# 3.14 Biochemistry and Molecular Biology of Pheromone Production

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## 3.14.1. Introduction and Overview

The elucidation of the structure of the first insect sex pheromone, bombykol ((*E*,*Z*)-10,12-hexadecadien-1-ol) (Butenandt *et al*., 1959) (**Figure 1**), from the silkworm moth, *Bombyx mori*, spanned more than 20 years and required a half million female abdomens. A few years later, (*Z*)-7-dodecenyl acetate (**Figure 1**) was identified as the sex pheromone of the cabbage looper, *Trichoplusia ni* (Berger, 1966). At about the same time Silverstein *et al*. (1966) identified three terpenoid alcohols, ipsenol, ipsdienol, and verbenol (**Figure 1**), as the pheromone of the bark beetle, *Ips paraconfusus*. This latter finding led to the recognition that most insect pheromones consisted of multicomponent blends. This has since been shown to be true for most insects, while single-component pheromones are rare. Rapid improvements in analytical instrumentation and techniques reduced the number of insects needed for pheromone extracts from a half million or more to where now individual insects can sometimes provide sufficient material for chemical analysis. Over the last four decades, extensive research on insect pheromones has resulted in the chemical and/or behavioral elucidation of pheromone components from well over 3000 insect species, with much of the work concentrating on sex pheromones from economically important pests.

An early issue addressed in pheromone production was the origin of pheromone components. Ultimately, all precursors for pheromone biosynthesis can be traced through dietary intake. A question asked in several systems was whether pheromone components were derived from dietary components that were altered only minimally, or whether they were synthesized *de novo*. This simple question proved surprisingly difficult to answer, and different answers were obtained for different groups of insects. It is now clear that most insect pheromone components are synthesized by insect tissue, with a

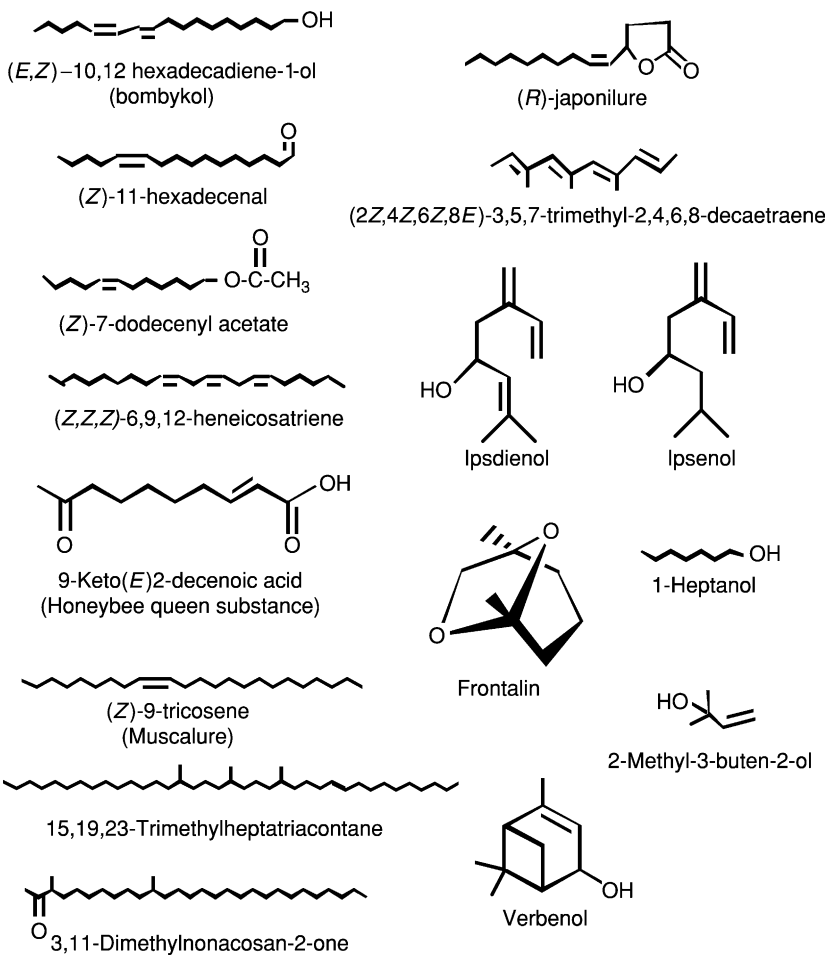


**Figure 1** Selected pheromone components representing fatty acid, hydrocarbon, and isoprenoid-derived components. Components were selected based on historical interest and work performed on their biosynthesis.

number of notable exceptions (reviews: Tillman *et al.*, 1999; Eisner and Meinwald, 2003).

By the mid-1980s, it had became apparent that the products of normal metabolism, particularly those of the fatty acid and isoprenoid pathways, were modified by a few pheromone-tissue specific enzymes to produce the myriad of pheromone molecules. The elegant work of the Roelofs laboratory (review: Bjostad *et al.*, 1987) demonstrated that many of the lepidopteran pheromones could be formed by the appropriate interplay of highly selective chain shortening and unique Δ11 and other desaturases, followed by modification of the carboxyl carbon. This work has been extended, and there now exists a clear understanding of the biosynthetic pathways for many of the lepidopteran pheromones (review: Jurenka, 2003). The honeybee also uses highly specific chain shortening of fatty acids to produce the major component of the queen pheromone (Plettner *et al.*, 1996, 1998). In some insects, fatty acid elongation followed by decarboxylation produces the hydrocarbon and hydrocarbon-derived pheromones (review: Tillman *et al.*, 1999). Recent work in bark beetles has shown that *Ips* and *Dendroctonus* spp. produce their monoterpenoid-derived pheromones ipsenol, ipsdienol, and frontalin by modification of isoprenoid pathway products (review: Seybold and Tittiger, 2003).

The work on the biosynthesis and endocrine regulation of pheromone production has emphasized sex and aggregation pheromones in lepidopteran, coleopteran, dipteran, and blattodean models. Research on representative species from these orders was motivated by their economic importance, the relatively large amount of pheromone produced by some members, and extension of ongoing studies on hydrocarbon and fatty acid biosynthesis in some species.

The production and/or release of sex pheromones are influenced by a variety of environmental factors (Shorey, 1974). In general, insects do not release

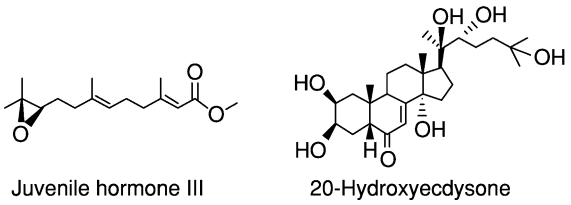


**Figure 2** Structures of juvenile hormone (JH) III and 20-hydroxyecdysone (20E). The other major hormone that regulates pheromone production, PBAN, is shown in **Figure 10**.

pheromones until they are reproductively competent, although exceptions occur. Pheromone production is usually age related and coincides with the maturation of ovaries or testes, and in some cases with feeding. The observation that females of certain species have repeated reproductive cycles and that mating occurs only during defined periods of each cycle led to the proposal that pheromone production might be under hormonal control (Barth, 1965). Early work on cockroaches established that females require the presence of functional corpora allata (CA) in order to produce sex pheromone. It is now recognized that juvenile hormone (JH) (**Figure 2**) regulates pheromone production in a number of species, especially among beetles (review: Seybold and Vanderwel, 2003) and cockroaches (review: Schal *et al*., 2003).

A unifying theme of this work on cockroaches and beetles was that the same hormone that regulated ovarian maturation (JH) also regulated pheromone production, coordinating sexual maturity with mating. Thus, in retrospect, it was not surprising that ovarian-produced 20-hydroxyecdysone (20E) (**Figure 2**), which plays an important role in reproduction in female Diptera, is also the key hormone inducing sex pheromone production in the female housefly, *Musca domestica* (review: Blomquist, 2003) and *Drosophila* (Wicker and Jallon, 1995a, 1995b).

It was recognized by the mid-1980s that female moths regulated pheromone production through a different mechanism than flies, cockroaches, and beetles (Raina and Klun, 1984), but it was not until 1989 that the structure of the pheromone biosynthesis activating neuropeptide (PBAN) was elucidated (Raina *et al*., 1989; Raina, 1993) and work is ongoing deciphering its mode of action (review: Rafaeli and Jurenka, 2003). In some species, there does not appear to be a hormone regulating pheromone production.

## 3.14.2. Pheromone Chemistry

The pheromones of over 3000 insect species are now known. The website Pherolist is an up-to-date compilation of lepidopteran pheromones and lists over 500 compounds identified from over 1700 lepidopteran species. These numbers are a huge increase from the 80 pheromone components from about 120 lepidopterous species known in 1985 (Tamaki, 1985) and illustrate both the rapid growth in pheromone chemistry and the increased ease with which pheromones are identified. The book *Pheromones of Non-Lepidopteran Insects Associated with Agricultural Plants* (Hardie and Minks, 1999) discusses the pheromones of a number of pest species other than lepidopterans along with pheromones of beneficial insects. The *Handbook of Insect Pheromones and Sex Attractants* (Mayer and McLaughlin, 1991) contains a complete list of the pheromones identified from insects up to 1991.

The majority of lepidopteran pheromone components arise from fatty acids, and have carbon numbers from 7 to 29, with 12, 14, and 16 carbon components predominating. Of the 540 compounds included on the Pherolist website in 2003, 377 (70%) have an even number of carbons and 185 (34%) are acetate esters. The chemical classes alcohols, aldehydes, hydrocarbons, and epoxides each have between 65 and 85 components represented. Many of the lepidopteran pheromones have one or more double bonds with specific blends of (*Z*) and (*E*) isomers.

Many of the dipteran pheromones are hydrocarbons of 21 carbons and longer, and function as short range or contact pheromones. The pheromone of the housefly consists of (*Z*)-9-tricosene (**Figure 1**) (Carlson *et al*., 1971), an epoxide and ketone derived from this hydrocarbon, and methyl-branched alkanes (review: Blomquist, 2003). Among the longest chain pheromones are the contact pheromones from the tsetse flies, which are often multimethyl branched hydrocarbons with up to 40 carbons (**Figure 1**) (Carlson *et al*., 1978, 1984, 1998; Carlson and Schlein, 1991). The role of hydrocarbons in chemical communication in insects has become increasingly recognized over the past two decades (Howard, 1993).

A number of coleopteran pheromones are isoprenoid in origin (Francke *et al*., 1995). The shortest chain pheromone described is 2-methyl-3-buten-2-ol from *Ips typographus* (Lanne *et al*., 1989). Many of the components either contain multiples of 5 carbons or are derived from such precursors. **Figure 1** shows a number of pheromone components that are of isoprenoid origin, and include ipsdienol, ipsenol, and frontalin. Frontalin contains 8 carbons but is clearly isoprenoid in origin (Barkawi *et al*., 2003). In addition to isoprenoids, the pheromones from coleopterans also consist of components derived from fatty acids and components of unknown orgin.

Many of the 4000+ described species of cockroaches use long-range volatile pheromones in mate-finding and cuticular contact pheromones in the final recognition process (Gemeno and Schal, 2004). Short-range volatile pheromones emitted by the male, coupled with nuptial tergal secretions, facilitate proper alignment of the pair prior to copulation (Nojima *et al.*, 1999). Pheromones also mediate intrasexual conflicts, especially when males establish dominance hierarchies and territories (Moore and Moore, 1999), and other behaviors. However, to date, few chemical structures have been elucidated, and the biosynthetic pathway of only one pheromone, the contact pheromone of the German cockroach, *Blattella germanica* has been described (major component is 3,11-dimethylnonacosan-2-one) (**Figure 1**) (Nishida *et al.*, 1974; Nishida and Fukami, 1983). Of particular note are the volatile sex pheromones of species in the genera *Periplaneta* and *Blatta* (periplanones) and 5-(2*R*,4*R*-dimethylheptyl)-3-methyl-2*H*-pyran-2-one (supellapyrone), the single-component sex pheromone of the brown-banded cockroach, *Supella longipalpa* (Charlton *et al.*, 1993; Leal *et al.*, 1995). Nevertheless, several cockroach pheromones have been investigated using behavioral and electrophysiological assays of crude body extracts. Interestingly, the pheromones of cockroach species that have served in important early studies on pheromone regulation (e.g., *Byrsotria fumigata*, *Blaberus* spp.) (see Barth, 1965; Barth and Lester, 1973) have yet to be identified.

## 3.14.3. Site of Pheromone Biosynthesis

There is much variability among insects in the anatomical location of pheromone production, just as there are many differences in the gross morphology and function of pheromone producing tissue. Complexity varies from simple unicellular glands distributed throughout the integument to elaborate internal cellular aggregates connected to a reservoir. Of the orders emphasized in this chapter (Lepidoptera, Coleoptera, Blattodea, and Diptera), the most common location for pheromone production is the abdomen. There are a number of excellent reviews of the ultrastructure of exocrine cells in general (Percy-Cunningham and MacDonald, 1987; Quennedey, 1998; Ma and Ramaswamy, 2003) and social insects in particular (Billen and Morgan, 1998). Definitive proof that pheromone production and release occur in certain tissues comes from studies where the isolated tissue has been shown to incorporate labeled precursors into pheromone components.

### 3.14.3.1. Location of Pheromone Production in Lepidoptera

The oxygenated lepidopteran pheromone components are usually produced and released from extrudable glands located between the 8th and 9th abdominal segments (Percy-Cunningham and MacDonald, 1987; Ma and Ramaswamy, 2003). The secretory cells in these glands typically contain a well-developed endoplasmic reticulum that is involved in fatty acid metabolism. Extensive studies examining the pheromone products and precursors from these glands in a number of species show that unusual specific fatty acids that had the same carbon number, double bond positions, and stereochemistry as the acetate, alcohol, or acetate ester pheromone components were present. The role of these glands in pheromone production has been clearly demonstrated with radiochemical studies (Bjostad *et al.*, 1987; Jurenka, 2003). Although a large number of moths utilize a gland located between the 8th and 9th segments, exceptions occur. It was shown in *Theresimima ampelophaga* (Zygaenidae) that the gland is located on the dorsal part of the 3–5th abdominal segments (Hallberg and Subchev, 1997).

In contrast, the site of synthesis of the hydrocarbon and hydrocarbon-derived pheromone components is more complicated. 2-Methylheptadecane is not synthesized in the pheromone gland of *Holomelina aurantiaca*, but rather is synthesized by epidermal tissue, transported by lipophorin, and then sequestered into and released from the pheromone gland (Schal *et al.*, 1998a). A similar situation exists for the gypsy moth, *Lymantra dispar*, in which the hydrocarbon precursor is synthesized in epidermal tissue, then transported by lipophorin to the pheromone gland where it is epoxidized and released (see Section 3.14.4.3.6) (Jurenka *et al.*, 2003).

### 3.14.3.2. Site of Pheromone Biosynthesis in Coleoptera

In many coleopteran species, pheromone production localizes to the abdomen, e.g., among the Scarabaeidae (Tada and Leal, 1997), Anobiidae (Levinson *et al.*, 1983), Dermestidae (Barak and Burkholder, 1977), and others (review: Plarre and Vanderwel, 1999; Tillman *et al.*, 1999). In some cases there are defined glands, whereas in others, groups of cells in defined anatomical locations may not form a gland, but are nevertheless a localized site of pheromone biosynthesis. Other anatomical locations for pheromone production have also been identified for some species. These include putative pheromone glands on the antennae of *Batrisodes oculatus* (de Mazo

and Vit, 1983) and on the forelimbs of *Tribolium castaneum* (Faustini *et al*., 1982).

Pheromone biosynthetic cells of many beetles are generally dermally derived, with one notable exception. Monoterpenoid pheromone components have been reported to be synthesized in the fat body of male boll weevils, *Anthonomus grandis* (Wiygul *et al*., 1990). The fat body is an unexpected location for boll weevil pheromone biosynthesis, because it requires transport of pheromone components into the lumen of the alimentary canal for release. This is unusual, because other pheromone glands and cells tend to be physically linked to release sites either by proximity or by specialized structures such as ducts or ductules (review: Ma and Ramaswamy, 2003). Recent work (Nural, Tittiger, Fu and Blomquist, unpublished data) demonstrate that isolated gut tissue from male *A. grandis* incorporated labeled acetate into components that coeluted on high-performance liquid chromatography (HPLC) with the four $C_{10}$ pheromone components. It is difficult to obtain *A. grandis* gut tract tissue free from adhering fat body, making it impossible to conclude with certainity that fat body tissue is not involved. The clear demonstration that several bark beetles synthesize their frass-associated isoprenoid pheromone components in midgut tissue (see below) along with the data from *A. grandis* suggests that frass-associated pheromones in general may be produced in gut tissue.

