and pheromone production are regulated by different hormones. But here also, the hormones that control pheromone production (e.g. PBAN) also affect other target tissues as myotropins, melanization agents, and diapause and pupariation factors. An interesting departure from the moth model occurs in migratory moth species in which reproduction is delayed by migration (low levels of JH production and sexual inactivity), and pheromone production and its release are JH dependent (Cusson *et al.*, 1994; Dingle, 2002). All these observations are consistent with our interpretation of Barth's model.

10.5 Anatomical sites of pheromone production and calling behavior

10.5.1 Volatile pheromones

Although early reports considered various organs in the head, thorax, and abdomen as possible sites of pheromone production in cockroaches, most of the more

recent research implicates specialized abdominal glands. Nevertheless, the biosynthetic tissue may be dorsal (tergal), ventral (sternal), genital (e.g. atrial glands), or associated with the digestive tract. Periplanones-A and -B, the sex pheromone components of *Periplaneta americana* (L.) (American cockroach, Blattidae), are a case in point. They are highly volatile and readily adsorb to females, their feces, and the cage in which virgin females are kept. This, coupled with the extraordinary behavioral sensitivity of males to periplanones, seriously confounded early behavioral bioassays of female extracts. Indeed, the exact location of the pheromone in the abdomen has yet to be resolved. There is general agreement that the greatest amount of pheromone activity is in the midgut (Takahashi *et al.*, 1976; Persoons *et al.*, 1979), and that it can be recovered from feces. However, female calling involves opening the genital vestibulum (=atrium; posterior genital pouch where the egg case is formed – terminology of McKittrick, 1964), without excretion of feces (Abed *et al.*, 1993b), and this glandular tissue (atrial glands) in fact attracts males (Seelinger, 1984). GC-MS analysis of extracts from the alimentary canal, the last two abdominal segments (which contain the atrial glands), and the anterior part of the abdomen confirmed that the atrial glands contain most of the periplanone-B, up to 60 ng (Abed *et al.*, 1993b). In *Blatta orientalis* L. (Oriental cockroach), a closely related species, the atrial glands also elicited the strongest male responses and of various tissues tested, only the atrial glands stimulated any males to court by raising their wings (Abed *et al.*, 1993a). The epithelium of the atrial gland consists of class-1 glandular cells, in which the secretion passes directly to the cuticle and not through a duct. However, Yang *et al.* (1998) extracted several abdominal tissues of *P. americana*, including the atrial glands, and concluded that both periplanone-A and periplanone-B were most abundant in the colon (0.34 and 8.31 ng, respectively), and that this tissue elicited the strongest electroantennogram (EAG) responses in males. Moreover, feces extracted from the colon elicited strong sexual responses in males, showing that fecal material contained pheromone before it could contact the atrial glands. Unfortunately, in these two species, as in other cockroaches that produce volatile sex pheromones, no definitive proof of the sites of pheromone biosynthesis is available through isotope tracing of pheromone precursors in isolated tissues.

The volatile sex pheromone of female *Blattella germanica* L. (German cockroach, Blattellidae) is produced in the 10th abdominal tergite, termed the pygidium. Because this pheromone has not been chemically identified, this conclusion is based upon surgical manipulations followed by bioassays with both live females and female extracts. In olfactometers, fewer males were attracted to the pygidium of males than to that of virgin females (Abed *et al.*, 1993c), and in two-choice assays females whose pygidium was ablated attracted only 13 percent of the males, whereas sham-operated females attracted 87 percent of the males (Liang and Schal, 1993a). Clear tergal modifications can be seen on the

anterior of the female pygidium but not in nymphs or males. The pheromone gland consists of three groups of cuticular depressions, one medial and two lateral, and each group contains numerous cuticular depressions, within which are 1–32 orifices (Liang and Schal, 1993c; Tokro *et al.*, 1993). Each orifice is ~0.5 μm in diameter and leads to a duct that penetrates through the cuticle and inserts deeply within a large secretory cell located in the modified epithelium beneath the cuticle. This class-3 secretory unit consists of a large secretory cell and a much smaller duct cell. Within the secretory cell a complex end-apparatus is elaborated which consists of numerous long microvilli. The secretory cells of mature, pheromone-producing virgin females are characterized by abundant mitochondria, SER, RER, a large nucleus, and numerous secretory vesicles. The latter, presumably containing pheromone, are discharged into the end-apparatus and up through the duct to the cuticular surface. Large amounts of secreted material can be found within the ducts and in the cuticular depressions (Liang and Schal, 1993c). The volatile pheromone of *B. germanica* is emitted while the female calls, a behavior during which the wings are elevated, abdomen lowered, and the genital vestibulum of the female occasionally exposed (Liang and Schal, 1993b). Calling females have been shown to release more pheromone and they are more likely to mate than non-calling females.

Similar pheromone glands are found in other cockroaches that produce volatile sex pheromones, but the glandular pores are usually more uniformly distributed on the cuticular surface and do not form the specialized regions evident in *B. germanica*. While calling, female *Supella longipalpa* (F.) (brownbanded cockroach, Blattellidae) release a volatile pheromone, 5-(2*R*′,4*R*′-dimethylheptyl)-3-methyl-2*H*-pyran-2-one (supellapyrone), that elicits sexual responses in males (Charlton *et al.*, 1993; Leal *et al.*, 1995). Although the genital vestibulum is exposed intermittently during calling, as in *Periplaneta* and *Blattella*, behavioral, electrophysiological, and morphological studies have localized the sex pheromone to the 4th and 5th tergites of virgin females (Schal *et al.*, 1992). The tergal glands of *S. longipalpa* females are composed of multiple class-3 secretory units, each leading through a long unbranched duct to a single cuticular pore, as in *Blattella*. Cuticular pores occur on all tergites, but their density is highest on the lateral margins of the 4th and 5th tergites.

Parcoblatta lata (Brunner) and *Parcoblatta caudelli* Hebard (Blattellidae) are forest-dwelling wood cockroaches endemic to North America. Sexually receptive females call, during which they release long-range sex pheromones to which males are attracted (Gemeno *et al.*, 2003). Based on a correlation between the density of cuticular pores that overlie class-3 glandular cells and behavioral and EAG analyses of tissue extracts, as in *S. longipalpa*, it was concluded that the sex pheromone is produced only in tergites 1–7 (Gemeno *et al.*, 2003). However, it remains to be demonstrated unequivocally that these cells produce pheromone, as in other species, because similar integumental glands are found

elsewhere on the body, and they have been shown to serve multiple functions, including pheromone production (Noirot and Quennedey, 1974).

Volatile sex pheromones are also released by male cockroaches, usually from tergal or sternal glands. Two types of behaviors are evident in males: (1) calling behavior, analogous to female calling, occurs in some species the absence of females; and (2) courtship behavior, during which tergal glands are exposed and release short-range attractants. The best studied example is *Nauphoeta cinerea* (Olivier) (lobster cockroach, Blaberidae). Glands on sternites 3–7 are exposed during calling, releasing a volatile male pheromone (Sreng, 1979a). The sternal glands are made up of four categories of cells including the class-3 glandular units seen in other species. Orifices of the class-3 cells are found only on the anterior zone of each sternite, but no specialized cuticular structures are evident (Sreng, 1979b, 1984, 1985). The sternal glands produce a mixture of the four components 3-hydroxy-2-butanone, 2-methylthiazolidine, 4-ethyl-2-methoxyphenol, and 2-methyl-2-thiazoline (Sreng, 1990; Sirugue *et al.*, 1992). Similar glands are also present in the closely related *Rhyparobia* (=*Leucophaea*) *maderae* (F.) (Madeira cockroach), but on sternites 2–7 (Sreng, 1984).

The tergal glands of *N. cinerea* also exhibit sexual dimorphism with more orifices on tergites 1 and 2 in males than in females (Brossut and Roth, 1977). These glands also lack cuticular modifications, other than individual cuticular pores, and are composed of five categories of cells, including class-3 glandular units, and three of the four compounds found in the sternal glands are also present in the tergal glands, but at much lower amounts. Presumably, these components of the pheromone are released during courtship, as the male raises his wings, and they stimulate the female to mount the male and feed from his tergal secretions. This wing-raising stance is common to male cockroaches of other species.

In some species the male tergal glands form specialized regions on the cuticular surface and produce a blend of close-range attractants, phagostimulants, and nutrients that the male deploys to place the female in a pre-copulatory position. For example, the tergal glands of *B. germanica* consist of traverse cuticular depressions on the 7th and 8th tergites, connected to numerous class-3 secretory cells (Sreng and Quennedey, 1976; Brossut and Roth, 1977), as in the female's pygidial gland (above). The tergal secretions include a number of volatile compounds, mainly carboxylic acids (>92 percent), but none have been tested for behavioral effects on females (Brossut *et al.*, 1975). Because males and nymphs also feed upon the exposed tergal secretions of courting males, these secretions appear to serve as non-specific food attractants.