The site of *de novo* monoterpenoid pheromone biosynthesis in bark beetles (especially *Ips* and *Dendroctonus* spp.) has been clearly demonstrated. Early work localized the pheromone and its release to the hindgut (Bordon *et al*., 1969; Byers, 1983), but did not determine the site of synthesis. Pheromone component precursors were localized in the abdomen–thorax border (Ivarsson *et al*., 1998), and the anterior midgut was determined as the site of *de novo* synthesis through the use of molecular probes to 3-hydroxy-3-methylglutaryl-CoA (HMG-R) reductase and radiotracer studies (**Figure 3**) (Hall *et al*., 2002a, 2002b). Electron microscopy studies show that pheromone-biosynthetic midgut cells have crystalline arrays of smooth endoplasmic reticulum, while nonpheromone-producing cells do not (Nardi *et al*., 2002). These studies also confirmed that bark beetle pheromones are synthesized by insect tissues, and not by microorganisms, as had been suggested by earlier studies (Brand *et al*., 1975, 1976). Pheromone components are secreted into the gut lumen through an apocrine mechanism, and all anterior midgut cells appear to be involved. Pheromone-biosynthetic cells are typically dedicated to that function, but anterior midgut cells of bark beetles apparently fulfill the dual roles of pheromone production and food digestion (Nardi *et al*., 2002).

Why midgut tissue? In considering the evolution of pheromone components, it is generally thought that pheromone molecules, as other signaling chemicals, originally had another purpose and were co-opted for signal function. Components were then shaped by selective forces acting on preexisting structures, and pheromones evolved so that the signal would be stronger and more easily discriminated. For bark beetles, it is thought that monoterpene components arose from the detoxification of oleoresin tree defensive components, and that many of the detoxification reactions involved hydroxylation reactions (Wood, 1982; Vanderwel and Oehlschlager, 1987). To lessen dependence upon host chemicals and increase host range, bark beetles then apparently evolved the ability to synthesize their monoterpenoid pheromones *de novo*, and with this, evolved the JH III regulation of pheromone production. As the ancient beetles chewed through the bark and phloem, they encountered tree terpenoids. Gut tissue was the first line of defense, and detoxification enzymes (cytochrome P450 hydoxylases) could have evolved to detoxify monoterpenes in this tissue. As *de novo* synthesis evolved, it is possible that production of pheromone arose in midgut tissue so that the hydroxylation reactions that were already present could be used.

#### 3.14.3.3. Site of Pheromone Production in Diptera

The hydrocarbon pheromone components of many Diptera evolved from, and still function as, cuticular lipids. Thus, it is expected that these pheromone components are synthesized in the same specialized epidermal cells (oenocytes) that produce cuticular hydrocarbons. After synthesis, pheromone components are secreted into the hemolymph, where they are transported by lipophorin, as has been demonstrated in *Drosophila* (Ferveur *et al*., 1997) and the housefly (Schal *et al*., 2001), before being deposited on the cuticular surface. During the process of grooming, relatively large amounts of (*Z*)-9-tricosene accumulate on the legs of female houseflies (Dillwith and Blomquist, 1982). The mechanism of how hydrocarbon pheromones, and hydrocarbons in general, are unloaded from lipophorin and transported across the cuticle is not known.

#### 3.14.3.4. Site of Pheromone Production in Blattodea

As in Coleoptera, early reports considered various organs in the head, thorax, and abdomen as possible sites of pheromone production in cockroaches, but

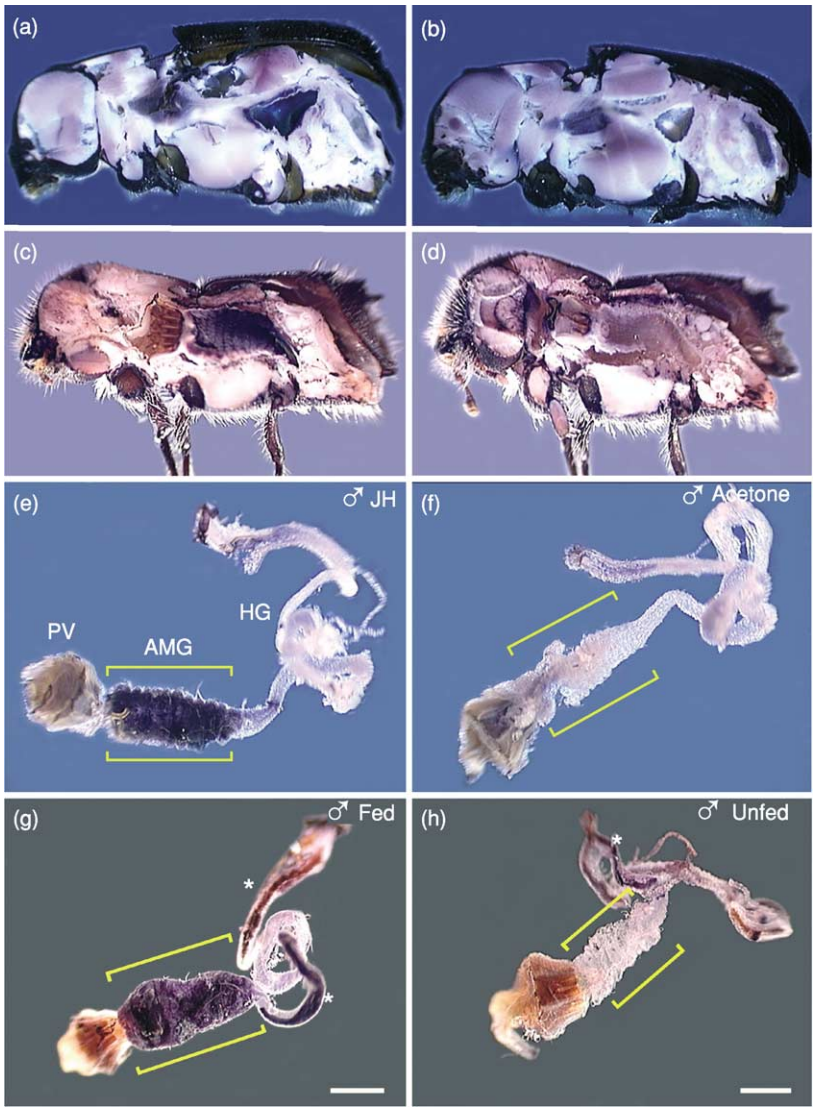


**Figure 3** Tissue localization of *HMG-R* expression. Exposed whole mounts show that HMG-R mRNA is observed in the midgut of JH III-treated male *Dendroctonus jeffreyi* (a) and *Ips pini* (c), but not in untreated insects (b, d). Panels (e) through (h) show whole mount hybridizations of isolated *I. pini* alimentary canals. *HMG-R* expression in the anterior midgut (AMG, marked by yellow brackets) correlates with pheromone production in starved, JH III-treated males (e) and fed males (g), while starved and untreated males (f, g), which do not produce monoterpenoid pheromone components, do not strongly express *HMG-R*. Asterisks mark nonspecific signal in the hindguts. PV, proventriculus; HG, hindgut. Scale bar = 0.5 mm. (Modified from Hall *et al.*, 2002a, 2002b.)

most of the more recent research implicates specialized abdominal glands. Periplanone-A and periplanone-B, the sex pheromone components of the American cockroach, *Periplaneta americana*, appear to be concentrated in the midgut and released in feces. However, female calling involves opening the genital vestibulum (i.e., atrial gland), without excretion of feces, and this glandular tissue contains most of the periplanone-B, up to 60 ng (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) concluded that both periplanone-A and periplanone-B were most abundant in the colon and that this tissue produced the strongest electroantennogram (EAG) responses. To date, no definitive proof of the sites of pheromone biosynthesis is available through isotopic tracing of pheromone precursors in isolated tissues.

The volatile sex pheromones of other cockroaches have been localized to abdominal tergites and sternites. Females of the German cockroach, *B. germanica*, produce the pheromone in the 10th abdominal

tergite (pygidium) (Liang and Schal, 1993; Tokro *et al*., 1993), *S. longipalpa* in the 4th and 5th tergites (Schal *et al*., 1992), and *Parcoblatta lata* (wood cockroach) females produce the pheromone in tergites 1–7 (Gemeno *et al*., 2003). In all three species, the tergal glands are composed of multiple class-3 secretory units, each leading through a long unbranched duct to a single cuticular pore. The secretory cells are characterized by abundant mitochondria, smooth endoplasmic reticulum (SER), rough endoplasmic reticulum (RER), a large nucleus, and numerous secretory vesicles that discharge through an end-apparatus with numerous long microvilli into the duct and to the cuticular surface. In all three species pheromone is released during a calling behavior.

Male cockroaches also release volatile sex pheromones from tergal or sternal glands. In *Nauphoeta cinerea* (lobster cockroach) glands on sternites 3–7 are exposed during calling, releasing a volatile male pheromone consisting of 3-hydroxy-2-butanone, 2-methylthiazolidine, 4-ethyl-2-methoxyphenol, and 2-methyl-2-thiazoline (Sreng, 1990; Sirugue *et al*., 1992). In some species the male tergal glands form specialized regions on the cuticular surface and they produce a blend of close-range attractants, phagostimulants, and nutrients that the male deploys to place the female in a precopulatory position. For example, the tergal glands of *B. germanica* consist of transverse cuticular depressions on the 7th and 8th tergites, connected to numerous class-3 secretory cells (Sreng and Quennedey, 1976; Brossut and Roth, 1977).

Cuticular contact pheromones mediate species and sex recognition and, in most cases, they function as courtship-inducing pheromones. They are thought to be distributed throughout the epicuticular surface. The blend of hydrocarbon-derived ketones of *B. germanica* females is produced by oenocytes, which are localized within the abdominal integument, separated from the hemocoel by a basal lamina (Liang and Schal, 1993). Fan *et al*. (2003) enzymatically dissociated the integument underlying the sternites into a cell suspension. After further Percoll gradient centrifugation, each fraction was assayed for incorporation of [$^{14}C_1$]propionate into methyl-branched hydrocarbons. Only oenocytes produced hydrocarbons and methyl ketone pheromones, whereas the much larger population of epidermal cells did not (Fan *et al*., 2003; Fan, unpublished data). As in the dipterans *M. domestica* and *Drosophila* and the moth *Holomelina*, the hydrocarbon-derived pheromones of *B. germanica* are produced by oenocytes and transported by lipophorin (see Section 3.14.5.4).

## 3.14.4. Biochemistry of Pheromone Production

### 3.14.4.1. Modification of "Normal Metabolism"

By the mid-1980s (Prestwich and Blomquist, 1987), the biosynthetic pathways of pheromones for a limited number of species had been determined, and work was progressing toward the characterization of some of the unique enzymes involved. It became apparent that the products and intermediates of normal metabolism, particularly those of the fatty acid and isoprenoid pathways, were modified by a few specific enzymes in pheromone gland tissue to produce the myriad of pheromone molecules. Many of the lepidopteran pheromones could be formed by the appropriate interplay of highly selective chain shortening and a unique Δ11 and other desaturases followed by modification of the carboxyl carbon (Bjostad *et al*., 1987). This work has been extended, and a clear understanding of the biosynthetic pathways for many of the lepidopteran pheromones is now known (Jurenka, 2003). The Δ11 and other pheromone-specific desaturases in Lepidoptera have been characterized at the molecular level (Knipple and Roelofs, 2003). Chain shortening of fatty acids is also involved in producing the queen pheromone in honeybees (Plettner *et al*., 1996, 1998). In some insects, fatty acid elongation followed by decarboxylation produces the hydrocarbon pheromones, and this process includes examples of lepidopterans (Jurenka, 2003), dipterans (Blomquist, 2003; Jallon and Wicker-Thomas, 2003), the German cockroach (Schal *et al*., 2003), and the social insects (Blomquist and Howard, 2003). More recent work in bark beetles has shown that *Ips* and *Dendroctonus* spp. produce their monoterpenoid-derived pheromones ipsenol, ipsdienol, and frontalin by modifications of isoprenoid pathway products (Seybold and Vanderwel, 2003). Until that work, it was considered very rare for animals to produce monoterpenoids ($C_{10}$ isoprenoids).

### 3.14.4.2. Pheromone Biosynthesis in Moths

Bjostad and Roelofs (1983) were the first to determine correctly how the major pheromone component for a particular moth was biosynthesized. They utilized the moth *T. ni* because the female produces a relatively large amount of pheromone (about 1 μg). They began by demonstrating that pheromone glands utilize acetate to produce the common fatty acids octadecanoate and hexadecanoate, which undergo Δ11 desaturation to produce *Z*11–18:acid and *Z*11–16:acid. However, the main pheromone component is *Z*7–12:OAc, which presumably is

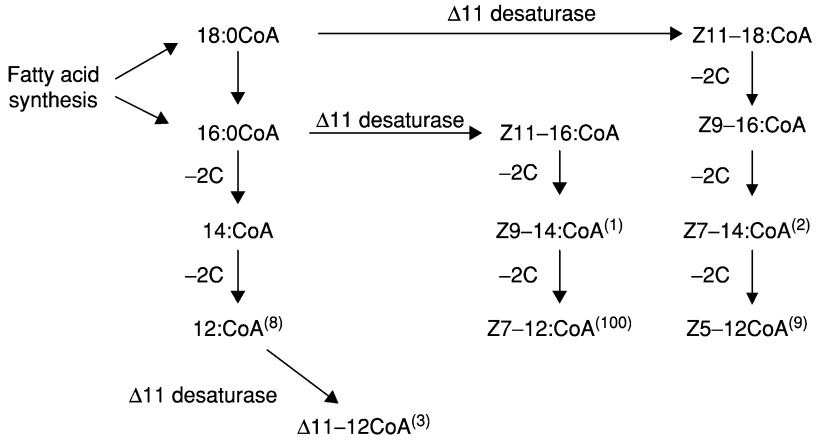


**Figure 4** Biosynthetic pathways for producing the intermediate CoA derivatives of the pheromone blend of the cabbage looper, *Trichoplusia ni*. The CoA derivatives followed by the superscript number in parenthesis are reduced to an alcohol and acetylated to form the acetate esters that make up the pheromone blend. The superscript numbers indicate the approximate ratio of components found in the pheromone gland (Bjostad *et al.*, 1984). (Reprinted with permission from Jurenka, R.A., **2003**. Biochemistry of female moth sex pheromones. In: Blomquist, G.J., Vogt, R.G. (Eds.), Insect Pheromone Biochemistry and Molecular Biology. Elsevier, San Diego, CA, pp. 53–80; © Elsevier.)

made from *Z*7–12:acid. To demonstrate how the fatty acid precursor *Z*7–12:acid was produced, [$^3H_{16}$]*Z*11–16:acid was applied to glands and found to be incorporated into both *Z*7–12:acid and *Z*7–12:OAc. They concluded that limited chain-shortening of *Z*11–16:acid could account for this incorporation. The other minor components are produced in a similar way (**Figure 4**). Thus the pheromone components are produced through a fatty acid biosynthesis pathway involving a Δ11 desaturase and limited chain-shortening enzymes. The appropriate chain length fatty acid is then reduced and acetylated to form the acetate ester. Subsequent research has demonstrated that similar pathways occur in a wide variety of female moths (Bjostad and Roelofs, 1984; Roelofs and Wolf, 1988).

Most female moths produce their pheromone through modifications of fatty acid synthesis pathways and thus techniques in fatty acid research were utilized to determine biosynthetic pathways (Bjostad *et al.*, 1987; Morse and Meighen, 1987b). The main techniques include thin-layer chromatography, gas chromatography (GC), and GC-mass spectrometry (MS) with the latter being the primary method utilized. The advantage of using GC–MS is that the label (stable isotopes of deuterium or carbon-13) can be explicitly shown to be present in the compound of interest. By monitoring for diagnostic ions that correspond to unlabeled and labeled products, it can be determined with considerable certainty that the label is associated with a particular compound. By utilizing different proposed intermediate labeled fatty acids a biosynthetic pathway can be deduced. For example, a recent study determined that *Z*11–16:Ald occurs by Δ11-desaturation of 16:CoA followed by reduction in the moths *Helicoverpa zea* and *H. assulta* (Choi *et al.*, 2002). However, *Z*9–16:Ald, the major pheromone component in *H. assulta*, was produced by a Δ9-desaturase using 16:CoA as a substrate, but in *H. zea* *Z*9–16:Ald was produced by Δ11-desaturation of 18:CoA to produce *Z*11–18:CoA that is then chain-shortened to *Z*9–16:CoA (Choi *et al.*, 2002). These types of studies have shown that the key enzymes of sex pheromone biosynthetic pathways are fatty acid biosynthesis, desaturases, chain-shortening enzymes, and specific enzymes to produce a functional group.

### 3.14.4.3. Enzymes Involved in Lepidopteran Pheromone Production

**3.14.4.3.1. Fatty acid synthesis** A combination of acetyl-CoA carboxylase and fatty acid synthase produce saturated fatty acids. Although no direct enzymatic studies have been conducted using pheromone gland cells, these enzymes are presumably similar to enzymes found in other cell types. Labeling studies conducted with acetate indicated that pheromone glands produce 16:acid and 18:acid saturated products (Bjostad and Roelofs, 1984; Tang *et al.*, 1989; Jurenka *et al.*, 1991b, 1994).

**3.14.4.3.2. Chain-shortening enzymes** Insects in general have the ability to shorten long-chain fatty acids to specific shorter chain lengths (Stanley-Samuelson *et al.*, 1988). This chain-shortening pathway has not been characterized at the enzymatic level in insects. It presumably is similar to the characterized pathway as it occurs in vertebrates which

is essentially a partial β-oxidation pathway located in peroxisomes (Hashimoto, 1996). The evidence for limited chain-shortening enzymes in pheromone glands was originally demonstrated by Bjostad and Roelofs (1983) using the cabbage looper moth, *T. ni* in which it was shown that *Z*11–16:acid labeled the intermediate fatty acid *Z*7–12:OAc. A similar study using *Argyrotaenia velutinana* demonstrated that deuterium-labeled 16:acid was chain-shortened to 14:acid, which was used to make *Z* and *E*11–14:acid (Bjostad and Roelofs, 1984). Since then considerable evidence in a number of moths has accumulated to indicate that limited chain shortening occurs in a variety of pheromone biosynthetic pathways.

Radio and stable isotopes were topically applied directly to an intact pheromone gland to provide evidence for chain shortening. The position of the label was on the terminal methyl carbon making it difficult for any type of rearrangement to occur (Wolf and Roelofs, 1983; Rosell *et al*., 1992). A more direct *in vitro* enzyme assay was utilized to demonstrate substrate preferences in a study using cabbage looper moths (Jurenka *et al*., 1994). This study was prompted by the finding of a mutant line of cabbage loopers that produced a greatly increased amount of *Z*9–14:OAc (Haynes and Hunt, 1990), which is a minor component of normal cabbage loopers. Increased amounts of *Z*9–14:OAc indicate that chain shortening was affected in the mutant cabbage loopers. To determine if chain shortening was affected, substrate specificities for both normal and mutant cabbage loopers were determined using an *in vitro* enzyme assay (Jurenka *et al*., 1994). Pheromone glands from normal cabbage loopers preferred to chain shorten *Z*11–16:CoA to *Z*7–12:CoA with two rounds of chain shortening, whereas pheromone glands from the mutant cabbage looper apparently have the ability to chain shorten by only one round. Therefore, *Z*11–16:CoA was chain shortened to *Z*9–14:CoA.

Changes in chain-shortening reactions have also been implicated in the alteration of pheromone ratios in several other species. In a laboratory selection pressure experiment using *A. velutinana*, the *Z*/*E* ratio of 11–14:OAc could not be changed much from a 92/8 ratio (Roelofs *et al*., 1986). However, it was found that the ratio of *E*9–12:OAc/*E*11–14:OAc could be selected (Sreng *et al*., 1989) and by comparing ratios of the 14-carbon/12-carbon pheromone components and *Z*/*E* isomers of each chain length it was determined that chain-shortening enzymes were selective for the *E* isomer (Roelofs and Jurenka, 1996). Another example where a change in chain shortening can account for a new pheromone blend is the larch budmoth, *Zeiraphera diniana*. The pheromone was first identified as *E*11–14:OAc (Roelofs *et al*., 1971), but it was later determined that some populations utilize *E*9–12:OAc, which is made by chain shortening of *E*11–14:acid (Guerin *et al*., 1984; Baltensweiler and Priesner, 1988). Another case where changes in chain shortening may have produced two populations of insects was found in the turnip moth, *Agrotis segetum*. A Swedish population has a ratio of *Z*9–14:OAc/*Z*7–12:OAc/*Z*5–10:OAc of 29/59/12, whereas, a Zimbabwean population has a ratio of 2/20/78. After conducting labeling studies, it was determined that chain-shortening enzymes could be affected to produce the alteration in pheromone ratios (Wu *et al*., 1998). These studies indicate that alteration in chain-shortening enzymes can have a major effect on pheromone blends.

**3.14.4.3.3. Desaturases** Desaturases introduce a double bond into the fatty acid chain. A variety of desaturases have been described that are involved in the biosynthesis of female moth sex pheromones. The desaturases identified so far include enzymes that act on saturated and monounsaturated substrates. These include Δ5 (Foster and Roelofs, 1996), Δ9 (Löfstedt and Bengtsson, 1988; Martinez *et al*., 1990), Δ10 (Foster and Roelofs, 1988), Δ11 (Bjostad and Roelofs, 1981, 1983), and Δ14 (Zhao *et al*., 1990) desaturases that utilize saturated substrates. The combination of these desaturases along with chain shortening can account for the majority of double bond positions in the various chain-length monounsaturated pheromones so far identified (Roelofs and Wolf, 1988). **Figure 5** illustrates the large number of monounsaturated compounds that can be generated through a combination of desaturation and chain shortening. Addition of various functional groups, acetate esters, alcohols, and aldehydes, increases the potential number of pheromone components. Notice that some of the intermediate compounds could be produced in two different ways. Therefore, although the desaturation and chain-shortening steps occur in a wide variety of moths, the order in which they occur and the type of desaturase must still be determined experimentally.

Some pheromone components are dienes and these can be produced by either the action of two desaturases or one desaturase and isomerization around the double bond. Some dienes with a 6,9-double bond configuration are produced using linoleic acid. Desaturases that utilize monounsaturated acyl-CoA substrates include Δ5 (Ono *et al*., 2002), Δ9 (Martinez *et al*., 1990), Δ11 (Foster and Roelofs, 1990), Δ12 (Jurenka, 1997), and Δ13

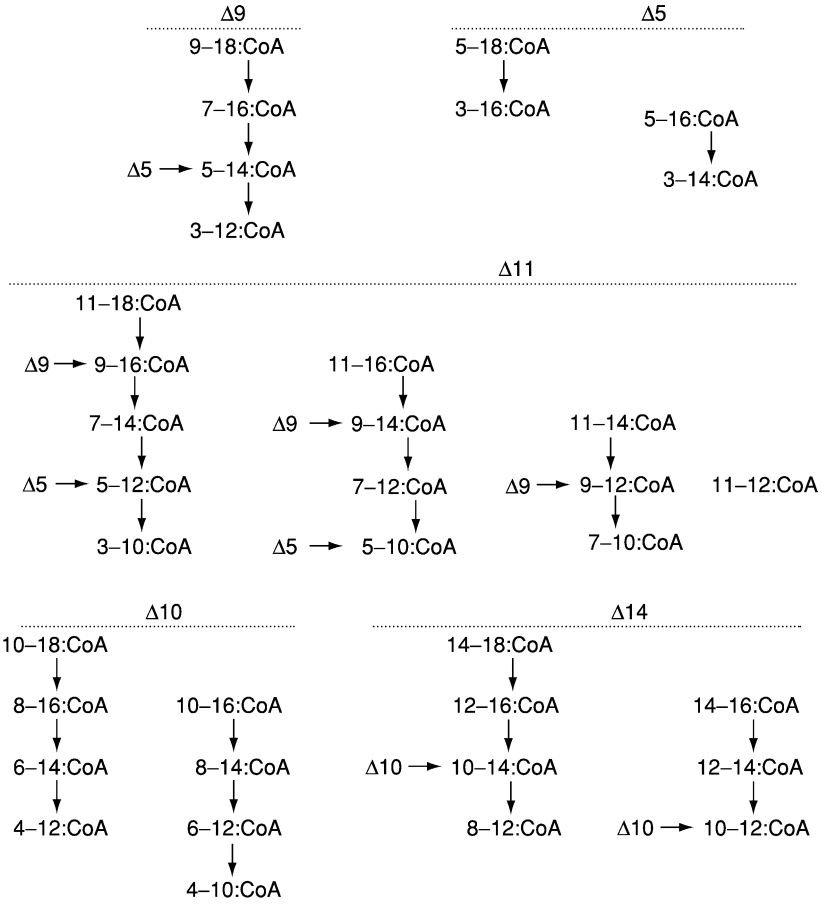


**Figure 5** Combination of desaturation and chain shortening can produce a variety of monounsaturated acyl-CoA precursors that can be modified to form acetate esters, aldehydes, and alcohols. The number followed by the Δ sign indicates a desaturase that introduces a double bond into the first indicated chain length acyl-CoA. The arrow pointing down indicates limited chain-shortening by two carbons. The arrow pointing to the right indicates that desaturation could produce the compound found within a chain-shortening pathway. This indicates that certain compounds could be produced in two different ways. Modification of all 16-, 14-, 12-, and 10-carbon acyl-CoA derivatives on the carbonyl carbon can account for the majority of monounsaturated acetate esters, aldehydes, and alcohols identified as sex pheromones. (Reprinted with permission from Jurenka, R.A., **2003**. Biochemistry of female moth sex pheromones. In: Blomquist, G.J., Vogt, R.G. (Eds.), Insect Pheromone Biochemistry and Molecular Biology. Elsevier, San Diego, CA, pp. 53–80; © Elsevier.)

(Arsequell *et al.*, 1990). These can act sequentially to produce the diene (Foster and Roelofs, 1990; Jurenka, 1997) or conjugated dienes could be produced by the action of one desaturase followed by isomerization (Ando *et al.*, 1988; Löfstedt and Bengtsson, 1988; Fang *et al.*, 1995a).

The biosynthesis of triene pheromone components has not been extensively investigated. Pheromones with a triene double bond system, that is *n*-3 (3,6,9-), are probably produced from linolenic acid (Millar, 2000). This was demonstrated in the salt-marsh caterpillar, *Estigmene acrea*, and the ruby tiger moth, *Phragmatobia fuliginosa* (Rule and Roelofs, 1989). Moths in the families Geometridae, Arctiidae, and Noctuidae apparently utilize linoleic and linolenic acid as precursors for their pheromones. Most of these pheromones are produced by chain elongation and decarboxylation to form hydrocarbons. Oxygen is added across one of the double bonds in the polyunsaturated hydrocarbon to produce an epoxide (Millar, 2000).

#### *3.14.4.3.3.1. Molecular biology of the desaturases*

More recent research has identified a variety of desaturases at the gene level with the first gene encoding a Δ11 desaturase being identified in the cabbage looper, *T. ni* (Knipple *et al.*, 1998). Since then a number of desaturase genes have been cloned and functionally expressed (Knipple and Roelofs, 2003). Expression in a strain of yeast lacking an endogenous desaturase was utilized to demonstrate functionality. This strain of yeast will not grow in a media lacking added unsaturated fatty acids but will grow if a functional desaturase is

inserted into the yeast genome. After growth, the fatty acids are analyzed to determine double bond positions and thus the desaturase can be characterized regarding double bond insertion and chain-length specificity.

Desaturase encoding cDNAs from pheromone glands that produce *Z*9, *Z*10, *Z*11, and *E*11 double bonds in 14- and 16-carbon acids have been identified so far. The *Z*9-desaturase is comparable to the metabolic desaturase found in the fat body. A *Z*10-desaturase was characterized from *Planotortrix octo* that produces Z10–16:acid (Hao *et al*., 2002), which would be chain shortened to produce the precursor to the pheromone *Z*8–14:OAc (Foster and Roelofs, 1988). Several Δ11-desaturases have been characterized including the ones from *T. ni* (Knipple *et al*., 1998) and *H. zea* (Rosenfield *et al*., 2001) that produce primarily *Z*11–16:acid. A single Δ11-desaturase was characterized from *A. velutinana* that produces both *Z*11– and *E*11–14:acid (Liu *et al*., 2002a). This desaturase is unique in that it produces both isomers and uses 14:acid as a substrate. Another unique Δ11-desaturase was identified from *Epiphyas postvittana* that not only produced *E*11–14:acid and *E*11–16:acid from saturated precursors, but also *E*9,*E*11–14:acid from a *E*9–14:acid precursor (produced by chain shortening *E*11–16:acid) (Liu *et al*., 2002b). Identification of desaturases in *Ostrinia nubilalis* and *O. furnicalis* produced the surprise finding that Δ11- and Δ14-desaturases are found in both moths (Roelofs *et al*., 2002). The Δ11-desaturase produced both *Z*11– and *E*11–14:acid and the Δ14-desaturase produced both *Z*14– and *E*14–16:acids. This finding has implications regarding the evolution of pheromone blends in moths (Baker, 2002).

**3.14.4.3.4. Specific enzymes to produce functional group on carbonyl carbon** Once a specific chain-length pheromone intermediate that has the appropriate double bonds is produced, the carbonyl carbon is modified to form a functional group. The majority of oxygenated pheromone components are acetate esters (or other esters), alcohols, and aldehydes. Production of these components requires the reduction of a fatty-acyl precursor to an alcohol which is a two-step reaction requiring a fatty acid reductase and an aldehyde reductase (Morse and Meighen, 1987b). Thus, alcohol formation goes through an aldehyde intermediate. Therefore, aldehydes could be produced by direct reduction of fatty acids. Another route for aldehyde formation is oxidation of alcohols. A cuticular oxidase has been characterized from pheromone glands of *H. zea* and *Manduca sexta* that produce aldehydes as pheromones (Teal and Tumlinson, 1988; Fang *et al*., 1995b). In those insects that utilize both an alcohol and an aldehyde as part of their pheromone, it is unclear how the production of both components occurs.

Production of acetate ester pheromone components utilizes an enzyme called acetyl-CoA:fatty alcohol acetyltransferase that converts a fatty alcohol to an acetate ester (Morse and Meighen, 1987a). Therefore, alcohols could be utilized as substrates for both aldehyde and acetate ester formation. Morse and Meighen (1987a) first demonstrated its presence in the spruce budworm, *Choristoneura fumiferana*, where it is involved in producing the acetate ester that serves as a precursor to the aldehyde pheromone (Morse and Meighen, 1987b). In some other tortricids, *A. velutinana*, *C. rosaceana*, and *Platynota idaeusalis,* an *in vitro* enzyme assay was utilized to demonstrate specificity of the acetyltransferase for the *Z* isomer of 11–14:OH (Jurenka and Roelofs, 1989). This specificity contributes to the final ratio of pheromone components. These results indicate that the family Tortricidae has members that have an acetyltransferase that is specific for the *Z* isomer of monounsaturated fatty alcohols. In contrast, several studies have shown no substrate preference for the acetyltransferase in other moths (Bestmann *et al*., 1987; Teal and Tumlinson, 1987; Jurenka and Roelofs, 1989). Therefore, this unique acetyltransferase apparently evolved within the Tortricidae.

**3.14.4.3.5. Production of specific pheromone blends** Most female moths utilize a blend of components produced in a specific ratio for pheromone attraction of conspecific males. A major question is how these species-specific ratios of components are produced. Research from several sources indicates that these ratios are produced by the inherent specificity of certain enzymes present in the biosynthetic pathways. The combination of these enzymes acting in concert produces the species-specific pheromone blend. Several examples will be utilized to illustrate this point.