The non-volatile fraction of the tergal secretion of male *B. germanica* contains lipids, proteins, and carbohydrates (Brossut *et al.*, 1975), but their identity and role(s) in courtship behavior have not been investigated. Recently, however, Nojima *et al.* (1999, 2002) identified several male compounds that serve as

phagostimulants, including a mixture of oligosaccharides (D-maltose, its analogs D-maltotriose and D-maltotetraose, four oligoglucosyl trehaloses with (1 → 4)-α-glycosidic linkages), and two trisaccharides. Activity of an artificial blend of these sugars is significantly enhanced by a polar lipid fraction that contains a mixture of phosphatidylethanolamines and phosphatidylcholines (lecithins) (Kugimiya *et al.*, 2002).

10.5.2 Contact pheromones

Cuticular contact pheromones mediate species and sex recognition and, in most cases, they function as courtship-inducing pheromones. Although they are probably present in most cockroach species, sex-specific contact pheromones have been identified in only two species, *B. germanica* females and *N. cinerea* males. They are thought to be distributed throughout the epicuticular surface and are perceived by means of antennal contact and with the mouth parts. Male-produced contact pheromones may function in male–male recognition and to inhibit courtship in other males (Fukui and Takahashi, 1983).

The best studied contact pheromone, indeed the only cockroach pheromone that has been investigated with isotope tracing, is the blend of hydrocarbon-derived ketones of *B. germanica* females that elicits courtship responses in males. The female German cockroach produces a pheromone blend that includes (3*S*,11*S*)-dimethylnonacosan-2-one as the major component, and three related compounds [29-hydroxy-(3*S*,11*S*)-dimethylnonacosan-2-one, 29-oxo-(3,11)-dimethylnonacosan-2-one, and 3,11-dimethylheptacosan-2-one] are also behaviorally active (Nishida *et al.*, 1974, 1976; Nishida and Fukami, 1983; Schal *et al.*, 1990b). Other putative pheromone components with a 3,11-dimethyl branching pattern are present on the female cuticular surface, including C_{31} and C_{33} methyl ketones (Schal, unpublished results), but their biological activity has not been confirmed. Critical to studies on the source of this pheromone was understanding its biosynthetic pathway (section 10.7.2.1, Figure 10.1) and evidence that a fatty acid synthetase and methyl-branched fatty acid precursors could be isolated from the abdominal epidermis but not from the fat body (Juarez *et al.*, 1992). Gu *et al.* (1995) incubated various tissues with [1–^{14}C]propionate, which labels methylmalonyl-CoA and methyl-branched hydrocarbons (Figure 10.1), and concluded that only the integument underlying abdominal sternites and tergites produced methyl branched hydrocarbons and the methyl ketone pheromone.

Oenocytes have been shown to biosynthesize hydrocarbons in several insect species in which the oenocytes are within the hemocoel and could be readily separated from other tissues (Diehl, 1973, 1975; Romer, 1980, 1991). These cells are characteristically very large, among the largest somatic cells in insects, and are rich in mitochondria and SER, as are steroidal cells in mammalian systems, suggesting participation in lipid synthesis (Rinterknecht and Matz, 1983). In the German and American cockroaches, however, the oenocytes are

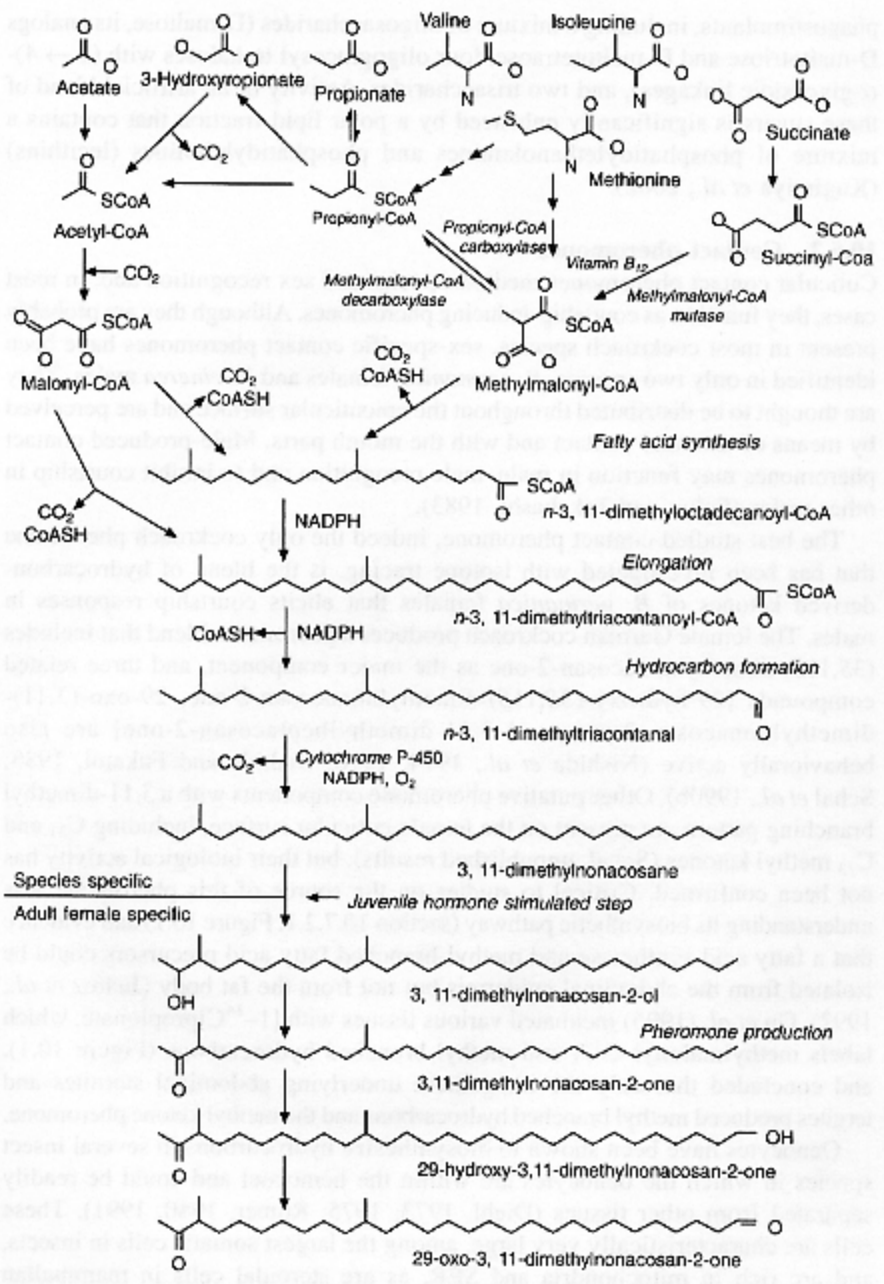

Figure 10.1 Metabolic pathways for the biosynthesis of 3,11-dimethylnonacosan-2-one and related sex pheromone components of the female contact sex pheromone of the German cockroach, *Blattella germanica*.

localized within the abdominal integument, separated from the hemocoel by a basal lamina (Kramer and Wigglesworth, 1950; Liang and Schal, 1993c). As a consequence, the definitive localization of hydrocarbon biosynthesis in these insects has been hampered by the inability to separate the integument into its component cell types. Recently, Fan *et al.* (2003) used enzymatic digestion of the basal lamina and the extracellular matrix to dissociate the integument underlying the sternites of female *B. germanica* into a cell suspension. Based upon ultrastructural details, two cell fractions were obtained: an "epidermal cell-enriched" sample that contained ~85 percent epidermal cells (<15 μm) and only 15 percent oenocytes (>30 μm), and an "oenocyte-enriched" sample that contained 60 percent oenocytes. Each cell suspension was then further separated into fractions in a Percoll gradient. In both gradients there was broad overlap between the two cell types, but nonetheless, epidermal cells predominated in more buoyant fractions, while most oenocytes were in fractions of higher density. Hydrocarbon synthesis was then measured in each fraction by incorporation of [1-^{14}C]propionate into methyl-branched hydrocarbons. Only very large cells produced hydrocarbons, whereas the much larger population of small cells did not (Fan *et al.*, 2003). These results demonstrate, for the first time using direct biochemical evidence, that hydrocarbons are produced by oenocytes and not by other cell types in an insect in which oenocytes are confined to the abdominal integument. They support the elegant genetic approach of Ferveur *et al.* (1997) showing that targeted expression of the *transformer* gene in oenocytes of male *Drosophila melanogaster* resulted in feminization of his hydrocarbon pheromone mixture.