The cabbage looper, *T. ni* uses *Z*7–12:OAc as the major sex pheromone component, which is produced by Δ11-desaturation of 16:CoA followed by two rounds of chain shortening, reduction, and acetylation. The Δ11-desaturase has been characterized (Wolf and Roelofs, 1986) and the gene isolated (Knipple *et al*., 1998). These studies indicate that 16:CoA and 18:CoA are substrates with 16:CoA as the preferred substrate and indeed *Z*11–16:acid is the most abundant monounsaturated fatty acid in pheromone glands (Bjostad *et al*., 1984). The next

step in the pathway is limited chain shortening and it was shown that *Z*11–16:CoA is the preferred substrate for these enzymes (Jurenka *et al*., 1994). After chain shortening the 14-carbon and 12-carbon intermediates are reduced to an alcohol and acetylated. The acetyltransferase enzyme is not specific and will accept a variety of substrates (Jurenka and Roelofs, 1989). From these observations, it can be inferred that the final ratio of pheromone components is produced by the specificity found within the Δ11-desaturase and chain-shortening enzymes.

A blend of seven acetate esters is used by *A. velutinana* that are produced in a biosynthetic pathway similar to the one just described, except that the Δ11-desaturase starts with 14:CoA as the substrate and produces both *Z* and *E* isomers of 11–14:CoA in about a 60/40 ratio (Wolf and Roelofs, 1987). Recent cloning of the Δ11-desaturase from redbanded leafroller (RBLR) females indicates that the expressed enzyme produces a ratio of *Z*/*E* of about 6/1 (Liu *et al*., 2002a). However, the final ratio of *Z*11– to *E*11–14:OAc is 92/8 (Miller and Roelofs, 1980). A selective increase in the *Z* isomer occurs within the biosynthetic pathway and it was determined that acetyl:CoA fatty alcohol acetyltransferase shows specificity for the *Z* isomer (Jurenka and Roelofs, 1989). Therefore, selective acetylation of *Z*11–14:OH and production of >60% *Z*11–14:CoA indicate that these enzymes have the inherent specificity to produce the 92/8 ratio of the major pheromone components *Z*11– and *E*11–14:OAc. Two minor pheromone components are produced by chain shortening *Z*11– and *E*11–14:OAc. The ratio of *Z*9– to *E*9–12:OAc is about 1 to 2. This indicates that the chain-shortening enzymes may prefer *E*11–14:CoA or that very little *Z*11–14:CoA is available to chain shorten. This combined information indicates that in *A. velutinana* pheromone glands, the final ratio of pheromone components can be produced through the concerted action of a Δ11-desaturase that produces at least a 60/40 ratio of *Z*/*E* intermediate isomers. The final ratio of acetate esters (92/8) is produced through the specificity for the *Z* isomer by the acetyltransferase. The minor components are produced by specificity in chain shortening.

Another insect that utilizes specific ratios of *Z*11– and *E*11–14:OAc is the European corn borer, *O. nubilalis*. Two strains are known in which one produces a ratio of *Z*/*E* of about 97/3 (*Z* strain) and the other produces an opposite ratio of *Z*/*E* of about 1/99 (*E* strain). Hybridization studies between the two strains indicated that offspring have an acetate ester ratio of *Z*/*E* of about 30/70 (Klun and Maini, 1982). The Δ11-desaturase from both strains produced a product with about 30/70 *Z*/*E* in an *in vitro* enzyme assay (Wolf and Roelofs, 1987). These results indicate that the final ratio of acetate ester isomers is produced after the desaturation step. The enzymes that follow the desaturase are a reductase to make an alcohol and an acetyltransferase to produce the acetate esters. Two studies have shown that the acetyltransferase is similar between the two strains (Jurenka and Roelofs, 1989; Zhu *et al*., 1996). However, labeled acids applied to glands *in vivo* were selectively incorporated into the correct pheromone ratio indicating that the reductase shows specificity (Zhu *et al*., 1996). Therefore, the final pheromone ratios produced by females of the European corn borer are made through the action of a Δ11-desaturase that can produce both *Z* and *E* isomers. The final acetate ester ratio is strain dependent and is produced through the specificity found in the reductase system.

The above three examples illustrate how a species-specific pheromone blend is produced by the concerted action of desaturases, chain-shortening enzymes, and a reductase and an acetyltransferase. The specificity inherent in certain enzymes in the pathway produces the final blend of pheromone components.

**3.14.4.3.6. Hydrocarbon pheromones** Moths in the families Geometridae, Arctiidae, Amatidae, Lymantriidae, Lyonetiidae, and some Noctuidae utilize hydrocarbons or epoxides of hydrocarbons as their sex pheromones. Biosynthesis of hydrocarbons occurs in oenocyte cells that are associated with either epidermal cells or fat body cells (Romer, 1991). Once the hydrocarbons are biosynthesized they are transported to the sex pheromone gland by lipophorin (Schal *et al*., 1998a). When the transport of hydrocarbon sex pheromones in arctiid moths was investigated in detail by Schal *et al*. (1998a), it was found that a very specific uptake was occurring at pheromone glands. Lipophorin was shown to contain both the sex pheromone and cuticular hydrocarbons; however, only the pheromone gland had the sex pheromone. Other studies have shown similar pathways in other moths (Jurenka and Subchev, 2000; Subchev and Jurenka, 2001; Wei *et al*., 2003).

Most moth sex pheromones that are straight chain hydrocarbons also usually have an odd number of carbons. Most of these are polyunsaturated with double bonds in the 3,6,9- or 6,9-positions, indicating that they are derived from linolenic or linoleic acid, respectively (Rule and Roelofs, 1989;

Millar, 2000). Linolenic and linoleic acid cannot be biosynthesized by moths so they must be obtained from the diet (Stanley-Samuelson *et al.*, 1988). A few even-chain-length hydrocarbon sex pheromones have been identified that also have 3,6,9- or 6,9-double bond configurations (Millar, 2000), indicating that they too are derived from linolenic or linoleic acids; however, it is not known how these even-chain hydrocarbons are formed.

A major class of sex pheromones that are derived from hydrocarbons are the polyene monoepoxides (Millar, 2000). These usually have double bonds in the 3,6,9-positions or 6,9-positions, again indicating that they are biosynthesized from linolenic or linoleic acids, respectively. Although the production of hydrocarbon occurs in oenocytes the epoxidation step takes place in the pheromone gland. This has been demonstrated in several studies utilizing deuterium labeled precursors. In a study on the Japanese giant looper, *Ascotis selenaria cretacea*, that uses 6,9–19:3,4Epox as a sex pheromone component, deuterium-labeled hydrocarbon precursor, D3–3,6,9–19:Hc, was topically applied to pheromone glands and found to be converted to the epoxide, indicating that epoxidation takes place in pheromone glands (Miyamoto *et al.*, 1999). By using a variety of polyene precursors, it was also determined that the monooxygenase regiospecifically attacked the *n*-3 double bond regardless of chain length or degree of unsaturation. This indicates that the epoxidation enzyme is regiospecific in this insect (Miyamoto *et al.*, 1999).

A study using the gypsy moth, *L. dispar*, illustrates the overall pathways involved in production of epoxide pheromone components (**Figure 6**) (Jurenka *et al.*, 2003). This insect uses disparlure, 2me-18:7,8Epox, as a pheromone component. Incubation of isolated abdominal epidermal tissue with deuterium-labeled valine resulted in incorporation into 2me-*Z*7–18:Hc. This indicates that the oenocyte cells associated with the epidermal tissues biosynthesize 2me-*Z*7–18:Hc using the carbons of valine to initiate the chain. The double bond is probably introduced by a Δ12-desaturase as determined by using specific deuterium-labeled intermediates. Hemolymph transport of 2me-*Z*7–18:Hc is indicated by the finding of this alkene in the hemolymph (Jurenka and Subchev, 2000). Demonstration that 2me-*Z*7–18:Hc is converted to the epoxide in the pheromone gland was shown by using deuterium-labeled 2me-*Z*7–18:Hc and incubation with isolated pheromone glands. Disparlure is a stereoisomer that has the 7*R*,8*S* or (+) configuration and chiral chromatography indicated that only the (+)-isomer was produced by pheromone glands (Jurenka *et al.*, 2003). These results indicate that hydrocarbon pheromones and their epoxides are produced through a pathway outlined in **Figure 6**.

### 3.14.4.4. Pheromone Biosynthesis in Beetles

Pheromone biosynthesis in the Coleoptera is as diverse as the taxa and the pheromone structures, and the utilization of several types of pheromone biosynthetic pathways has been demonstrated (Vanderwel and Oehlschlager, 1987; Vanderwel, 1994; Seybold and Vanderwel, 2003; Tittiger, 2003). Extensive work has been done on the biosynthesis of coleopteran pheromones, and the major

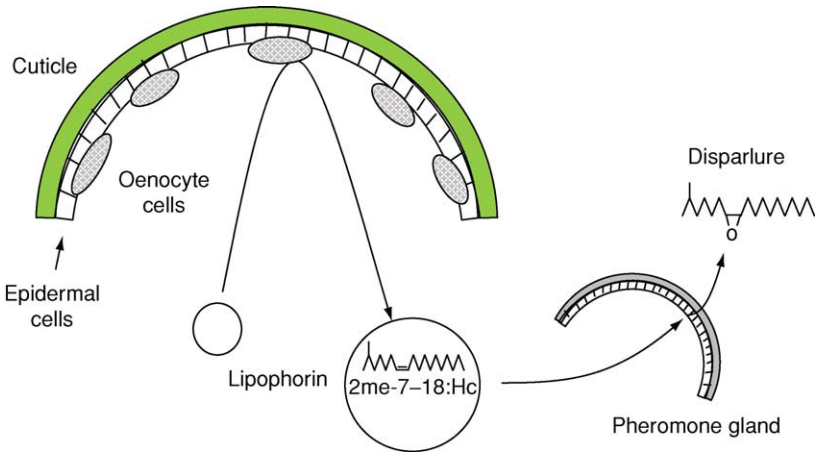


**Figure 6** Production of the sex pheromone in the gypsy moth, *Lymantria dispar*. The oenocyte cells located in the abdomen biosynthesize the alkene hydrocarbon precursor to the pheromone, 2me-*Z*7–18:Hc. It is transported through the hemolymph by lipophorin. The alkene is taken up by pheromone gland cells where it is acted upon by an epoxidase to produce the pheromone disparlure, 2me-18:7,8Epox. (Reprinted with permission from Jurenka, R.A., **2003**. Biochemistry of female moth sex pheromones. In: Blomquist, G.J., Vogt, R.G. (Eds.), Insect Pheromone Biochemistry and Molecular Biology. Elsevier, San Diego, CA, pp. 53–80; © Elsevier.)

systems that have been investigated are described below. Beetles can generate pheromone either by modification of dietary host compounds or *de novo* biosynthesis, with the latter accounting for the majority of beetle pheromone components.

**3.14.4.4.1. Isoprenoid pheromones from bark beetles**
Most of the knowledge about beetle pheromone biosynthesis and endocrine regulation (see Section 3.14.4.5.2) comes from studies of various bark beetles, especially *Ips* and *Dendroctonus* species (Scolytidae). Some bark beetles may modify fatty acyl or amino acid precursors (Vanderwel and Oehlschlager, 1987; Birgersson *et al*., 1990); however, the majority of pheromone components are isoprenoid (Schlyter and Birgarson, 1999: Seybold *et al*., 2000).

Understanding of the origin of bark beetle pheromone components has undergone a paradigm shift in the last decade. Until recently, it was widely accepted that bark beetles, in contrast to most other insects (Tillman *et al*., 1999), obtained their pheromone components by simple modification of of host tree dietary precursors (reviews: Bordon, 1985; Vanderwel and Oelschlager, 1987; Vanderwel, 1994). This model was based on various studies that showed the association of monoterpene synthesis with conifers (Croteau, 1981), the abundance of potential precursors in the host tree (Gershenzon and Croteau, 1991), the demonstration that monoterpenoid precursors such as myrcene and α-pinene could be converted to the pheromone components ipsdienol, ipsenol, and *cis* and *trans* verbenol (Hughes, 1974; Renwick *et al*., 1976a, 1976b; Byers, 1981), and the conclusive demonstration that deuterated myrcene was converted to ipsenol and ipsdienol in *I. paraconfusus* (Hendry *et al*., 1980).

In the past decade and a half, however, significant evidence has emerged supporting the *de novo* biosynthesis of most bark beetle pheromone components. First, doubts were raised about the role of myrcene in ipsdienol and ipsenol production when it was noted that myrcene may not be present in sufficient quantity in some host trees to account for the amount of pheromone that was produced (Byers, 1981; Byers and Birgersson, 1990). Second, *Ips* beetles treated with the HMG-R reductase inhibitor compactin show a marked decrease in ipsdienol production (Ivarsson *et al*., 1993). Third, myrcene-treated male *I. pini* produce ipsdienol with a racemic enantiomeric composition whereas JH III-treated males produce ipsdienol with a 87–96% (−) enantiomeric composition (Lu, Blomquist, and Seybold, unpublished data). The naturally occurring enantiomeric composition of ipsdienol from California *I. pini* is 95–98% (−) (Seybold *et al*., 1995a). Fourth, JH III-treated male *I. pini* incorporate labeled acetate and mevalonate into ipsdienol (Seybold *et al*., 1995b; Tillman *et al*., 1998). 2-Methyl-3-buten-2-ol is similarly synthesized *de novo* in *I. typographus* (Lanne *et al*., 1989). More recently, key genes in pheromone production, including HMG-R and HMG-CoA synthase (HMG-S) have expression patterns consistent with their roles in regulating *de novo* isoprenoid pheromone biosynthesis (Tittiger *et al*., 1999; Tillman, Blomquist, and Seybold, unpublished data). A geranyl diphosphate synthase (GPPS) cDNA from *I. pini* was also isolated, functionally expressed, and modeled (Gilg-Young, Welch, Tittiger, and Blomquist, unpublished results). The existence of this novel enzyme argues strongly for the evolution of *de novo* pheromone biosynthetic capacity in bark beetles. Taken together, the data emerging from *Ips* species overwhelmingly indicate the *de novo* production of monoterpenoid pheromone components.

These data are supported by studies in other beetles that similarly prove or imply *de novo* pheromone component biosynthesis. Radiotracer studies demonstrated the *de novo* biosynthesis of frontalin in a number of *Dendroctonus* species (Barkawi *et al*., 2003). Expression patterns of HMG-R and HMG-S in *D. jeffreyi* correlate tightly with frontalin production (Tittiger *et al*., 2000, 2003). The four monterpene alcohols and aldehydes in the boll weevil, *A. grandis*, are also synthesized from mevalonate and acetate (Mitlin and Hedin, 1974; Thompson and Mitlin, 1979).

The capacity for *de novo* biosynthesis does not preclude the conversion of host precursors to pheromone components. For example, the incorporation of acetate and mevalonolactone into ipsdienol and ipsenol may proceed through the conversion of geranyl diphosphate to myrcene, which could be directly hydroxylated to ipsdienol and *E*-myrcenol, and indirectly, perhaps through a ketone intermediate, to ipsenol (Martin *et al*., 2003). In this scheme, host myrcene ingested during feeding would enter the *de novo* pathway downstream of geranyl diphosphate. Similarly, cotton plant monoterpenes (myrcene and limonene) could enter a *de novo* biosynthetic pathway to grandlure in *A. grandis*. The question then arises as to whether *de novo* biosynthesis or host precursor conversion is the preferred route to pheromone production. Male *I. pini* exposed to myrcene vapors produce a racemic mixture of ipsdienol, whereas the naturally occurring pheromone of Western *I. pini* is about 95 : 5 (−)/(+) ratio (Lu, Blomquist, and Seybold, unpublished data). This suggests that myrcene is not the

direct precursor to ipsdienol, but that its hydroxylation is a true detoxification reaction. Perhaps a highly specific hydroxylase may synthesize ipsdienol *de novo*, while a hydroxylase with a different specificity detoxifies myrcene. Other possibilities also exist, particularly considering that ipsdienone appears to be a precursor to ipsdienol (Ivarsson *et al.*, 1997). To make ipsdienol, it is possible that geranyl diphosphate is hydroxylated and then dephosphorylated, bypassing myrcene as an intermediate. Thus, though the final biosynthetic steps remain uncharacterized, *de novo* biosynthesis is clearly the most important route to ipsdienol and ipsenol in *I. pini* and *I. paraconfusus*. Further examples should arise as other species are studied.