10.6 Pheromone production regulated by gland development and cellular plasticity

Developmental regulation of pheromone biosynthesis must be universal among insects because organogenesis of a sex-specific gland occurs only in the pre-imaginal stage. However, this mechanism has received meager attention from researchers. In short-lived insects that emerge as reproductively competent adults it is expected that functional competence of the gland for pheromone production might be regulated by the same factors that control pheromone gland development (e.g. ecdysteroids – see Tang *et al.*, 1991, for example). In insects whose pheromone gland acquires functional competence during an imaginal maturation period, developmental regulation might involve factors that also control adult reproductive readiness. Examples include many insects that mature sexually for several days after eclosion, and adult insects that enter diapause or migrate before the onset of reproduction. Competence for the production of pheromone, once attained, may be maintained for the life of the insect. Little is known of the cellular processes that occur during the stimulation of pheromone production and its

suppression after mating, even in well-studied moths. Recent studies of the silkmoth *Bombyx mori* suggest that lipid droplets within pheromone secreting cells increase in size in the pre-adult stage (Fónagy *et al.*, 2000). In the young adult female, the lipid droplets declined in size but increased in number, and these changes could be prevented by decapitation and stimulated by PBAN. Fónagy *et al.* (2001) also showed that these changes followed a diel pattern in relation to pheromone production. In mid-photophase, when females produced pheromone and called, small lipid droplets predominated, whereas large lipid droplets accumulated at other times. Mated females, which ceased pheromone production, also accumulated large droplets. Together, these results suggest that the lipid droplets contain pheromone precursors that undergo daily and physiological cycles of accumulation and depletion (Fónagy *et al.*, 2001). Indeed, Matsumoto *et al.* (2002) confirmed that triacylglycerols were a major component of the lipid spheres, and the pheromone precursor Δ10,12-hexadecadienoate predominated. It is likely that other ultrastructural changes in pheromone gland cells follow these diel and physiological cycles. Likewise, it is expected that such changes will be discovered in insects that exhibit cycles of sexual receptivity, including some short-lived moths.

In long-lived insects, in which reproduction is interrupted by long periods of sexual inactivity, developmental regulation of the sex pheromone gland can result in alternating cycles of acquisition and subsequent loss of competence through maturation and retrogression of the glandular machinery, respectively. In cockroaches, pheromone production is controlled by both developmental differentiation of gland cells before the imaginal molt and by cyclic maturational changes in the gland that coincide with the ovarian cycle.

The best studied examples of differentiation and maturation of sex-specific pheromone glands are the tergal and sternal glands of *N. cinerea* and *B. germanica* males. Both possess class-3 glandular units composed of two cells – a secretory cell and a duct cell (section 10.5.1) (Noirot and Quennedey, 1974). But after apolysis and before the imaginal molt the immature gland contains four concentric cells, including also an enveloping cell and a ciliary cell in addition to the adult cells (Sreng, 1985, 1998; Sreng and Quennedey, 1976). During several days after the adult molt the gland matures by undergoing apoptosis (programmed cell death): the ciliary cell gives rise to a part of the microvillar end-apparatus, then dies, whereas the enveloping cell forms an upper portion of the duct, then it too dies (Sreng, 1998). Although the factors regulating sex pheromone gland differentiation in the pre-adult stage are not known, it is thought that differentiation proceeds in response to a low JH III titer throughout the last stadium and a rise in ecdysteroids late in the intermolt period.

Regulation of the maturational stage of pheromone gland differentiation in the adult is better understood. Before day-5 the immature sternal glands of *N. cinerea* males contain numerous pycnotic nuclei (from the dying enveloping and

ciliary cells) and produce little pheromone (Sreng, 1998; Sreng *et al.*, 1999). By day-5, however, each glandular unit consists of only a glandular cell and a duct cell, and its pheromone content increases significantly. Sreng (1998) proposed that brain factors mediate these maturational changes. He showed that decapitation or allatectomy (removal of the CA) of *N. cinerea* males before a critical point during maturation completely blocked the apoptotic process. Nevertheless, pheromone accumulated in the sternal gland of decapitated or allatectomized males, albeit at a slower rate, suggesting that pheromone production is less dependent on brain factors than is cellular differentiation. Moreover, because pheromone release was probably hindered in the undifferentiated glands, further accumulation of pheromone would result. The brain factor was shown not to be PBAN-like neuropeptides because injection of brain extracts or synthetic PBAN did not restore gland differentiation or stimulate greater pheromone production (Sreng *et al.*, 1999). JH III administered to allatectomized males, on the other hand, restored both cellular differentiation of the gland and pheromone production.

Female *B. germanica* employ similar class-3 glands to produce a volatile sex pheromone that is yet to be identified. Ultrastructural studies and behavioral and electrophysiological assays have shown that, as in males, the pheromone gland undergoes cellular maturation during the period of sexual maturation and attainment of sexual receptivity (Abed *et al.*, 1993c; Liang and Schal, 1993c; Torko *et al.*, 1993; Schal *et al.*, 1996). The secretory cells of newly formed glands in the imaginal female are small (~5–10 μm in diameter) and they contain few, primarily electron-lucid secretory vesicles, few short microvilli around the end-apparatus, and little material within the end-apparatus. The amount of behaviorally and EAG-active material in the gland is low after the imaginal molt (day-0) but it increases with age and peaks on day-6, corresponding to the physiological stage when females become sexually receptive and begin to emit the pheromone (Liang and Schal, 1993c; Schal and Chiang, 1995). In tandem, the glandular epithelium thickens and the size of pheromone-secreting cells increases; mature day-6 glands are characterized by large secretory cells (about three-fold larger than on day-0) containing a large number of spherical or oval electron-lucid and electron-dense secretory vesicles, an end-apparatus lined with numerous long microvilli, and a large spherical nucleus (Abed *et al.*, 1993c; Liang and Schal, 1993c). Often, large amounts of secreted material can be found inside the cuticular depressions into which the ducts empty.

The mature pheromone gland of *B. germanica* undergoes a cyclical pattern of cellular hypertrophy and retrogression in relation to successive reproductive cycles. Both the thickness of the glandular tissue and the size of each secretory cell decline markedly after mating, when pheromone production is no longer needed, secretory vesicles become small and sparsely distributed, and the gland remains atrophied during gestation (Abed *et al.*, 1993; Liang and Schal, 1993c; Torko *et al.*, 1993; Schal *et al.*, 1996). EAG and behavioral assays confirmed

that the pheromone content of the gland declines after mating and remains low throughout gestation (Abed *et al.*, 1993; Liang and Schal, 1993c; Schal *et al.*, 1996). But as a new vitellogenic cycle begins after the infertile egg case of virgins or the viable egg case of mated females is deposited, the pheromone gland undergoes rapid regrowth and proliferation of cellular machinery, the epithelium thickens again, and extracts of active glands from both virgin and mated females in the second reproductive cycle are EAG and behaviorally active. This pattern has been related to the pattern of JH III production (and the titer of JH III in the hemolymph) (Liang and Schal, 1993c; Schal *et al.*, 1996), but no experimental manipulations of hormone titers have been conducted to verify the hypothesis that JH III controls the cellular plasticity of the pheromone gland. Interestingly, this appears to be the only known case in insects where pheromone production is modulated in relation to the reproductive cycle (and JH), independently of the mating status of the female. The pattern of chemical signaling in *B. germanica* females is thus controlled primarily through regulated pheromone emission during calling, not through regulation of pheromone production. Calling behavior, too, is modulated by the titer of JH III (section 10.8.1).

To our knowledge, this pattern of cellular plasticity has not been described in any other insect species. It is tempting to speculate that this mechanism of regulating pheromone production is unusual and has evolved to conserve cell energy in insects that require long-term arrestment of pheromone synthesis during periods of sexual inactivity (e.g. during a long gestation period, diapause, migration). However, the CA – the endocrine glands that produce JH – also exhibit cycles of growth and retrogression of CA cells and these changes play a major role in the regulation of JH III biosynthesis during reproductive cycles in both *Blattella* and *Supella* females (Chiang *et al.*, 1991; Chiang and Schal, 1994). Female *Supella* retain the egg case for less than one day, and thus exhibit rapid reproductive cycles. It is therefore plausible that developmental regulation of pheromone biosynthesis is not restricted to species with long periods of gestation. In *Supella*, a major function of developmental changes in the pheromone gland might be in the long-term suppression of pheromone production after mating, rather than in tracking changes in JH III titers at each ovarian cycle as in *Blattella* (Smith and Schal, 1990a). Comparative studies of oviparous cockroaches – with rapid, short reproductive cycles – and ovoviviparous cockroaches – with slow, protracted reproductive cycles – would be enlightening in this regard.

10.7 Pheromone production regulated by juvenile hormone

The previous section highlighted the pivotal role that JH plays not only in pheromone gland differentiation, but also in its cyclic maturation in relation to the reproductive cycle. Most aspects of the female reproductive cycle in cockroaches

are coordinately controlled by JH. Removal of the CA abolishes vitellogenin synthesis, its uptake by oocytes, and in some species, both pheromone production and sexual receptivity are suppressed by this operation. Barth and Lester (1973) and Schal and Smith (1990) reviewed the early literature on endocrine regulation of pheromone production in cockroaches.

10.7.1 Volatile pheromones

Unfortunately, no studies are available on the regulation of volatile pheromone production using analytical or radiochemical approaches. The most detailed studies have used behavioral and EAG responses of males as estimates of the relative amount of pheromone in females or their pheromone glands. The best studied species are *B. germanica* and *S. longipalpa*. Virgin *Supella* females initiate pheromone production 4 days after the imaginal molt (Smith and Schal, 1990a), in relation to increasing JH III biosynthesis by the CA (Smith *et al.*, 1989). Likewise, *Blattella* females begin to accumulate pheromone in the pygidial glands in relation to increasing rates of JH III synthesis (Liang and Schal, 1993c). Ablation of the CA of newly emerged adult females prevented pheromone production in both species, and pheromone production could be restored by reimplantation of active CA or by treatment with JH III or JH analogues. Interestingly, although growth of the vitellogenic oocytes is controlled by and highly correlated with JH III biosynthetic rates, direct or even intermediary involvement of the ovaries in regulating calling and pheromone production in both species was excluded by ovariectomies (Smith and Schal, 1990a).

It is not known whether JH exerts its pheromonotropic effects directly on secretory cells of the pheromone gland, or whether it acts indirectly by stimulating the synthesis and/or release of pheromonotropic neuropeptides. Notably, although cockroach brain extracts could induce PBAN-like pheromonotropic activity in moth pheromone glands (Raina *et al.*, 1989), injections of brain extracts of cockroaches or synthetic *Hez*-PBAN failed to induce pheromone production in allatectomized *Nauphoeta* males (Sreng *et al.*, 1999) or in *Supella* females (Schal, unpublished results). Moreover, lack of pheromone production in mated females that periodically produce large amounts of JH III suggests that JH plays a "permissive" role (Schal *et al.*, 1997). That is, its presence is *required* for pheromone to be produced, but even when the JH III titer is high pheromone production can be suppressed by neural or humoral pheromonostatic factors.

10.7.2 Contact pheromones

B. germanica has served as an important model for delineating the regulation of non-volatile cuticular pheromones. In this section we review findings on the biochemical pathways and the role of JH in facilitating the production of (3*S*,11*S*)-dimethylnonacosan-2-one, the major component of the courtship-inducing female pheromone (see reviews in Schal *et al.*, 1991, 1996; Blomquist *et al.*, 1993; Tillman *et al.*, 1999).

10.7.2.1 Biosynthetic pathway

Central to investigations of the biosynthetic pathway and regulation of the contact pheromone of *B. germanica* was the observation that the major cuticular hydrocarbon in all life stages of this species is an isomeric mixture of 3,7-, 3,9- and 3,11-dimethylnonacosane (Jurenka *et al.*, 1989). The presence of only the 3,11-isomer in the cuticular dimethyl ketone fraction and only in adult females prompted Jurenka *et al.* (1989) to suggest that production of the pheromone might result from the female-specific oxidation of its hydrocarbon analog. This scheme follows the well-established conversion of hydrocarbons to methyl ketone and epoxide pheromones in the housefly (Blomquist *et al.*, 1984; Ahmad *et al.*, 1987).

This model has since been validated with several independent approaches. Biochemical studies on the biosynthesis of methyl-branched alkanes showed that the methyl branches are added during the early stages of chain elongation (Chase *et al.*, 1990). Using carbon-13 labeling and NMR analyses Chase *et al.* (1990) showed that carbons 1 and 2 of acetate are incorporated as the chain initiator, and that the carbon skeleton of propionate serves as the methyl branch donor (Figure 10.1). Further, propionate and succinate labeled the hydrocarbons and the methyl ketone pheromone, as did the amino acids valine, isoleucine and methionine, all of which can be metabolized to propionate. NMR studies confirmed that these substrates were metabolized to methylmalonyl-CoA for incorporation into the methyl branch unit of hydrocarbons (Chase *et al.*, 1990), as in the housefly (Dillwith *et al.*, 1982; Halarnkar *et al.*, 1987), the American cockroach (Halarnkar *et al.*, 1985), the cabbage looper moth (de Renobales and Blomquist, 1983), and the termite *Zootermopsis* (Chu and Blomquist, 1980).

Methyl-branched fatty acids are intermediates in branched alkane biosynthesis (Juarez *et al.*, 1992). Thus, [1-^{14}C]propionate labeled methyl-branched fatty acids of 16–20 carbons, but did not label straight chain-saturated and monounsaturated fatty acids (Chase *et al.*, 1990).

Chase *et al.* (1992) examined the hypothesis that the methyl ketone sex pheromone arises from the insertion of an oxygen into the preformed 3,11-dimethyl alkane. When high-specific activity, tritiated,3,11-dimethyl nonacosane was topically applied on the cuticle of *B. germanica* females, it readily penetrated the cockroach and radioactivity from the alkane was detected in both 3,11-dimethylnonacosan-2-ol and 3,11-dimethylnonacosan-2-one. When tritiated 3,11-dimethylnonacosan-2-ol was applied to the cuticle it was readily and highly efficiently converted to the corresponding methyl ketone pheromone. But, surprisingly, the dimethyl ketone pheromone was derived from the corresponding alcohol not only in females, as expected, but also in males. These results suggest that the sex pheromone of *B. germanica* arises via a female-specific hydroxylation of 3,11-dimethylnonacosane and a subsequent non-sex-specific oxidation, probably involving a polysubstrate monooxygenase system, to the (3*S*,11*S*)-dimethyl-

nonacosan-2-one pheromone (Figure 10.1). Chase *et al.* (1992) also suggested that a similar hydroxylation and subsequent oxidation at the 29-position of 3,11-dimethylnonacosan-2-one might give rise to 29-hydroxy- and 29-oxo-(3,11)-dimethylnonacosan-2-one, the other components of the contact pheromone blend, but this hypothesis has yet to be tested. It is quite likely, as well, that the same mechanism converts the C_{27} dimethyl alkane to the corresponding methyl ketone pheromone, and perhaps its 27-hydroxy- and 27-oxo- analogues.

10.7.2.2 Juvenile hormone-mediated contact pheromone production

JH plays an essential regulatory role in the metabolism of (3*S*,11*S*)-dimethylnonacosane to (3*S*,11*S*)-dimethylnonacosan-2-one, probably by increasing the activity of a female-specific polysubstrate monooxygenase. This subject has been reviewed in relation to pheromone biosynthesis in other insects (Schal *et al.*, 1996; Blomquist *et al.*, 1993; Tillman *et al.*, 1999). The pattern of synthesis and accumulation of pheromone on the female's cuticle suggested the involvement of JH. Incorporation of radiolabel from injected [1-^{14}C]propionate into the methyl-branched sex pheromone is low in previtellogenic and in gravid females, when the JH III titer is also low, and high in vitellogenic females, when the JH III titer is greatly elevated (Schal *et al.*, 1994; Sevala *et al.*, 1999). Likewise, the amount of pheromone on the cuticle increases in relation to the vitellogenic cycle and the titer of JH III (Schal *et al.*, 1990a). But most convincing is the experimental evidence from allatectomized females, females treated with JH analogs, and from dietary manipulations that influence both hormone titer and pheromone production (Schal *et al.*, 1990a, 1991, 1994; Chase *et al.*, 1992). Females treated so as to reduce their JH III titer (allatectomy, anti-allatal drugs such as precocene, starvation, or implantation of an artificial egg case into the genital vestibulum, which inhibits JH biosynthesis) produced less pheromone (Schal *et al.*, 1990a, 1991, 1994). But pheromone production was greatly stimulated by treatments with JH III or with JH analogs. Because only the hydroxylation of 3,11-dimethylnonacosane to 3,11-dimethylnonacosan-2-ol is regulated in a sex-specific manner, it appears that this step is under JH III control (Figure 10.1) (Chase *et al.*, 1992).

All life stages of the German cockroach produce 3,11-dimethylnonacosane, suggesting that production of a sex-specific contact pheromone may be less dependent on differentiation of sexually dimorphic pheromone glands, as is the case for volatile pheromones, and more so on the endocrine milieu of the adult female. Normally, adult male cockroaches produce much less JH III than do females, and males have a much lower titer of JH III in the hemolymph (Piulachs *et al.*, 1992; reviewed in: Tobe and Stay, 1985; Wyatt and Davey, 1996). However, when newly emerged males were exposed to filter papers treated with the JH mimic hydroprene they exhibited a six-fold elevation in female pheromone on their cuticle (Schal, 1988). Although substantial, this limited stimulation indicates

either a limited capacity to express the putative female-specific polysubstrate monooxigenase or a loss of JH receptor sites in the adult male oenocytes. Nevertheless, the parallels with estrogen induction of vitellogenin synthesis in the male liver of oviparous vertebrates, and JH induction of vitellogenin synthesis in male cockroaches (e.g. Mundall *et al.*, 1983) are striking. The emerging model suggests, at a minimum, that the endocrine milieu of the vitellogenic female contributes significantly to the production of female-specific contact pheromones in cockroaches.

Yet another feature distinguishes JH control of contact pheromone production from control of volatile pheromone production in cockroaches. Although based solely on behavioral data, lack of JH III in females completely suppresses production of attractant pheromones, as for instance in *S. longipalpa* (section 10.7.1). The contact pheromone of *B. germanica*, on the other hand, is still biosynthesized in allatectomized females, albeit at a lower rate, probably because regulation of contact sex pheromone production operates at several levels, including the regulated production of its 3,11-dimethylnonacosane precursor (section 10.7.2.3).