A proposed biosynthetic scheme for a number of coleopteran isoprenoid pheromone components is presented in **Figure 7**. Hemiterpene pheromone components of the bark beetles are similarly synthesized *de novo*. Lanne *et al.* (1989) demonstrated the incorporation of labeled acetate, glucose, and mevalonate into 2-methyl-3-buten-2-ol in *I. typographus*. This also argues for the *de novo* synthesis of 3-methyl-3-buten-1-ol and 3-methyl-2-buten-1-ol.

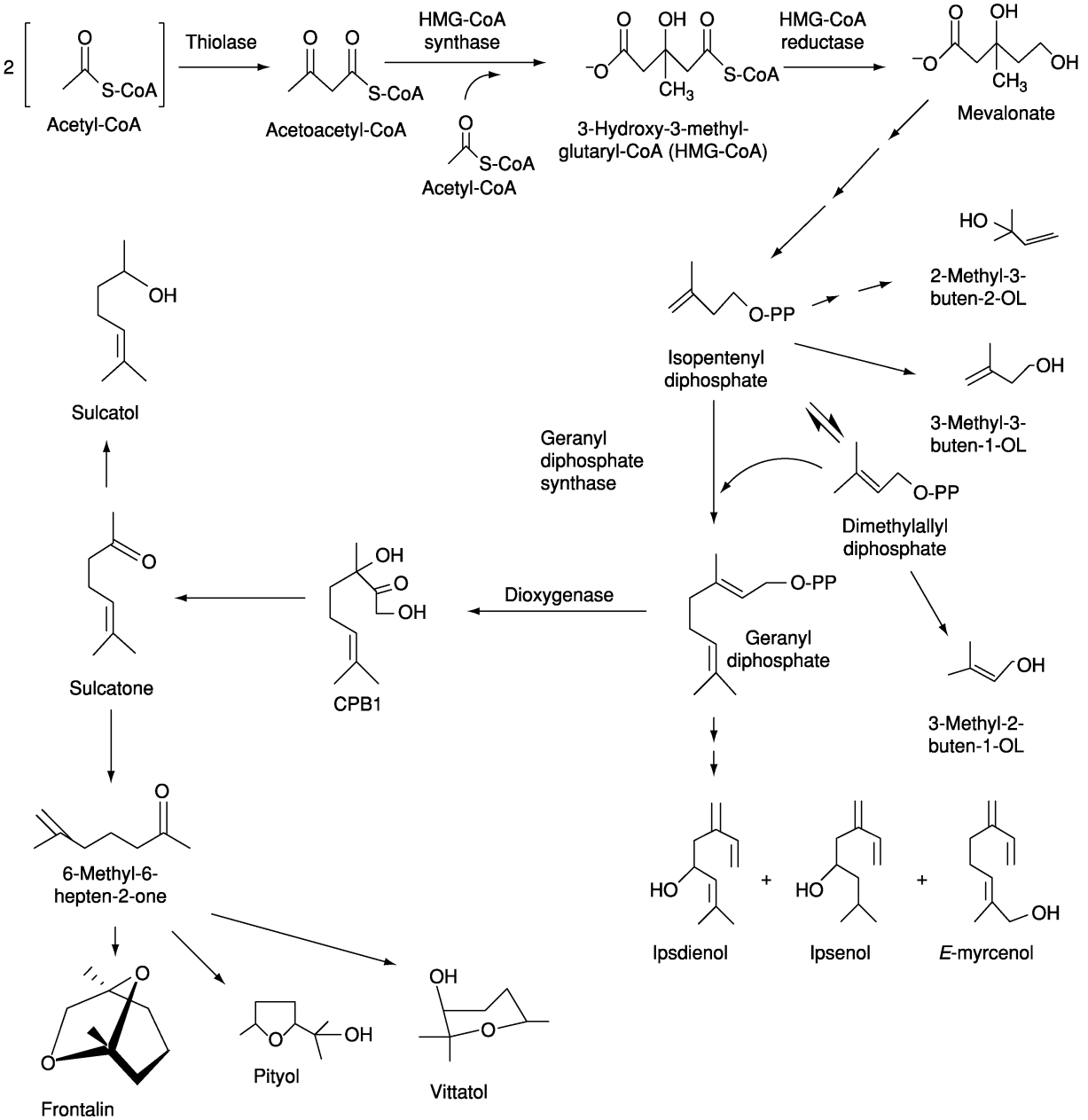


**Figure 7** Proposed biosynthetic pathways for a number of hemi- and monoterpenoid pheromone components in the Coleoptera.

The mevalonate pathway intermediate, dimethylallyl diphosphate, likely provides the carbon skeleton for 3-methyl-2-buten-1-ol by dephosphorylation. The other five-carbon intermediate, isopentenyl diphosphate, could be directly converted to 3-methyl-3-buten-1-ol, and perhaps through several steps, to 2-methyl-3-buten-2-ol.

Ipsdienol and ipsenol production from geranyl diphosphate appears to involve relatively straightforward though not yet fully characterized biochemical modifications. The production of frontalin, and the recently identified male-produced pheromone of the Colorado potato beetle, *Leptinotarsa decemlineata* (*S*)-3,7-dimethyl-2-oxo-oct-6-ene-1,3-diol (CPB1) (Dickens *et al*., 2002), is less clear. Barkawi *et al*. (2003) demonstrated that acetate and mevalonolactone are precursors to frontalin, proving the isoprenoid origin of this common *Dendroctonus* pheromone component. he carbon skeleton of frontalin probably arises from geranyl disphosphate via a putative dioxygenase, which converts geranyl diphosphate to sulcatone. Sulcatone is an obvious precursor to sulcatol, which is a pheromone component of some bark beetles (*Gnathotrichus sulcatus*) (Byrne *et al*., 1974). In *Dendroctonus* spp., geranyl diphosphate-derived sulcatone may be converted to 6-methyl-6-hepten-2-one (6-MHO), a known intermediate to frontalin (Perez *et al*., 1996). Alternative cyclizations of 6-MHO in other beetles could lead to the pheromone components pityol and vittatol. Significant amounts of sulcatone are also found in Colorado potato beetle males during their synthesis of CPB1 (Dickens *et al*., 2002). It is easy to envision CPB1 as an intermediate in the dioxygenase reaction (**Figure 7**).

The picture emerging from these studies is that isoprenoid pheromone component production in beetles is mostly *de novo*, with carbon being diverted from the mevalonate pathway at geranyl diphosphate. Geranyl diphosphate may be directly modified through dephosphorylation and cyclizations (cotton boll weevil) or hydroxylations (*Ips* spp.). Alternatively, some bark beetles, such as *Dendroctonus* spp., apparently have a dioxygenase that oxidizes geranyl diphosphate or geraniol to produce a ketodiol (CPB1) or sulcatone, which acts as a precursor to some pheromone components such as sulcatol, pityol, and frontalin.

**3.14.4.4.2. Fatty acid-derived pheromones** Numerous beetle genera use modified fatty-acyl compounds as pheromone components. Less is known about their biosynthesis compared to that of isoprenoid pheromones, but the same general strategy of modifying or combining existing biosynthetic pathways is conserved.

For some beetles, the modifications are relatively minor. For example, *Attagenus* spp. (Dermestidae) myristic acid may be desaturated at the Δ5 and Δ7 positions to produce tetradecadienoic acid pheromone components. The stereochemistries of the double bonds apparently provide specificity between species (Fukui *et al*., 1977). It is unclear whether the short-chain fatty acid precursors to these pheromone components are synthesized through normal fatty acid elongations, or are the β-oxidation products of longer fatty acids. For other beetles, modifications can become more complex. Female *Tenebrio molitor* produce 4-methyl-1-nonanol from propionyl-, malonyl-, and methylmalonyl-precursors (Islam *et al*., 1999). This is an example of carbon being shunted away from fatty-acyl elongation before long fatty acids are completed. The use of methylmalonate to produce methyl-branched hydrocarbons is well established in other insect systems (Blomquist, 2003; Schal *et al*., 2003), though it is unknown if beetles have a secondary fatty acyl synthase which, similar to that in houseflies, incorporates methylmalonyl-CoA precursors efficiently.

The flexibility of the fatty acid biosynthetic pathway is extended in some nitidulid beetles (*Carpophilus* spp.), where males use propionate and butyrate (presumably as methylmalonate and ethylmalonate) to make methyl- and ethyl-branched triene and tetraene pheromone components (**Figure 1**), apparently also via the fatty acid biosynthetic pathway (Bartelt *et al*., 1992). The branched hydrocarbons generally have 10–12 carbon backbones with conjugated double bonds. In contrast to other systems, where pheromone component biosynthesis is highly specific, *Carpophilus* spp. males produce a mixture of related structures, some of which act as pheromones and some of which do not. Since di-substituted tetraenes are less abundant than mono- or unsubstituted tetraenes, it appears that nonacyl units placed in the growing hydrocarbon chains represent "mistakes" made by a synthesis machinery with a low stringency for substrate selection (Bartelt, 1999). Such nonspecific hydrocarbon biosynthesis may serve speciation, since changes in antennal receptivity may accommodate preexisting compounds (Bartelt, 1999). Interestingly, the desaturated nature of these hydrocarbons is not due to fatty acyl desaturases, but due to the inactivity of enoyl-ACP reductase during biosynthesis so that the enoyl-ACP intermediate formed during elongation is not reduced (Petroski *et al*., 1994). This

suggests that carbon is shunted out of the fatty acid biosynthetic pathway when the chains are of the correct length, similar to the situation in *T. molitor*.

Rather than modifying the normal biosynthetic pathway to produce pheromone components, some beetles modify normal products of the pathway. For example, lactone pheromone components of some scarab beetles are produced by the stereospecific alterations of long chain fatty acids. Female *Anomala japonica* (Scarabaeidae) are perhaps best studied among scarab beetles for the biosynthesis of japonilure and buibuilactone, which involves the successive Δ9 desaturation, hydroxylation, two rounds of β-oxidation to shorten the chain length, and cyclization of stearic and palmitic acids (Leal *et al*., 1999). Of all these, only the hydroxylation step appears to be stereospecific (Leal, 1998). This step is important because different enantiomers have different functions in different *Anomala* species (Leal *et al*., 1999).

**3.14.4.4.3. Host precursor modifications** While most bark beetle isoprenoid pheromones are clearly synthesized *de novo*, there is strong evidence that some pheromone components are indeed the result of modifying host precursor molecules. α-Pinene is produced naturally by pine trees, and can be hydroxylated to *cis*- and *trans*-verbenol (**Figure 1**) by *Ips* beetles (Renwick *et al*., 1976a). A further oxidation of verbenol yields verbenone in *Dendroctonus ponderosae* (Hunt and Bordon, 1989). Similarly, Jeffrey pine trees contain relatively low levels of monoterpenoids, but high levels of heptane. Female *Dendroctonus jeffreyi* that attack these trees produce 1-heptanol and 2-heptanol, and 1-heptanol acts as a sex pheromone (Paine *et al*., 1999).

### 3.14.4.5. Pheromone Biosynthesis in Diptera

***3.14.4.5.1.1. Housefly pheromone biosynthesis: C23 sex pheromone components*** A combination of *in vivo* and *in vitro* studies using both radio- and stable-isotope techniques established the biosynthetic pathways for the major sex pheromone components in the housefly (**Figure 8**) (Dillwith *et al*., 1981, 1982; Dillwith and Blomquist, 1982; Blomquist *et al*., 1984b; Vaz *et al*., 1988). (*Z*)-9-tricosene is formed by the microsomal elongation of 18 : 1-CoA to 24 : 1-CoA using malonyl-CoA and NADPH, and the elongated fatty acyl-CoA is then reduced to the aldehyde and converted to the hydrocarbon one carbon shorter (**Figure 8**) (Reed *et al*., 1994, 1995; Mpuru *et al*., 1996). A cytochrome P450 enzyme is involved in the metabolism of the alkene to the corresponding epoxide and ketone (Ahmad *et al*., 1987). It appears that the same enzyme that catalyzes formation of an epoxide from the double bond between carbons 9 and 10 of the alkene of (*Z*)-9-tricosene also hydroxylates it at position *n*-10. The secondary alcohol thus formed is then converted to the unsaturated ketone (Guo *et al*., 1991).

***3.14.4.5.1.2. Mechanism of hydrocarbon formation: decarboxylation?*** The mechanism of hydrocarbon formation has proven elusive. In an elegant set of experiments in the 1960s and early 1970s, Kolattukudy and coworkers demonstrated that fatty acyl-CoAs were elongated and then converted to hydrocarbon by the loss of the carboxyl group (reviews: Kolattukudy *et al*., 1976; Kolattukudy, 1980). In the 1980s and early 1990s, the hypothesis was put forward that very long chain fatty acyl-CoAs were reduced to aldehyde and then decarbonylated to hydrocarbon and carbon monoxide, and that this reaction did not require any cofactors or $O_2$. Evidence for this decarbonylation mechanism was obtained from a plant (Cheesbrough and Kolattukudy, 1984), an alga (Dennis and Kolattukudy, 1991, 1992), a vertebrate (Cheesbrough and Kolattukudy, 1988), and an insect (Yoder *et al*., 1992).

In the work on hydrocarbon formation in the housefly, it was found that the acyl-CoA is reduced to the aldehyde, with the conversion of the aldehyde to hydrocarbon requiring NADPH and molecular oxygen, and that the products were hydrocarbon and carbon dioxide (demonstrated by radio-gas–liquid chromatography) (Reed *et al*., 1994, 1995). Antibodies to housefly cytochrome P450 and to P450 reductase inhibited hydrocarbon formation in microsomes, as did exposure to CO, and the latter could be partially reversed by white light. GC-MS analyses of specifically deuterated substrates showed that the protons on positions 2,2 and 3,3 of the acyl-CoA were retained during conversion to hydrocarbon, and that the proton on position 1 of the aldehyde was transferred to the adjacent carbon and retained during hydrocarbon formation. Furthermore, several peroxides could substitute for $O_2$ and NADPH and support hydrocarbon production. All this evidence strongly supports a cytochrome P450 involvement in hydrocarbon synthesis (Reed *et al*., 1994).

The mechanism of hydrocarbon formation remains controversial, with evidence obtained favoring both a decarbonylation and a decarboxylation mechanism. The resolution of the problem awaits cloning, expressing, and assaying the enzymes involved.