10.7.2.3 Pheromone production regulated by food availability

There are numerous examples in insects of sequestration or metabolism of plant compounds for their own chemical defenses (Eisner and Meinwald, Chapter 12, in this volume). For sex pheromones, on the other hand, *de novo* biosynthesis is much more common than the sequestration of pheromones or their precursors from plant compounds. Nevertheless, some pheromones, especially those which are produced in large quantities (micrograms), require large amounts of food-derived biosynthetic substrate, and a deficiency of building blocks may significantly interfere with pheromone production. Production of the methyl ketone contact pheromone in the female German cockroach depends on the biosynthesis of considerable amounts of the parent methyl-branched hydrocarbon, production of which is regulated to a large extent by availability of food, not JH (Schal *et al.*, 1994). All life stages of *B. germanica* produce and secrete onto the cuticle large amounts of 3,11-dimethylnonacosane. In both nymphs and adults, surges in hydrocarbon synthesis occur during feeding stages that follow each molt, but hydrocarbon synthesis declines dramatically when nymphs cease to feed in preparation for the next molt or when adult females reduce their food intake at the end of the vitellogenic phase (Schal *et al.*, 1994, 1996; Young *et al.*, 1997, 1999a). Therefore, hydrocarbon production is high in the adult female when the ecdysteroid titer is low and it wanes well before any appreciable decline in the JH III titer, suggesting that neither of these two hormones directly regulates hydrocarbon biosynthesis. Rather, hydrocarbon production in both nymphs and adults appears to change only in relation to food intake. Young *et al.* (1999a) showed a clear causal relationship between these two processes in last instar *B. germanica*: starved nymphs ceased to produce hydrocarbons, whereas refed nymphs

resumed hydrocarbon biosynthesis. Interestingly, this pattern was reflected in short-term *in vitro* culture of tergites in a nutrient-rich medium, suggesting that starvation reduced not only the availability of substrates for hydrocarbon biosynthesis but also the *capacity* of oenocytes to produce hydrocarbons.

A complex interaction between food intake and JH III titer controls contact pheromone production in adult female *B. germanica*. In normal females, food consumption facilitates both the production of hydrocarbons by the oenocytes and JH III biosynthesis by the CA (Schal *et al.*, 1993; Osorio *et al.*, 1998; Holbrook *et al.*, 2000). A high JH III titer, in turn, stimulates the conversion of the hydrocarbon to contact pheromone (Figure 10.1, section 10.7.2.2). At ovulation and throughout the ensuing 3-week gestation period, food intake is minimal and the JH III titer generally remains at or near undetectable levels; much less hydrocarbon and pheromone are produced. Production of all three lipids resumes after parturition when a new vitellogenic and feeding cycle commences.

Considerable insight into this system has been gained from studies of allatectomized females. Young allatectomized females ate less and therefore synthesized less hydrocarbons during the first few days of adult life (Schal *et al.*, 1994). However, for several interrelated reasons, large amounts of hydrocarbons accumulated over time in the hemolymph of allatectomized females. First, in the absence of JH III females did not provision their eggs, and therefore vast amounts of nutrients accumulated in the hemolymph. Second, food intake was not suppressed in older allatectomized females because this normally requires the presence of an egg case, resulting in a further build-up of nutrients in the hemolymph. Third, the relatively low rate of hydrocarbon biosynthesis in older allatectomized females far exceeded that of gravid females of the same age. And finally, large amounts of hydrocarbons are normally deposited into the vitellogenic oocytes (Fan *et al.*, 2002), but because ovarian uptake of hydrocarbons was impeded in allatectomized females, the hemolymph titer of hydrocarbons increased well beyond normal levels (Schal *et al.*, 1994). Fan *et al.* (2002) demonstrated that in normal females, as the oocytes matured and hydrocarbon uptake was reduced, a higher fraction of hemolymph hydrocarbons (section 10.8.2.3) was directed to the epicuticular surface. Thus, as allatectomized females accumulated more hydrocarbons in their hemolymph, the amount of cuticular pheromones also increased. In fact, the amount of 3,11-dimethylnonacosan-2-one increased beyond normal levels in both the hemolymph and the cuticle, suggesting that excess 3,11-dimethylnonacosane was metabolized to pheromone.

These patterns suggest that under normal conditions, feeding in adult females is modulated in a stage-specific manner, regulating the amount of 3,11-dimethylnonacosane that is available for pheromone production. Early in the vitellogenic cycle JH mediates the metabolism of 3,11-dimethylnonacosane to 3,11-dimethylnonacosan-2-one and other pheromone components. Later in the reproductive cycle, however, the oocytes sequester large amounts of hydrocarbons

and much less pheromone is produced. This system illustrates the complex interactions among multiple mechanisms that regulate the biosynthesis of lipids that serve multiple functions. Hydrocarbons serve not only as pheromone precursors, but also as water repellents on the cuticular surface and as significant maternal provisions to progeny.

10.8 Transport and emission of pheromones

10.8.1 Volatile pheromones

Little is known of the cellular processes that deliver volatile pheromones from secretory cells to the cuticular surface, even in the intensively researched Lepidoptera. Based on results from ultrastructural studies of the moth *Heliothis virescens*, Raina *et al.* (2000) recently speculated that secretory cells of the pheromone gland somehow deliver pheromone or its precursors to hollow cuticular hairs. During calling behavior the female exposes the gland and also the cuticular hairs that exude pheromone droplets. Raina *et al.* (2000) further posit that as the female retracts the ovipositor more pheromone is "squeezed" onto the exposed surface, thus recharging the cuticular hairs.

In cockroaches, electron microscopy studies often show accumulation of secretion in the end-apparatus, ducts, and around the cuticular pores of class-3 exocrine glands in both males and females. Whether these are carrier proteins that deliver hydrophobic pheromones to the surface, or other excretions, is not known. Recent studies in *Leucophaea maderae* have identified and sequenced an epicuticular protein, Lma-p54, that is expressed specifically in the tergites and sternites of adult males and females, but not in nymphs (Cornette *et al.* in 2002). The sequence of this protein is closely related to aspartic proteases, but because it appears to be enzymatically inactive the authors speculate that it serves as a ligand-binding protein. Cornette *et al.* (2002) further hypothesize that Lma-p54, alone or together with a ligand, serves in sexual recognition. Other ligand-binding proteins, namely the lipocalins Lma-p22 and Lma-p18, have been isolated only from male tergal secretions of *L. maderae* (Korchi *et al.*, 1999; Cornette *et al.*, 2001). These exciting findings suggest that carrier proteins might be involved in transport of volatile pheromones to the cuticle but functional studies will be needed to verify this hypothesis. It is worth noting that Bla g4, an allergen that was suggested to serve a similar role in *B. germanica*, is expressed in male reproductive tissues and appears to play no role in pheromone transport (Schal, unpublished results).

Attractant sex pheromones are usually emitted while the female or male cockroach performs a species-specific calling behavior, as in most lepidopterans. In females, calling is generally characterized by elevated tegmina and wings, a recurved abdomen, and occasional exposure of the genital vestibulum (see Gemeno

and Schal, 2003; Gemeno *et al.*, 2003). Although it has been shown for several species that pheromones are released during this behavior, the precise connection between calling and pheromone emission remains unknown. In some species, calling exposes pheromone gland openings that are normally obscured by the wings or by more anterior tergites or sternites (in most males, e.g. *B. germanica*, *S. longipalpa*, *N. cinerea*). In other species, however, including females of *B. germanica* and *S. longipalpa*, and especially wingless females of *Parcoblatta*, the tergal openings of the pheromone glands do not seem to become appreciatively more exposed during calling. Moreover, in *Supella* and *Parcoblatta* there is no reservoir or storage space for pheromone on the cuticular surface, suggesting that pheromone is emitted "on demand" only when needed. In these species, calling behavior is expressed exclusively by sexually receptive females and volatiles collected from calling females are much more attractive to males than volatiles collected from non-calling females of the same age (Smith and Schal, 1990a; Liang and Schal, 1993b; Gemeno *et al.*, 2003). This suggests that the postures employed by calling females must have a direct effect on the release of the pheromone by the individual glands. Both pheromone emission and calling behavior are physiologically regulated, but the exact mechanisms by which calling behavior effects the transfer of pheromone from secretory cells to the exterior of the body is unclear. It is worth noting, in this context, that the pheromone glands are extensively innervated (Liang and Schal, 1993c) and it is possible that the same neuronal signals that control calling also control pheromone release.

JH III regulates calling behavior in both *B. germanica* and *S. longipalpa*. In both species, transection of the nerves connecting the CA to the brain, an operation that significantly accelerates the rate of JH III biosynthesis by the CA (Gadot *et al.*, 1989; Schal *et al.*, 1993), also hastens the age when calling first occurs (Smith and Schal, 1990a; Liang and Schal, 1994). In *B. germanica*, the central role of JH has been confirmed by ablation of the CA and with rescue experiments with a JH analogue (Liang and Schal, 1994). In this species, JH also is required for females to become sexually receptive and accept courting males (Schal and Chiang, 1995).

10.8.2 Contact pheromones

The view that newly synthesized integumental lipids, including contact pheromones, pass directly from oenocytes to the epicuticle has recently been critically re-examined. An alternative model has gained support (Figure 10.2): a lipoprotein-mediated indirect transport pathway plays a major, if not exclusive, role in delivering lipids to the cuticular surface. This hypothesis is supported by the following observations (reviewed in Schal *et al.*, 1998b). First, the hydrocarbons in the hemolymph of several insect species are similar to the hydrocarbon profile of the respective cuticle (e.g. Chino and Downer, 1982; Katase and Chino, 1984; reviewed in Schal *et al.*, 1998b). Second, in some insects the male and female

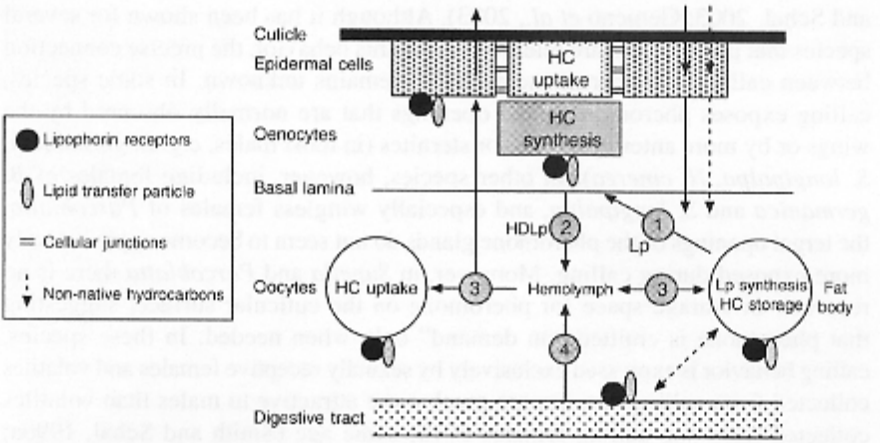

Figure 10.2 A conceptual model depicting transport of hydrocarbons (HC) and hydrocarbon metabolites from the oenocytes that produce them to the cuticular surface, fat body, oocytes, and digestive tract. Arrows represent directions of movement of lipophorin (Lp) and hydrocarbons. Lipophorin is biosynthesized in the fat body, but it is not known whether nascent Lp is loaded with hydrocarbons before it is released into the hemolymph (1) Epidermal cells and oenocytes are enclosed within a basal lamina. Oenocytes biosynthesize hydrocarbons, which are then loaded into HDLp (2) HDLp delivers hydrocarbons to the cuticular surface, oocytes, and fat body (3) After a molt, some exuvium hydrocarbons can also be reclaimed through the digestive tract (4) Non-native hydrocarbons can enter through the cuticle or the digestive tract, and removed through fat body metabolism, as well as through the digestive system. The involvement of lipid transfer particle (LTP) in uptake or unloading of hydrocarbons in any of these tissues remains unknown. It is also not known if HDLp enters target tissues (epidermis, fat body) and how hydrocarbons traverse the cuticle. The recognition of non-native hydrocarbons by the transport system and target tissues has not been studied. Based in part on a similar scheme for diacylglycerol transport (Arrese *et al.*, 2000; Canavoso *et al.*, 2001).

hemolymph carries different hydrocarbons, some of which serve as sex pheromones, and the composition of hemolymph is determined by the types of sex-specific hydrocarbons biosynthesized by the insect (e.g. cockroach – Gu *et al.*, 1995; *Drosophila* – Pho *et al.*, 1996; houseflies – Mpuru *et al.*, 2001; moths – Schal *et al.*, 1998a; Jurenka and Subchev, 2000). Third, in insects with oenocytes localized within the hemocoel (see section 10.5.2) a hemolymph transport pathway is clearly required to deliver lipids to the cuticular surface. However, in all species, hydrocarbons also need to be delivered to integument that does not synthesize hydrocarbons as well as to internal tissues, such as the ovaries (Fan *et al.*, 2002), a process that requires hemolymph transport regardless of where the oenocytes are located.

10.8.2.1 Hydrocarbon transport in hemolymph: role of lipophorin

Hydrocarbons and contact pheromones can be transported from biosynthetic tissues (e.g. abdominal integument) to tissues that do not synthesize them (e.g. head, wings, legs) either by physical translocation on the cuticular surface or by transport through hemolymph. To test the hypothesis that hemolymph is required for transport of lipids to tissues that do not synthesize them Gu *et al.* (1995) severed the veins that enter the fore-wings. The amount of hydrocarbons that appeared on the wings was significantly lower than on intact fore-wings of the same insects. Because the wings do not biosynthesize hydrocarbons, these results show that hydrocarbon transport from the abdominal oenocytes to various other body parts is hemolymph mediated.

It turns out that transport of hydrophobic ligands, including hydrocarbons and their semiochemical metabolites, requires plasma lipoproteins. The seminal work of Chino and co-workers showed that hydrocarbons associate with an M_r ~600 kDa high-density lipophorin (HDLp). In most insects HDLp is characterized by two constituent apoproteins, apoLp-I and apoLp-II, ~240 kDa and ~70 kDa, respectively (Chino *et al.*, 1981; Chino, 1985; Kanost *et al.*, 1990; Law *et al.*, 1992; Van der Horst *et al.*, 1993; Blacklock and Ryan, 1994; Soulages and Wells, 1994; Arrese *et al.*, 2000; Ryan and Van der Horst, 2000; Canavoso *et al.*, 2001). HDLp serves multiple transport functions. Its shell consists of the two apoproteins, phospholipids, and cholesterol, while the core contains non-polar lipids. Flying insects generally have large amounts of diacylglycerol which Lp delivers from the midgut to the fat body for storage, and to flight muscles for utilization as metabolic fuel. An important feature of Lp is its dynamic nature; it can range from a low-density particle (LDLp), resulting from an exchangeable association of apoLp-III (~17–20 kDa) and diacylglycerol with HDLp, to a very high-density complex (VHDLp) that is depleted of lipids. Neither of the latter forms has been isolated from cockroaches.

HDLp also carries cholesterol and carotenoids, and it has specific, high-affinity binding sites for JH III in Coleoptera, Isoptera, Diptera, Hymenoptera, and Dictyoptera (Trowell, 1992; Sevala *et al.*, 1997). In addition to its recognized central roles in transporting hydrophobic ligands, Lp is also a plasma coagulogen, involved in clotting of insect hemolymph (Ferstl *et al.*, 1988) and it appears to mediate immune responses, presumably by modulating cell adhesion responses of hemocytes (Coodin and Caveney, 1992; Dettloff *et al.*, 2001) and formation of lipopolysaccharide–Lp complexes which constitute an anti-bacterial defense system (Kato *et al.*, 1994). The multiplicity of its functions and its use by different developmental stages suggest that both the concentration of HDLp in hemolymph and its lipid composition are probably related to the stage-specific function that it serves. For example, the hemolymph concentration of HDLp, which serves as a JH-binding protein, is unrelated to the JH III titer, but more correlated with the titer of the much more abundant hemolymph hydrocarbons (Sevala *et al.*, 1999).

In the cockroaches *P. americana* and *B. germanica* hydrocarbons are synthesized by the abdominal epidermis, and virtually all newly synthesized hydrocarbons that enter the hemolymph are bound to HDLp (Figure 10.2) (Chino *et al.*, 1981; Gu *et al.*, 1995). In both species, hydrocarbons constitute up to 50 percent of the total lipids in HDLp and they are qualitatively the same as the cuticular hydrocarbons. Moreover, in both species, newly synthesized hydrocarbons can only be transferred from the integument to an incubation medium if HDLp is present, and other hemolymph lipoproteins, such as vitellogenin, cannot mediate this transfer (Katase and Chino, 1982, 1984; Chino and Downer, 1982; Fan *et al.*, 2002a). Abundance of epicuticular hydrocarbons on body parts that do not synthesize hydrocarbons (e.g. wings, legs) lends further support to the idea that HDLp serves as the sole vehicle for transport of species-specific hydrocarbons from the oenocytes to the cuticle. However, while Lp must deliver hydrocarbons to integument that does not synthesize hydrocarbons, its role in delivering hydrocarbons and pheromones to integumental tissue that synthesizes hydrocarbons (i.e. sternites and tergites) is not clear.

10.8.2.2 Loading of hydrocarbons into lipophorin

Newly synthesized hydrocarbons are loaded from oenocytes into HDLp and delivered to the cuticle, oocytes, and to the fat body for storage (Figure 10.2). In non-feeding stages that do not synthesize hydrocarbons, as, for example, late in the last stadium, the fat body appears to load hydrocarbons into HDLp for delivery to the cuticle (Young and Schal, 1997; Young *et al.*, 1999b). In newly ecdysed insects, hydrocarbons reclaimed from the ingested exuvium are loaded into HDLp from the midgut (Young and Schal, unpublished results). Thus, at least three tissues take part in loading HDLp with hydrocarbons – the oenocytes, midgut, and fat body.