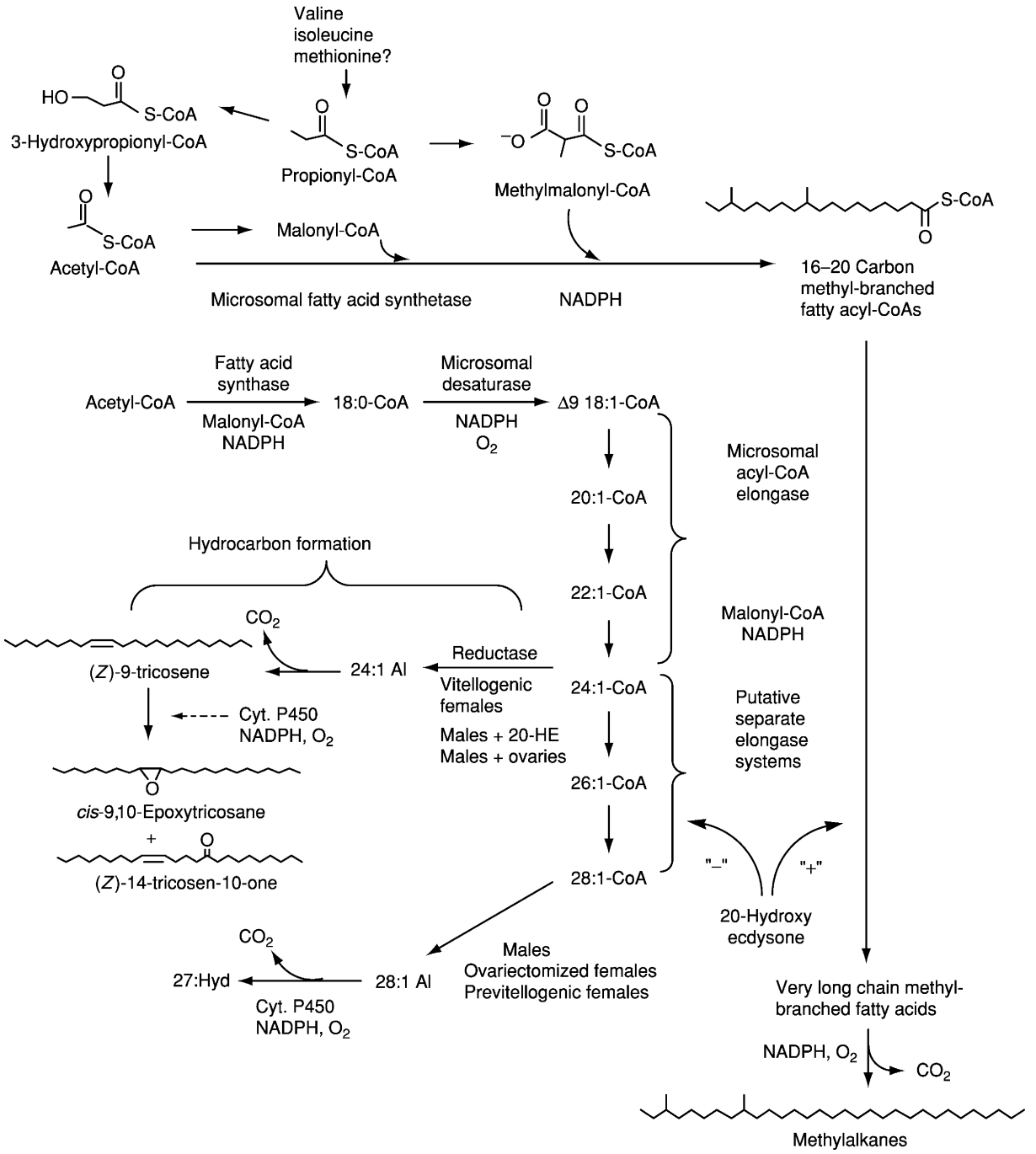


**Figure 8** Biosynthetic pathways showing the putative steps at which ecdysteroids regulate the hydrocarbon and hydrocarbon-derived pheromone components of the female housefly, *Musca domestica*. (Reprinted with permission from Blomquist, G.J., **2003**. Biosynthesis and ecdysteroid regulation of housefly sex pheromone production. In: Blomquist, G.J., Vogt, R.G. (Eds.), Insect Pheromone Biochemistry and Molecular Biology. Elsevier, New York, pp. 131–252; © Elsevier.)

*3.14.4.5.1.3. Biosynthesis of the methylalkane pheromone components* Methylalkanes are formed by the substitution of methylmalonyl-CoA in place of malonyl-CoA at specific points during chain elongation. Carbon-13 nuclear magnetic resonance (NMR), mass spectrometry, and radiochemical studies (Dwyer *et al.*, 1981; Dillwith *et al.*, 1982; Chase *et al.*, 1990) demonstrated that the methylmalonyl-CoA was added during the initial steps of chain elongation in insects using what appears to be a novel microsomal fatty acid synthase (FAS) (**Figure 8**). A microsomal FAS was

first suggested from studies on *T. ni* for the formation of methyl-branched very long chain alcohols (de Renobales *et al*., 1989). In this insect, high rates of methyl-branched very long chain alcohol synthesis were observed in the mid-pupal stages at times when soluble FAS activity was very low or undetectable. The FAS of most organisms is soluble (cytoplasmic).

A microsomal FAS was also implicated in the biosynthesis of methyl-branched fatty acids and methyl-branched hydrocarbon precursors of the German cockroach contact sex pheromone (Juarez *et al*., 1992; Gu *et al*., 1993). A microsomal FAS present in the epidermal tissues of the housefly is a likely candidate responsible for methyl-branched fatty acid production (Blomquist *et al*., 1994). The housefly microsomal and soluble FASs were purified to homogeneity (Gu *et al*., 1997) and the microsomal FAS was shown to preferentially use methylmalonyl-CoA in comparison to the soluble FAS. GC–MS analyses showed that the methyl-branching positions of the methyl-branched fatty acids of the housefly (Blomquist *et al*., 1994) were in positions consistent with them being the precursors of the methyl-branched hydrocarbons.

The methylmalonyl-CoA unit that is the precursor to methyl-branched fatty acids and hydrocarbons arises from the carbon skeletons of valine and isoleucine, but not succinate (Dillwith *et al*., 1982). Propionate is also a precursor to methylmalonyl-CoA, and in the course of these studies, a novel pathway for propionate metabolism in insects was discovered. Many insect species, including the housefly, do not contain vitamin $B_{12}$ (Wakayama *et al*., 1984), and therefore cannot catabolize propionate via methylmalonyl-CoA to succinate. Instead, as first demonstrated in the housefly (Dillwith *et al*., 1982), insects metabolize propionate to 3-hydroxypropionate and then to acetyl-CoA, with carbons 3 and 2 of propionate becoming carbons 1 and 2 of acetyl-CoA (**Figure 8**) (Halarnkar *et al*., 1986).

**3.14.4.5.2. Pheromone biosynthesis in *Drosophila***

The other major dipteran system in which pheromone production has been extensively studied is that of *Drosophila*. Jallon and Wicker-Thomas (2003) provide an excellent and detailed review of the chemistry, biochemistry, molecular biology, and genetics of pheromone production in *Drosophila*. *Drosophila*, as do *Musca*, use long chain, sex-specific hydrocarbons as close range and contact sex pheromones. *Drosophila melanogaster* Canton-S mature females have abundant (*Z*,*Z*)-7,11-heptacosadiene, which is absent on males and was shown to effectively stimulate wing vibration in conspecific males when applied to a dummy (Antony and Jallon, 1982; Ferveur and Sureau, 1996). In some *Drosophila*, 7-tricosene is involved in chemical communication. A series of studies established that the hydrocarbon pheromones are produced in the oenocytes (Ferveur *et al*., 1997), transported through the hemolymph via lipophorin (Pho *et al*., 1996), and then deposited on the cuticle.

A series of experiments assaying the incorporation of various precursors into hydrocarbons established that *Drosophila* use an elongation–decarboxylation pathway for hydrocarbon production (Pennanec'h *et al*., 1997). An interesting question in *Drosophila* pheromone production is the origin of the 7,11 double bonds. Biochemical evidence suggested that either a Δ7-desaturase working on myristate, or a Δ9-desaturase working on palmitate give rise to the double bond in the 7-position, which could then be elongated to produce vaccenate (*n*-7, 18 : 1). Vaccenate could then be elongated and decarboxylated to produce 7-tricosene, and, with an additional desaturation step, to produce 7,11-27:2Hyd.

Wicker-Thomas *et al*. (1997) isolated a cDNA encoding a desaturase (*desat1*) in *D. melanogaster*. The expressed *desat1* protein is a Δ9-desaturase that preferentially used palmitate, resulting in *n*-7 fatty acids. A *desat2*, located close to *desat1*, appears to be responsible for the 5,9-dienes present in Tai females (Jallon and Wicker-Thomas, 2003). The identification of the desaturase genes responsible for pheromone production in fruit flies will greatly benefit from the characterization of the *D. melanogaster* genome. Understanding of the genetics combined with molecular biology undoubtedly will result in a more complete understanding of the mechanisms and genes involved and regulated in pheromone production in *Drosophila*.

### 3.14.4.6. Biosynthesis of Contact Pheromones in the German Cockroach

Upon antennal contact with a female, the male German cockroach rotates his body 180° and raises his wings, thus exposing specialized tergal glands that attract the female and place her into a precopulatory position (Nojima *et al*., 1999). The nonvolatile contact pheromone responsible for this behavior was identified as (3*S*,11*S*)-dimethylnonacosan-2-one (**Figure 1**) (Nishida *et al*., 1974), and an alcohol (29-hydroxy-3*S*,11*S*-dimethylnonacosan-2-one) and an aldehyde (29-oxo-3,11-dimethylnonacosan-2-one) derivatives with the same 3,11-dimethylketone skeleton (Nishida and Fukami, 1983). A fourth pheromone component, 3,11-dimethylheptacosan-2-one, is less active than its $C_{29}$ homolog (Schal *et al*., 1990b).

The route of biosynthesis and its physiological regulation have been reviewed previously (Blomquist *et al*., 1993; Tillman *et al*., 1999; Schal *et al*., 2003). Central to investigations of the biosynthetic pathway was the observation that the major cuticular hydrocarbon in all life stages of the German cockroach 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 propose that production of the pheromone might result from the sex-specific oxidation of its hydrocarbon analog only in adult females. This scheme follows the well-established conversion of hydrocarbons to methyl ketone and epoxide pheromones in the housefly (see above) (Blomquist *et al*., 1984b; 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 9**). Further, propionate and succinate labeled methyl-branched 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*., 1986), American cockroach (Halarnkar *et al*., 1985), 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, [$^{14}C_1$]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) investigated the hypothesis that the 3,11-dimethyl ketone sex pheromone arises from the insertion of an oxygen into the preformed 3,11-dimethyl alkane. When high-specific activity, tritiated 3,11-dimethylnonacosane (mixture of stereoisomers), 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. Likewise, 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 nonsex-specific oxidation, probably involving a polysubstrate monooxygenase system, to the (3*S*,11*S*)-dimethylnonacosan-2-one pheromone (**Figure 9**). 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 3,11-dimethylheptacosane to the corresponding methyl ketone pheromone, and perhaps its 27-hydroxy- and 27-oxo- analogs.

The contact sex pheromone of female *B. germanica* remains the only cockroach pheromone whose biosynthetic pathway has been investigated with radio- and stable-isotope tracers.

#### 3.14.4.7. Biosythesis of the Honeybee Queen Pheromone

The queen substance used for "queen control" inside the nest is also the substance used by virgin queens to attract drones for mating. It is the best understood of the sexual pheromones of the social insects. Callow and Johnston (1960) and Barbier and Lederer (1960) identified ([*E*]-9-oxodec-2-enoic acid) (9-ODA) in queen mandibular glands. 9-Hydroxy-2(*E*)-decenoic acid (9-HDA) is also present (Callow *et al*., 1964) and together both attract drones. Recent work (Keeling *et al*., 2003) identified a number of additional compounds that function synergistically with the 9-ODA and 9-HDA, making this the most complex pheromone blend known for any organism.

In an elegant set of experiments, Plettner *et al*. (1996, 1998) elucidated the biosynthetic pathways for the honeybee queen mandibular pheromone (QMP) components 9-ODA and 9-HDA and compared their biosyntheses to that of worker produced 10-hydroxy-2(*E*)-decenoic acid and the corresponding diacid. Using carbon-13 and deuterated precursors, Plettner *et al*. (1996, 1998) demonstrated (1) the *de novo* synthesis of stearic acid in worker mandibular glands, (2) the hydroxylation of

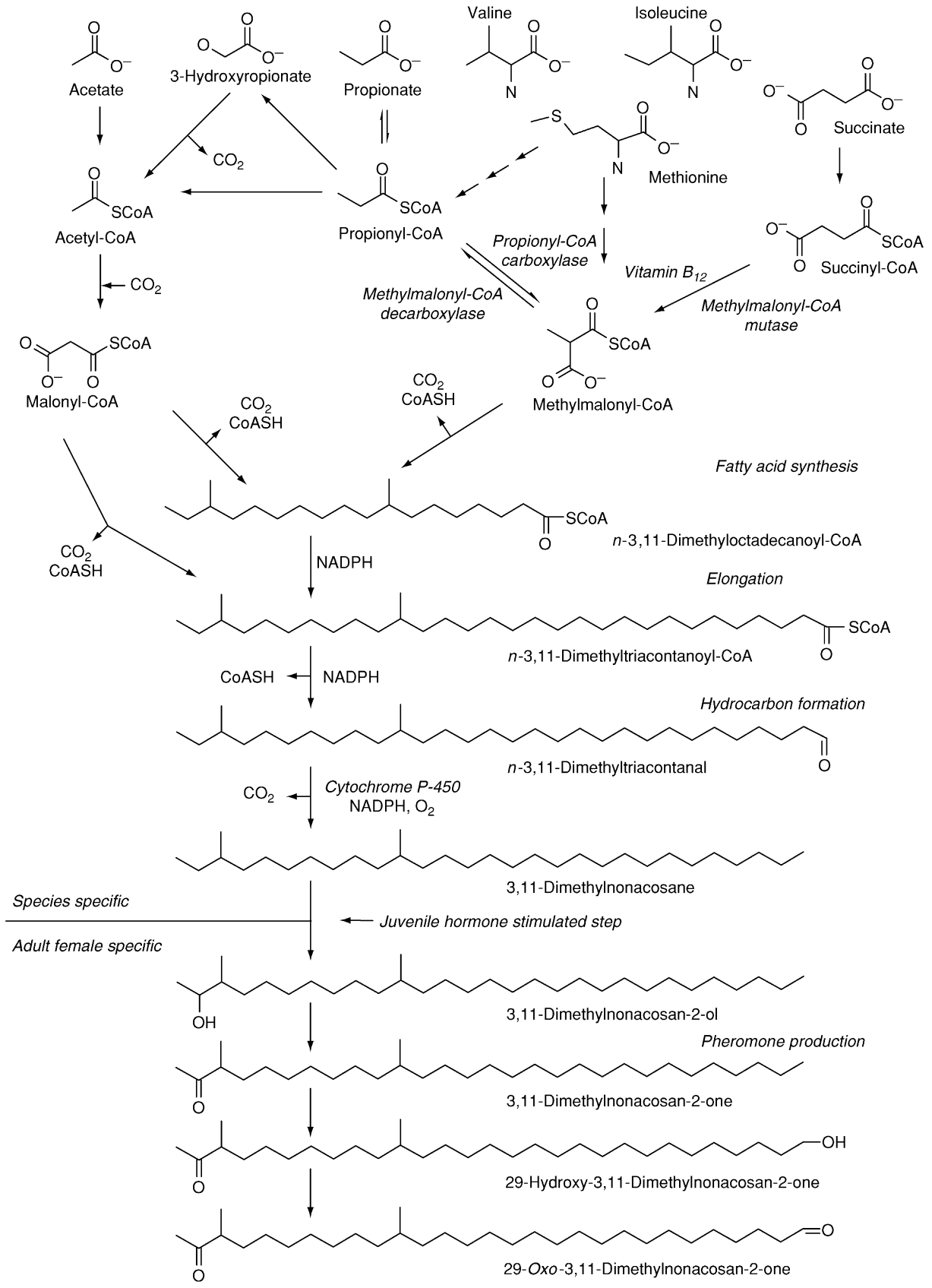


**Figure 9** Proposed biosynthetic pathways for the major pheromone components of the German cockroach, *Blattella germanica*. The JH III regulated step appears to be the hydroxylation of the dimethylalkane.

stearic acid at the *n*-(workers) and *n*-1 (queens) positions, (3) chain shortening through β-oxidation to the 10- and 8-carbon hydroxy acids, and (4) oxidation of *n*- and *n*-1 hydroxy groups to give diacids and 9-keto-2(*E*)-decenoic acid, respectively. Stearic acid was shown to be the main precursor of the pheromone molecules as it was converted to $C_{10}$ hydroxy acids and diacids more efficiently than either 16 or 14 carbon fatty acids.

## 3.14.5. Endocrine Regulation of Pheromone Production

### 3.14.5.1. Barth's Hypothesis

Juvenile hormone's central role in mate-finding was recognized in 1965 when Barth proposed that neuroendocrine control of pheromone production would be common in 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. Cockroaches and beetles are quintessential examples of this life-history syndrome. 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. The discovery of PBAN in moths (see Section 3.14.4.5.2) appeared in conflict with this hypothesis, but Barth's model (Barth and Lester, 1973) clearly accounted for cases in moth species where adults feed and oocyte maturation requires the participation of JH or other neuroendocrine factors. Schal *et al.* (2003) proposed a reconsideration of the hypothesis, taking into account the coordination of reproductive developmental processes with mating-related events. Accordingly, in long-lived insects, such as cockroaches, pheromone production is expected to be synchronously regulated 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 control of 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, alternatively, 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 such 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). All these observations are consistent with our interpretation of Barth's model.