The mechanisms by which hydrocarbons are taken up by HDLp are poorly understood. In some insects a very high-density lipid transfer particle (LTP) plays a crucial role in mediating diacylglycerol transfer from the midgut to HDLp (Canavoso and Wells, 2001). LTP also carries hydrocarbons (Blacklock and Ryan, 1994), and Takeuchi and Chino (1993) suggested that it may catalyze hydrocarbon transport in the American cockroach. Hydrocarbons comprise 40 percent of the lipids in LTP of *P. americana*, while only 28 percent of the lipids in HDLp are hydrocarbons; nevertheless, LTP carries much less total hydrocarbons than does HDLp (Takeuchi and Chino, 1993). Although the capacity of LTP to transfer hydrocarbons between Lp particles was clearly demonstrated in the American cockroach (Takeuchi and Chino, 1993), results from *in vitro* experiments with purified HDLp of *P. americana* and *B. germanica* have shown that HDLp accepts hydrocarbons from oenocytes without involvement of LTP (Katase and Chino, 1982, 1984; Fan *et al.*, 2002). In fact, purified HDLp (without LTP) is no less efficient in hydrocarbon transport than hemolymph, which also contains

LTP. Although these results suggest that LTP might not be required for the exchange of hydrocarbons, two confounding features of this system have yet to be investigated. First, it is possible that despite extensive washing of tissues and cells, LTP could remain bound to tissues. Second, LTP might be produced by either oenocytes or epidermal cells. Van Heusden and Law (1989) showed that although diacylglycerol could be transferred *in vitro* from fat body to purified *M. sexta* HDLp, pre-incubation of fat body with LTP antibody reduced the amount of diacylglycerol loaded onto HDLp. Canavoso and Wells (2001) also significantly reduced diacylglycerol transfer from labeled midgut sacs to Lp by pretreatment with LTP antibody; when LTP was added, diacylglycerol exchange was restored. Of course, it is also possible that hydrocarbon uptake by HDLp does not require LTP. In *M. sexta*, for example, cholesterol transfer occurs by a mass action mechanism, without involvement of LTP. Pretreatment of the midgut with anti-LTP IgG did not inhibit the transfer of cholesterol from midgut to HDLp, showing that the uptake of cholesterol is fundamentally different from diacylglycerol uptake (Yun *et al.*, 2002).

Whatever the mechanism(s) of hydrocarbon and contact pheromone uptake by HDLp from various tissues, it appears that oenocyte-containing tissues can exchange their hydrocarbons with both native and heterospecific HDLp, indicating that the uptake of hydrocarbons into Lp lacks species specificity (Katase and Chino, 1984; Fan and Schal, unpublished results). Interestingly, this appears to be the case for diacylglycerol loading as well (Chino and Kitazawa, 1981).

10.8.2.3 Hydrocarbon exchange from lipophorin into tissues

Unlike vertebrate lipoproteins, insect Lp is generally considered to be a reusable particle that shuttles lipids among tissues without entering cells (Chino and Kitazawa, 1981; Van Heusden *et al.*, 1991). Although delivery of hydrocarbons and pheromones to cells has not been studied in any insect, this process clearly involves hemolymph HDLp. When *B. germanica* sternites, which synthesize hydrocarbons, or pronotum, which does not synthesize hydrocarbons, were co-incubated with hydrocarbon-prelabeled sternites, radiolabeled hydrocarbons were transferred into the unlabeled tissue only when HDLp was present in the incubation medium (Schal, unpublished results). The accumulated evidence, though insufficient, supports the idea that the hydrocarbons and contact pheromone components of *B. germanica* are produced by oenocytes within the abdominal integument, carried by HDLp, and deposited in the cuticle (Figure 10.2). Whether epidermal cells are involved in the deposition process, and if so, how, remains to be determined.

The best evidence of hydrocarbon transfer into cells is with oocytes. Oocyte development in cockroaches, as in most oviparous animals, is characterized by a dramatic growth period during which all maternally derived macromolecules are sequestered. Vitellogenin uptake by the oocyte has been extensively studied

in insects, and it occurs via receptor-mediated endocytosis (Sappington and Raikhel, 1995, 1998; Raikhel and Snigirevskaya, 1998). Recently, Lp uptake by receptor-mediated endocytosis has also been demonstrated in mosquitoes (Sun *et al.*, 2000; Cheon *et al.*, 2001). In general, however, insect eggs contain larger amounts of lipid than can be accounted for by internalized Lp, suggesting that a selective lipid uptake mechanism, with Lp as a reusable lipid shuttle, is the primary mechanism operating in insect oocytes (Chino *et al.*, 1977).

The eggs of *B. germanica* contain the same types of hydrocarbons as the hemolymph, HDLp, and cuticle of the adult female. Only 150 μg of hydrocarbons accumulate on the epicuticular surface whereas up to 450 μg accumulate within the female during the period of egg maturation (Fan *et al.*, 2002). The internal hydrocarbons are divided primarily between the ovaries, fat body, and 150 μg of HDLp-bound hydrocarbons in the hemolymph. During oocyte maturation ovarian hydrocarbons increase by more than 65-fold – from 3.5 μg on day-1 to 232 μg on day-8 (Fan *et al.*, 2002). However, after oviposition on day-9, ovarian hydrocarbons decline to only 8.2 μg, demonstrating that hydrocarbons were associated with the ovulated oocytes. Radiotracing results indicate that they serve as components of the cuticular lipids of the embryos and first instars (Fan and Schal, unpublished results).

When ovaries were co-incubated with sternites that had been treated with [^{3}H]3,11-dimethylnonacosane, the amount of labeled hydrocarbon in the ovary increased with increasing concentration of hemolymph, reaching a plateau at 10 percent hemolymph (Fan *et al.*, 2002). Moreover, the uptake of hydrocarbons by the ovary was a function of HDLp concentration in the medium, and the maximum uptake occurred at a concentration of about 1 mg/ml. Because the concentration of HDLp in the hemolymph is about 10 mg/ml (Sevala *et al.*, 1999), the *in vitro* results suggest a remarkable consistency in peak rates of uptake and normal concentrations of HDLp.

Incubated oocytes accept hydrocarbons at similar rates from hemolymph and from purified HDLp, suggest that LTP might not be required for uptake of hydrocarbons (Fan *et al.*, 2002). However, this idea will have to await careful experiments with LTP antibodies, as detailed above. In *M. sexta* LTP plays a role in delivery of lipids to the developing oocytes and the conversion of adult HDLp to VHDLp in the egg (Liu and Ryan, 1991).

The small amounts (relative to vitellin) of Lp found within mature oocytes do not account for the large amount of hydrocarbons in ovulated eggs of *B. germanica* (Fan *et al.*, 2002). This suggests that the maturing oocytes endocytose some HDLp, but the vast majority of egg lipid is probably internalized by other mechanisms. Nevertheless, endocytosis of HDLp, including all its lipid cargo, explains why some, albeit little, 3,11-dimethylnonacosan-2-one contact pheromone has also been isolated from mature ovaries (Gu *et al.*, 1995).

10.9 Termination of pheromone production – neural control

Pheromone production and calling occur only in sexually receptive adult female cockroaches that experience a rising JH III titer. Therefore, lack of sexual signaling in sexually immature adult females can be attributed to low JH III titers, and indeed, exogenous JH accelerates the onset of both calling and pheromone production in young females (section 10.7.1). However, the termination of pheromone production and calling (as well as sexual receptivity) in older mated females is not caused by a decline in JH III. Rather, specific male-derived inhibitory factors transferred to the female during copulation terminate these processes. In spite of increasing rates of JH III synthesis immediately after mating (Gadot *et al.*, 1989; Smith *et al.*, 1989), the emission of sex pheromone is terminated after copulation through two successive events, with only minor differences between *Supella* and *Blattella* females. The first event involves the physical insertion of a spermatophore into the genital chamber, followed by a second stage involving presence of sperm in the spermathecae. In *S. longipalpa*, the transient insertion of a normal or spermless spermatophore (from a vasectomized male) into the female's genital chamber was sufficient to completely suppress calling during the first ovarian cycle (Smith and Schal, 1990b). However, subsequent absence of sperm from the spermatheca signals females to resume calling within several days, during the next gonadal maturation cycle. In *B. germanica*, on the other hand, insertion of a spermatophore suppressed calling for a day or two, but calling resumed during the same ovarian cycle if sperm and or associated secretions failed to be transferred to the female (Liang and Schal, 1994).

In most insects, peptides and other seminal substances transferred from the male to the female during copulation cause a transient or permanent switch to a "matedness" state (reviewed in Rafaeli, 2002). The best studied example is *Drosophila melanogaster*, where male-derived peptides not only mediate sperm transfer, storage, and protection from microbial attack within the female, but also stimulate JH production, egg production, and ovulation, while causing a reduction in sexual receptivity, longevity, and female survival (Wolfner, 1997, 2002). In other insects, notably lepidopterans, males also transfer to females specific pheromonostatic factors, including JH and peptides, most of which act through humoral pathways (Rafaeli, 2002).

In contrast, chemical factors from the male appear not to be involved in the switch to "matedness" in cockroaches, because injection into virgin females of sperm, spermatophores, spermatophore extracts, or hemolymph from mated females failed to suppress calling. Instead, the relevant inhibitory signals in cockroaches appear to be mechanosensory, involving pressure from the spermatophore, because implantation of artificial spermatophores also terminated calling (Smith and Schal, 1990b). However, because females that mated with castrated or vasectomized males resumed calling, sperm appear to be involved in the second stage of the

switch to matedness. In both *Supella* and *Blattella*, an intact VNC was required for the inhibitory signals from mating to effectively suppress calling behavior (Smith and Schal, 1990b; Liang and Schal, 1994) as was shown in *Nauphoeta cinerea* females, where an intact VNC was required to turn off sexual receptivity after mating (Roth, 1962). Thus, it appears that neural signals resulting from mechanical distention of sexual organs in the female ascend the VNC and inhibit calling.

Virgin females of both *Supella* and *Blattella* did not call while carrying infertile oothecae, even after treatment with JH analogues (Smith and Schal, 1990a; Liang and Schal, 1993b). This inhibition was also due to signals that ascend the VNC, because gravid virgin or mated females with a transected VNC resumed calling behavior. However, because the VNC also transmits signals that suppress JH biosynthesis in gravid females (Roth and Stay, 1962; Chiang *et al.*, 1991; Gadot *et al.*, 1991), calling behavior in gravid *B. germanica* with transected VNC could also be explained by activation of the CA and a rise in the JH titer. To distinguish between direct suppression of calling by neural directives and indirect suppression by inhibiting a rise in JH, Liang and Schal (1994) treated gravid females with a JH analog and either removed the ootheca or transected the VNC. Intact virgin gravid females did not call even when treated with exogenous JH, but these 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 the biosynthesis of JH III. Both signals ascend the intact VNC (Gadot *et al.*, 1991). At this point, however, it is not known whether these neural pathways also affect pheromone production.

10.10 Concluding remarks and future directions

Almost a decade before the term "pheromone" was coined, Roth and Willis (1952) conducted seminal experiments that characterized volatile and contact pheromones in cockroaches. Louis Roth's research integrated studies of endocrinology and behavior, and the influence of this approach was reflected in Barth's early articulation of the interplay between the endocrine system and sexual behavior. In later years cockroaches continued to serve as important models for invertebrate endocrinology (Scharrer, 1987; Tobe and Stay, 1985), but research on pheromones lagged, in part due to technical difficulties in sex pheromone identification. Below, we highlight some of the many issues yet to be resolved in the physiological and behavioral regulation of sex pheromone production and emission in cockroaches.

1 *Identification of cockroach sex pheromones.* More than two decades have passed since the identification and chemical synthesis of periplanone-B in

P. americana (Persoons *et al.*, 1979; Still, 1979), and only one additional female volatile sex pheromone has been identified outside the *Periplaneta* group, in *S. longipalpa* (Charlton *et al.*, 1993; Leal *et al.*, 1995). Obviously, more cockroach sex pheromones need to be identified to provide material for comparative studies on biosynthetic pathways and their endocrine and neural control. It would be of particular interest to identify sex pheromones of solitary, nocturnal cockroach species that do not associate with humans. They are most likely to communicate with volatile sex pheromones over longer distances. Indeed, field studies have shown that calling occurs in a variety of tropical species representing two of the largest cockroach families, Blattellidae and Blaberidae (Schal and Bell, 1985).

2 *Studies of biosynthetic pathways.* A major impediment to studies on regulation of pheromone production in cockroaches is a lack of information on biosynthetic pathways. Only one such pathway, that for the contact sex pheromone of the German cockroach, has been well characterized. Although specific steps in this pathway, especially the terminal oxidation of the alkane to a ketone, require further elucidation, this system has served as a fruitful source of information about sex-specific biochemical events in sexually receptive females. No such information is available on the volatile sex pheromones of *Periplaneta* or *Supella*. Therefore, recent studies of pheromone production have used behavioral and electrophysiological assays of male response to female odors, much as was done 40 years ago. While valuable, these assays cannot distinguish between pheromone that accumulated in pheromone glands and that which was newly synthesized in response to experimental intervention. Obviously, investigating pheromones with labeled precursors is also challenging without information on the biosynthetic pathways.

3 *Tissues and cells that produce pheromones.* Periplanone-B was identified 24 years ago, but its site of production remains in doubt, and even recent studies have implicated several unrelated tissues. Association of periplanone-B with the digestive tract, coupled with the pervasiveness of coprophagy in cockroaches, can result in tissue contamination when only static analytical methods are employed. A biochemical test of *de novo* pheromone production *in vitro* will be necessary for an unambiguous determination of the site of pheromone production. This should be a feasible approach with periplanone, but elucidation of its biosynthetic pathway will profoundly facilitate such studies. Pheromone production in other cockroaches has been localized primarily in the dorsal and ventral tegmina and intersegmental membranes of the abdomen. However, the precise tissue and cells that produce pheromones remain unknown in most species.

4 *Cellular plasticity of pheromone glands.* Reproduction in female cockroaches depends on the timely acquisition and processing of resources that are then provisioned to many synchronously maturing oocytes. As virgin females advance

through successive stages of the gonadotrophic cycle, they experience various checkpoints that determine whether they should carry on or resorb their oocytes. Reproduction in oviparous species with rapid reproductive cycles (e.g. *Supella*) can also be arrested by external sensory and internal physiological signals. Cockroaches appear to have evolved a tight link between the gonadotrophic cycle and glandular maturation (both CA and pheromone glands). Thus, even short 2–3-day periods of sexual inactivity result in a concomitant regression of the cellular machinery within these glands. This is especially clear in ovoviviparous females that experience long periods of sexual inactivity. Such periods are followed in cockroaches by a proliferation of the cellular machinery involved in pheromone production. Because cockroaches are long-lived, many such cycles can occur within pheromone secreting cells. The factors that regulate these cellular cycles remain to be elucidated.

5 *Role of JH III in pheromone production and calling behavior.* In addition to its fundamental role as a gonadotropic hormone, in many cockroaches JH III plays a pivotal permissive role in pheromone production, calling behavior, and sexual receptivity of the female. Without it, these components of mate-finding are not expressed or are terminated. In most such examples, however, JH appears to exert its effects as a priming hormone – its effects require a long time to become evident and JH appears to prepare cells to respond to it or to other regulatory agents (see Wyatt and Davey, 1996 for discussion). To date, the only example of a possible regulatory (i.e. "releaser") action of JH on pheromone production in cockroaches is in *B. germanica*, where JH III induces the metabolism of 3,11-dimethylnonacosane to 3,11-dimethyl-nonacosan-2-ol, a precursor of the contact pheromone. How this is accomplished and what enzymes catalyze this change remains unknown.

It is imperative to demonstrate whether JH III operates directly on pheromone glands or whether it facilitates the activity of other pheromonotropic regulators. Studies of mated females, which produce JH III but not pheromone, have made it abundantly clear that unknown regulatory elements downstream of the action of JH must be involved. These factors probably interact with inhibitory signals from the terminal abdominal ganglion that ascend the VNC and control CNS activity. A concerted effort is needed to identify neuropeptides and other factors that activate and terminate pheromone production, emission, and sexual receptivity.

6 *Transport of pheromones.* Two major routes for translocation of pheromones have been considered in this chapter: (a) from the secretory cell directly through the cuticle overlying it; and (b) indirectly through the hemolymph. Cockroaches share with even the most studied lepidopterans an almost complete lack of information on the former pathway. Transport of hydrocarbons and contact sex pheromones, on the other hand, has been extensively studied in cockroaches, commencing with the work of Chino and colleagues. It has

become clear that HDLp serves as pheromone carrier not only in cockroaches, but also in termites, flies, and moths. However, details of this pathway remain unknown. How are pheromone components loaded into HDLp? How are they imported into cells? Are Lp receptors involved? Does LTP catalyze these transfers? And finally, how are different types of hydrocarbons sorted among various tissues? Resolution of these questions in cockroaches will certainly further our understanding of similar mechanisms in other insects because it appears that in some moths as well hydrocarbon pheromones are transported by lipophorin (Schal *et al.*, 1998a).

7 *Role and mechanics of calling behavior.* Calling behavior in female cockroaches is associated with the release of volatile sex pheromones. However, we have only a rudimentary understanding of how the female calling posture results in the release of pheromone and what role atrial glands play in calling and courtship. Although the orifices of class-3 pheromone glands are always exposed, only when the female calls is the pheromone emitted. The relationship between the motor patterns that characterize calling behavior and the release of pheromone needs to be studied. Do these movements facilitate transport of the pheromone products along the ducts? What is the function of exposing the genital vestibulum during calling in females whose pheromone appears to be produced only by the tergites?

8 *Role of neural directives.* Insertion of the spermatophore in the genital chamber has a multiplicity of effects on the female's endocrinology and behavior: it stimulates JH production and inhibits pheromone production, calling, and sexual receptivity. Sperm, likewise, inhibits further sexual behaviors, while the ootheca also inhibits JH biosynthesis. The prevalent model is that these actions are conveyed to the CNS through the VNC. But it is not known whether mechanoreceptors transduce this information, how, and what events transpire within the CNS to prevent the expression of sexual behaviors.

9 *Cockroaches as integrative models.* Cockroaches have served as early models for investigations of mechanisms that regulate pheromone production. Research on ovoviviparous blaberids, over four decades ago, and more recent studies with the blattellids *B. germanica* and *S. longipalpa* have shown that in cockroaches, several mechanisms are integrated to promote or suppress production and emission of pheromones. Almost all physiological and behavioral aspects of female reproduction are coordinately regulated and paced by JH. Food intake and mating also intervene in the regulation of pheromone production and emission in females, in part by modulating the production of JH. Mechanoreceptive signals from the spermatophore and sperm appear to play roles in inhibiting pheromone production and emission. And pheromone-producing glands undergo cycles of competence for pheromone production. Because they are long-lived and reproduce cyclically, cockroaches will remain an important model system for integrative studies of regulation of pheromone production.

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