The three hormones that regulate pheromone production in insects are shown in **Figure 2** and **Table 1**. PBAN has been studied in female moths and alters enzyme activity through second messengers at one or more steps during or subsequent to fatty acid synthesis during pheromone production (Rafaeli and Jurenka, 2003). In contrast, 20E and JH induce or repress the synthesis of specific enzymes at the transcription level. The action of JH has been studied most thoroughly in the German cockroach and in bark beetles, and this work is discussed below. Ecdysteroid regulation of pheromone production occurs in Diptera, and has been most extensively studied in the housefly, *M. domestica*.

### 3.14.5.2. PBAN Regulation in Moths

**3.14.5.2.1. PBAN** Most female moths release sex pheromones in a typical calling behavior in which the pheromone gland is extruded to release pheromone during a particular time of the photoperiod. In most cases pheromone biosynthesis coincides with calling behavior and the synchronization of these events is achieved by neuroendocrine mechanisms present in the female that in turn are influenced by various environmental and physiological events such as temperature, photoperiod, host plants, mating, hormones, neurohormones, and neuromodulators. We now know that the main neuroendocrine mechanism that regulates pheromone production in moths is pheromone biosynthesis activating neuropeptide (PBAN).

**Table 1** Amino acid sequences of the pyrokinin/PBAN family of peptides; the FXPRLamide motif is shown in bold (X standing for S, T, G, or V)

| *Function* | *Species* | *Peptide sequence* | *Reference* |
|---|---|---|---|
| PBAN | *Helicoverpa zea* | LSDDMPATPADQEMYRQDPEQIDSRTKY**FSPRL**amide[a] | Raina *et al.* (1989) |
| | *Helicoverpa assulta* | LSDDMPATPADQEMYRQDPEQIDSRTKY**FSPRL**amide[b] | Choi *et al.* (1998) |
| | *Bombyx mori* | LSEDMPATPADQEMYQPDPEEMESRTRY**FSPRL**amide[a] | Kitamura *et al.* (1989) |
| | *Lymantria dispar* | LADDMPATMADQEVYRPEPEQIDSRNKY**FSPRL**amide[a] | Masler *et al.* (1994) |
| | *Agrotis ipsilon* | LADDTPATPADQEMYRPDPEQIDSRTKY**FSPRL**amide[b] | Duportets *et al.* (1999) |
| | *Mamestra brassicae* | LADDMPATPADQEMYRPDPEQIDSRTKY**FSPRL**amide[b] | Jacquin-Joly *et al.* (1998) |
| | *Spodoptera littoralis* | LADDMPATPADQELYRPDPDQIDSRTKY**FSPRL**amide[b] | Iglesias *et al.* (2002) |
| Pheromontropic peptides | *Bombyx mori* | α II**FTP**K**L**amide[b] | Kawano *et al.* (1992) |
| | *Helicoverpa zea* | α VI**FTP**K**L**amide[b] | Ma *et al.* (1994) |
| | *Helicoverpa assulta* | α VI**FTP**K**L**amide[b] | Choi *et al.* (1998) |
| | *Agrotis ipsilon* | α VI**FTP**K**L**amide[b] | Duportets *et al.* (1999) |
| | *Mamestra brassicae* | α VI**FTP**K**L**amide[b] | Jacquin-Joly *et al.* (1998) |
| | *Spodoptera littoralis* | α VI**FTP**K**L**amide[b] | Iglesias *et al.* (2002) |
| | *Bombyx mori* | β SVAKPQTHESLE**FI****PRL**amide[b] | Kawano *et al.* (1992) |
| | *Helicoverpa zea* | β SLAYDDKSFENVE**FTPRL**amide[b] | Ma *et al.* (1994) |
| | *Helicoverpa assulta* | β SLAYDDKSFENVE**FTPRL**amide[b] | Choi *et al.* (1998) |
| | *Agrotis ipsilon* | β SLSYEDKMFDNVE**FTPRL**amide[b] | Duportets *et al.* (1999) |
| | *Mamestra brassicae* | β SLAYDDKVFENVE**FTPRL**amide[b] | Jacquin-Joly *et al.* (1998) |
| | *Pseudaletia separata* | β KLSYDDKVFENVE**FTPRL**amide[b] | Matsumoto *et al.* (1992) |
| | *Spodoptera littoralis* | β SLAYDDKVFENVE**FTPRL**amide[b] | Iglesias *et al.* (2002) |
| | *Bombyx mori* | γ TMS**FSPRL**amide[b] | Kawano *et al.* (1992) |
| | *Helicoverpa zea* | γ TMN**FSPRL**amide[b] | Ma *et al.* (1994) |
| | *Helicoverpa assulta* | γ TMN**FSPRL**amide[b] | Choi *et al.* (1998) |
| | *Agrotis ipsilon* | γ TMN**FSPRL**amide[b] | Duportets *et al.* (1999) |
| | *Mamestra brassicae* | γ TMN**FSPRL**amide[b] | Jacquin-Joly *et al.* (1998) |
| | *Spodoptera littoralis* | γ TMN**FSPRL**amide[b] | Iglesias *et al.* (2002) |
| Diapause hormone | *Bombyx mori* | TDMKDESDRGAHSERGALC**FGPRL**amide[a] | Imai *et al.* (1991) |
| | *Helicoverpa zea* | NDVKDGAASGAHSDRLGLW**FGPRL**amide[b] | Ma *et al.* (1994) |
| | *Helicoverpa assulta* | NDVKDGAASGAHSDRLGLW**FGPRL**amide[b] | Choi *et al.* (1998) |
| | *Agrotis ipsilon* | NDVKDGGADRGAHSDRGGMW**FGPR**lamide[b] | Duportets *et al.* (1999) |
| | *Spodoptera littoralis* | NEIKDGGSDRGAHSDRAGLW**FGPRL**amide[b] | Iglesias *et al.* (2002) |
| Pyrokinins | *Leucophaea maderae* | ETS**FTPRL**amide[a] | Holman *et al.* (1986) |
| | *Locusta migratoria* | (I) pEDSGDGWPQQP**FVPRL**amide[a] | Schoofs *et al.* (1991) |
| | | (II) pESVPT**FTPRL**amide[a] | Schoofs *et al.* (1993) |
| Myotropins | *Locusta migratoria* | (I) GAVPAAQ**FSPRL**amide[a] | Schoofs *et al.* (1990a) |
| | | (II) EGD**FTPRL**amide[a] | Schoofs *et al.* (1990b) |
| | | (III) RQQP**FVPRL**amide[a] | Schoofs *et al.* (1992) |
| | | (IV) RLHQNGMP**FSPRL**amide[a] | Schoofs *et al.* (1992) |
| | *Schistocerca gregaria* | MT1 GAAPAAQ**FSPRL**amide[a] | Veelaert *et al.* (1997) |
| | | MT2 TSSLFPH**PRL**amide[a] | Veelaert *et al.* (1997) |
| | *Periplaneta americana* | PK1 HTAG**FI****PRL**amide[a] | Predel *et al.* (1997) |
| | | PK2 SPP**F**A**PRL**amide[a] | Predel *et al.* (1997) |
| | | PK3 LVP**F**R**PRL**amide[a] | Predel *et al.* (1999) |
| | | PK4 DHLPHDVY**SPRL**amide[a] | Predel *et al.* (1999) |
| | | PK5 GGGGSGETSGMW**FGPRL**amide[a] | Predel *et al.* (1999) |
| | | PK6 SESEVPGMW**FGPRL**amide[a] | Predel and Eckert (2000) |
| | *Periplaneta fuliginosa* | PK4 DHLSHDVY**SPRL**amide[a] | Predel and Eckert (2000) |
| | *Drosophila melanogaster* | CAP2b-3 TGPSASSGLW**FGPRL**amide[b] | Choi *et al.* (2001) |
| | | PK-2 SVP**F**K**PRL**amide[b] | Choi *et al.* (2001) |
| | | ETH-1 DDSSPGFFLKITKNV**PRL**amide[b] | Park *et al.* (1999) |
| | | hug γ pELQSNGIPAYRVR**TPRL**amide[b] | Meng *et al.* (2002) |
| | *Penaeus vannamei* (Crustacea) | DFA**FSPRL**amide[a] | Torfs *et al.* (2001) |
| | | ADFA**F**N**PRL**amide[a] | Torfs *et al.* (2001) |

[a]Identified from the amino acid sequence of a purified peptide.
[b]Deduced from the cloned gene sequence.

The neuropeptide PBAN was localized to the subesophageal ganglion (SEG) (Raina and Menn, 1987), which facilitated purification and sequencing (Kitamura *et al*., 1989; Raina *et al*., 1989). The first PBAN identified had 33 amino acids with a C-terminal amidation and the core sequence FXPRLamide (where X represents S, T, G, or V) is required for activity, which places PBAN in a family of peptides with the C-terminal FXPRLamide motif (**Table 1**). The first member of this family to be identified was called leucopyrokinin based on its ability to stimulate hindgut contraction in the cockroach *Leucophaea maderae* (Holman *et al*., 1986). Additional functions for this family include induction of embryonic diapause in *B. mori* (Imai *et al*., 1991), induction of melanization in lepidopteran larvae (Matsumoto *et al*., 1990), and acceleration of puparium formation in several flies (Zdarek *et al*., 1998). In addition it was determined that the white shrimp, *Penaeus vannamei*, has two peptides that can induce myotropic activity (Torfs *et al*., 2001). These results demonstrate the ubiquity and multifunctional nature of this family of peptides.

Localization of PBAN-like immunoreactivity in the central nervous system of adult moths indicated that several neurons in the SEG contain PBAN-like activity. These were found as clusters along the ventral midline, one each in the presumptive mandibular, maxillary, and labial neuromeres (Kingan *et al*., 1992). All three groups of neurons have axons that project into the corpus cardiacum (CC) (Kingan *et al*., 1992; Davis *et al*., 1996). Two pairs of maxillary neurons send processes within the SEG that include posterior projections into the paired ventral nerve cord (VNC) and travel its entire length to terminate in the terminal abdominal ganglion (TAG). Arborizations arising from these paired projections were found in each segmental ganglion (Davis *et al*., 1996). In addition the segmental ganglia have neurons that contain PBAN-like activity and these neurons can release peptides into the hemolymph (Ma and Roelofs, 1995c; Davis *et al*., 1996; Ma *et al*., 1996).

**3.14.5.2.2. Molecular genetics of PBAN** The gene encoding PBAN was first characterized from *H. zea* and *B. mori* (Imai *et al*., 1991; Davis *et al*., 1992; Kawano *et al*., 1992; Sato *et al*., 1993; Ma *et al*., 1994). The full-length cDNA was found to encode PBAN plus four additional peptide domains with a common C-terminal FXPRL sequence motif including that of the diapause hormone of *B. mori*. Three additional peptides with the common C-termini and sequence homology to those of *H. zea* and *B. mori* have been deduced from cDNA isolated from pheromone glands of *Mamestra brassicae* (Jacquin-Joly and Descoins, 1996), *H. assulta* (Choi *et al*., 1998), *Agrotis ipsilon* (Duportets *et al*., 1999), *Spodoptera littoralis* (Iglesias *et al*., 2002), *H. armigera* (Zhang *et al*., 2001), and *Adoxophyes* sp. (Lee *et al*., 2001). The posttranslational processed peptides can be found in the SEG (Sato *et al*., 1993; Ma *et al*., 1996). The matrix-assisted laser desorption ionization (MALDI) MS data indicated that PBAN was found to a greater extent in the mandibular and maxillary clusters than in the labial cluster (Ma *et al*., 2000). The other neuropeptides were found in all clusters. In addition some larger peptide fragments were found indicating alternative processing of the precursor protein (Ma *et al*., 2000).

**3.14.5.2.3. PBAN mode of action** Through the development of a sensitive *in vitro* bioassay, studies on *H. armigera* and *H. zea* demonstrated that brain extracts and synthetic *H. zea* PBAN could stimulate the production of the main pheromone component (Soroker and Rafaeli, 1989; Rafaeli *et al*., 1990, 1991, 1993; Rafaeli, 1994; Rafaeli and Gileadi, 1996). The response obtained was specific to the pheromone gland and independent of other abdominal tissues (Rafaeli, 1994; Rafaeli *et al*., 1997b). Evidence has since accumulated regarding several other species of moths including *B. mori*, *Spodoptera litura*, *O. nubilalis*, *Plodia interpunctella*, and *Thaumetopoea pityocampa* (Arima *et al*., 1991; Fónagy *et al*., 1992; Fabriàs *et al*., 1995; Ma and Roelofs, 1995b; Rafaeli and Gileadi, 1995a; Jurenka, 1996). In addition, functional and viable pheromone gland cell-clusters were obtained from the intersegmental membrane of *B. mori* using papain enzymatic digestion (Fónagy *et al*., 2000).

The signal transduction events that occur after PBAN binds to a receptor have been studied in several model moth species (**Figure 10**). The main difference found between these species so far is whether or not 3′,5′,cyclic-AMP (cAMP) is used as a second messenger. In the case of the heliothines and several others, cAMP is a second messenger. Alternatively, cAMP is thought not to act in pheromone gland cells of *B. mori* and *O. nubilalis* (Fónagy *et al*., 1992; Ma and Roelofs, 1995b). Instead, in these insects, it is thought that an increase in cytosolic calcium directly activates downstream events leading to stimulation of the biosynthetic pathway.

Extracellular calcium is essential for pheromonotropic activity in all moths studied to date (Fónagy *et al*., 1992, 1999; Ma and Roelofs, 1995a; Matsumoto *et al*., 1995). It is suggested that free calcium,

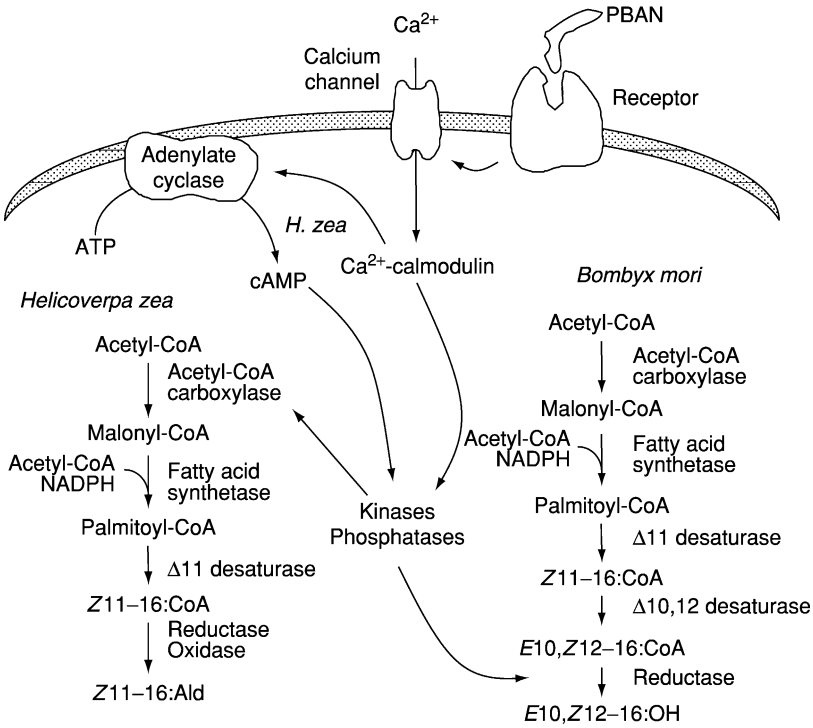


**Figure 10** Proposed signal transduction mechanisms that stimulate the pheromone biosynthetic pathway in *Helicoverpa zea* and other heliothines as compared with that in *Bombyx mori*. It is proposed that PBAN binds to a receptor present in the cell membrane. Binding to the receptor somehow induces a receptor-activated calcium channel to open causing an influx of extracellular calcium. This calcium binds to calmodulin and in the case of *B. mori* will directly stimulate a phosphatase that will dephosphorylate and activate a reductase in the biosynthetic pathway. This activated reductase will then produce the pheromone bombykol. In *H. zea* and other heliothines like *H. armigera* the calcium-calmodulin will activate adenylate cyclase to produce cAMP that will then act through kinases and/or phosphatases to stimulate acetyl-CoA carboxylase in the biosynthetic pathway. (Reprinted with permission from Rafaeli, A., Jurenka, R.A., **2003**. PBAN regulation of pheromone biosynthesis in female moths. In: Blomquist, G.J., Vogt, R.G. (Eds.), Insect Pheromone Biochemistry and Molecular Biology. Elsevier, San Diego, CA, pp. 107–136; © Elsevier.)

entering the cell, binds to calmodulin to form a complex thereby activating adenylate cyclase and/or phosphoprotein phosphatases (**Figure 10**). Calmodulin was characterized from pheromone glands of *B. mori* (Iwanaga *et al.*, 1998) and was shown to have an identical amino acid sequence to *Drosophila* calmodulin (Smith *et al.*, 1987). In *B. mori*, it is suggested that the $Ca^{2+}$/calmodulin complex directly or indirectly activates a phosphoprotein phosphatase (Matsumoto *et al.*, 1995). This phosphatase will then activate an acyl-CoA reductase in the biosynthetic pathway. Two genes encoding calcineurin heterosubunits were identified from the pheromone gland of *B. mori* and were found to be homologous to the catalytic subunit and regulatory subunits of other animal calcineurins (Yoshiga *et al.*, 2002). The calcineurin complex will apparently dephosphorylate an acyl-CoA reductase, which catalyzes the formation of bombykol in *B. mori*.

**3.14.5.2.4. Enzymes affected in the pheromone biosynthetic pathway** PBAN has been shown to stimulate the reductase that converts an acyl-CoA to an alcohol precursor (**Figure 10**) in several moths including *B. mori* (Arima *et al.*, 1991; Ozawa *et al.*, 1993), *T. pityocampa* (Gosalbo *et al.*, 1994), *S. littoralis* (Martinez *et al.*, 1990; Fabriàs *et al.*, 1994), and *M. sexta* (Fang *et al.*, 1995b; Tumlinson *et al.*, 1997). In *A. velutinana* (Tang *et al.*, 1989), *H. zea* (Jurenka *et al.*, 1991a), *Cadra cautella*, *S. exigua* (Jurenka, 1997) and *M. brassicae* (Jacquin *et al.*, 1994), it was demonstrated that PBAN controls pheromone biosynthesis by regulating a step during or prior to fatty acid biosynthesis (**Figure 10**). Circumstantial evidence in *A. segetum* (Zhu *et al.*, 1995) and *H. armigera* (Rafaeli *et al.*, 1990) also points to the regulation of fatty acid synthesis by PBAN. In one study using the moth *Sesamia nonagrioides* it was shown that the acetyltransferase enzyme might be regulated by PBAN (Mas *et al.*, 2000). There appears to be no particular pattern as to which enzyme within the pheromone biosynthetic pathway will be regulated by PBAN. However, in the majority of moths studied it is either the reductase or fatty acid synthesis that is stimulated.

Several families of moths utilize hydrocarbons and/or their epoxides as sex pheromones (Millar, 2000). It is thought that PBAN does not regulate the production of hydrocarbon sex pheromones as demonstrated in *Scoliopteryx libatrix* (Subchev and Jurenka, 2001). However, PBAN is probably regulating the production of epoxide sex pheromones. This was demonstrated in *Ascotis selenaria cretacea* where decapitation resulted in pheromone decline and it could be restored by injecting PBAN (Miyamoto *et al*., 1999). Decapitation also decreases the epoxide pheromone titer in the gypsy moth, *L. dispar*, and injection of PBAN can restore pheromone production (Thyagaraja and Raina, 1994). However, decapitation did not decrease the levels of the hydrocarbon precursor in the gypsy moth (Jurenka, unpublished data). These findings indicate that PBAN may regulate the epoxidation step in those moths that utilize epoxide pheromones but not the production of the alkene precursor or alkene pheromones.

**3.14.5.2.5. Mediators and inhibitors of PBAN action** Juvenile hormones play an important role in reproductive development of many moth species (see **Chapters 3.7** and **3.9**). Although JH probably does not regulate pheromone biosynthesis directly it has been shown to be involved in the release of PBAN in the migratory moths *Pseudaletia unipuncta* and *A. ipsilon* (Cusson and McNeil, 1989; Gadenne, 1993; Cusson *et al*., 1994; Picimbon *et al*., 1995). In addition, JH has been shown to prime the pheromone glands in pharate adults of the nonmigratory moth *H. armigera* (Fan *et al*., 1999). JH II, in an *in vitro* assay, primed pheromone glands of pharate adults to respond to PBAN and induced earlier pheromone production by intact newly emerged females (Fan *et al*., 1999). This induction could be mediated by JH upregulation of a putative PBAN receptor in pharate adults (Rafaeli *et al*., 2003).

The corpus bursae has been implicated in the mediation of PBAN stimulation of pheromone biosynthesis in some tortricids (Jurenka *et al*., 1991c). A peptide was partially purified from corpus bursae that could stimulate pheromone production (Fabriàs *et al*., 1992). To date a bursal factor has only been demonstrated in *A. velutinana* and the related tortricids *C. fumiferana* and *C. rosaceana* (Delisle *et al*., 1999).

The role of the nervous system in pheromone biosynthesis in moths is not clearly understood. In several moths including *L. dispar* (Tang *et al*., 1987; Thyagaraja and Raina, 1994), *H. virescens* (Christensen *et al*., 1991), *S. littoralis* (Marco *et al*., 1996), and *M. brassicae* (Iglesias *et al*., 1998) an intact VNC was reported as necessary for pheromone biosynthesis. Christensen and coworkers (Christensen *et al*., 1991, 1992, 1994; Christensen and Hildebrand, 1995) proposed that the neurotransmitter octopamine might be involved as an intermediate messenger during the stimulation of sex pheromone production in *H. virescens*. These workers suggested that octopamine was involved in the regulation of pheromone production and that PBAN's role lies in the stimulation of octopamine release at nerve endings. However, contradicting results concerning VNC transection and octopamine-stimulated pheromone production were reported in the same species as well as in other moth species (Jurenka *et al*., 1991c; Ramaswamy *et al*., 1995; Rafaeli and Gileadi, 1996; Park and Ramaswamy, 1998; Delisle *et al*., 1999). A modulatory role for octopamine was suggested by research conducted on *H. armigera* (Rafaeli and Gileadi, 1995b; Rafaeli *et al*., 1997a, 1999). Octopamine and several octopaminergic analogs inhibited pheromone production in studies using both *in vitro* and *in vivo* bioassays in two species of moths (Rafaeli and Gileadi, 1995b, 1996; Rafaeli *et al*., 1997, 1999; Hirashima *et al*., 2001). The role of the VNC in pheromone production still requires clarification. It has been suggested, that in some moth species (*S. littoralis*) both humoral and neural regulation occurs (Marco *et al*., 1996). Given the diversity of moths it may not be surprising to find several mechanisms regulating pheromone biosynthesis.

### 3.14.5.3. JH Regulation in Beetles

**3.14.5.3.1. General** In the Coleoptera, pheromone production or release is controlled by JH III (review: Tillman *et al*., 1999). JH, or JH analogs, stimulate pheromone production in *T. molitor* (Menon, 1970), *T. castaneum* (both Tenebrionidae), some members of the Cucujidae (review: Plarre and Vanderwel, 1999), *A. grandis* (Curculionidae) (Wiygul *et al*., 1990), and various Scolytidae (see Section 3.14.5.3.2). Various factors may work upstream as cues to stimulate JH biosynthesis. Feeding stimulates pheromone production or release in many species (Vanderwel and Oehlschlager, 1987). Sexual maturity and population density may also be important, e.g., for some Scolytidae (Byers, 1983) and Cucujidae (Plarre and Vanderwel, 1999). The effect of population density may be mediated by sensitivity to pheromone concentrations. Antennectomy can raise pheromone biosynthetic rates in *A. grandis* (Dickens *et al*., 1988) and *L. decemlineata* (Dickens *et al*., 2002), suggesting that detection of pheromone components may inhibit their production. Various combinations of physiological and

environmental factors therefore regulate JH titers, which in turn stimulate pheromone biosynthesis and/or release.

**3.14.5.3.2. JH regulation in bark beetles (Scolytidae)** Aggregation pheromone biosynthesis in bark beetles generally begins shortly after the beetle arrives at a new host tree (Wood, 1982; Seybold *et al*., 2000). Unfed beetles can be artificially stimulated to produce pheromones by treatment with JH III. For example, males are the pioneers of *Ips* spp., and starved male *I. paraconfusus* and *I. pini* can be induced to synthesize the pheromone components ipsenol and ipsdienol if JH III or a JH analog is applied topically (Bordon *et al*., 1969; Chen *et al*., 1988; Ivarsson and Birgersson, 1995; Tillman *et al*., 1998). Similarly, starved *Dendroctonus* spp. can be induced to synthesize pheromone components following treatment with JH (Bridges, 1982; Conn *et al*., 1984). Feeding apparently stimulates the synthesis of JH III in the CA (Tillman *et al*., 1998), resulting in elevated JH titers that trigger pheromone biosynthesis in midgut cells (Hall *et al*., 2002a, 2002b).

Pheromone biosynthesis requires a shift in metabolic priorities, particularly for those beetles that produce large quantities of pheromone. JH must actuate this shift, and since pheromone components are synthesized *de novo* (Seybold *et al*., 1995b), induction of the pheromone biosynthetic pathway involves elevated expression of at least some related genes. This was first demonstrated in *I. paraconfusus*, where JH-treated male beetles have increased HMG-R mRNA levels compared to controls (Tittiger *et al*., 1999). Male *I. pini* and *D. jeffreyi* HMG-R mRNA levels also rise in response to topical JH III applications (**Figure 11**) (Tittiger *et al*., 2003; Tillman, Lu, Tittiger, Blomquist, and Seybold, unpublished data). In all species studied, the response is dose and time dependent and correlates with pheromone component biosynthesis. This is consistent with the hypothesis that JH controls HMG-R, which functions as a regulator of the mevalonate (pheromone biosynthetic) pathway. HMG-S mRNA levels in male *D. jeffreyi* also respond to topical JH applications similarly to HMG-R, though at a more modest level (**Figure 11**) (Tittiger *et al*., 2000). In *I. pini*, mRNA for a GPPS gene is also elevated in JH III-treated beetles (Gilg-Young, Welch, Tittiger, and Blomquist, unpublished data). This enzyme diverts carbon from the normal mevalonate pathway into the pheromone biosynthetic pathway, and is thus also a likely regulatory step.

The HMG-S and HMG-R studies in *D. jeffreyi* show how putative regulatory enzymes are

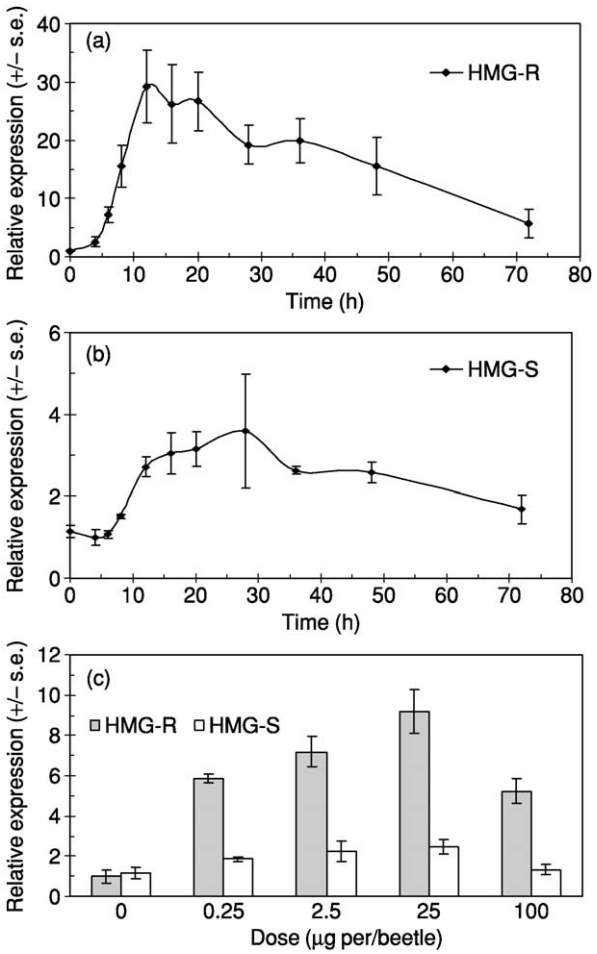


**Figure 11** Regulation of *HMG-R* and *HMG-S* expression in male *Dendroctonus jeffreyi* by JH III. The time course (a, b) and dose response (c) for each gene in mature (emerged) males was investigated by Northern blotting. All values are relative to starved, untreated males. Each point represents the mean +/− standard error of three replicates, five isolated thoraces/sample. (Reprinted with permission from Tittiger, C., Barkawi, L.S., Bengoa, C.S., Blomquist, G.J., Seybold, S.J., **2003**. Structure and juvenile hormone-mediated regulation of the HMG-CoA reductase gene from the Jeffrey pine beetle, *Dendroctonus jeffreyi*. *Mol. Cell. Endocrinol. 199*, 11–21; © Elsevier.)

controlled at the transcriptional level by JH III (though posttranscriptional regulation is probably also important; see below). Their coordinate induction implies that other mevalonate/pheromone biosynthetic genes may also be stimulated by JH III. This question has been addressed mostly in *I. pini*; indeed, more is known about pheromone biosynthetic gene regulation in this insect than in any other beetle, due in part to microarray-based expression profiling. Microarray analyses of male and female JH-treated and untreated *I. pini* give an idea of the profound change that male midgut cells undergo in order to synthesize pheromone, with numerous genes being upregulated or downregulated

following JH III treatment (Keeling and Tittiger, unpublished data). All identified mevalonate pathway genes in a recent expressed sequence tags (ESTs) project (BeetleBase, 2003; Eigenheer *et al*., 2003) have elevated expression levels compared to controls (Keeling, Bearfield, Blomquist, and Tittiger, unpublished data). These include enzymes at predicted control points (HMG-R and GPPS) as well as others that would not be expected to have a strong regulatory role (e.g., isopentenyl diphosphate (IPP) isomerase). Thus, induction of identified pheromone-biosynthetic genes is apparently coordinate. By extension, genes encoding unidentified enzymes in the pheromone biosynthetic pathway are also likely JH regulated. The putative pheromone precursor, ipsdienone, was recovered from thoracic sections of JH-treated *I. paraconfusus* (Ivarsson *et al*., 1998), and a male-specific myrcene synthase activity is also elevated in tissues of JH III-treated *I. pini* (Martin *et al*., 2003). These data provide biochemical evidence that JH III affects enzyme activities converting geranyl diphosphate to ipsdienol, with myrcene and ipsdienone as probable intermediates. Confirmation that the corresponding genes are also JH regulated awaits their identification, and characterization of the relevant enzymes.

In the studies mentioned above, the amount of JH III penetrating the cuticle and acting on the midguts is unknown. A more biologically relevant question is whether feeding similarly stimulates pheromone biosynthetic gene expression. Recent real-time polymerase chain reaction (PCR) studies of RNA recovered from midguts from fed male and female *I. pini* confirm that HMG-R and HMG-S are induced in both sexes by feeding to levels similar to those observed in JH III-treated insects (Keeling and Tittiger, unpublished data). This is curious, because female midguts do not synthesize monoterpenoid pheromones, and female JH III titers do not naturally rise upon feeding (Tillman *et al*., 1998). Other factors therefore must cause elevated *HMG-R* and *HMG-S* expression in female midguts. These experiments underscore the complexity of gene regulation in bark beetles, and remind us that mevalonate pathway genes almost certainly respond to other, possibly nonpheromone-related factors. Whatever the case, feeding clearly induces expression of certain genes in both male and female tissues, with males having an additional response characterized by increased rates of pheromone biosynthesis.

Juvenile hormone may act as a both trigger and stimulus for pheromone production. The basal HMG-R and HMG-S mRNA levels are from fivefold to eightfold higher in the midguts of males than of females, implying a greater capacity of mevalonate pathway flux in male cells compared to female cells (Keeling and Tittiger, unpublished data). Also, female GPPS mRNA levels, which are not clearly induced by feeding in female *I. pini* midguts, are approximately 16-fold lower than those in male midguts, and GPPS is induced by feeding and JH III treatment in males (Gilg-Young, Welch, Keeling, Bearfield, Blomquist, and Tittiger, unpublished data). This suggests that male midguts may be primed for pheromone biosynthesis, possibly due to an earlier, developmental cue, even before JH III triggers the process, and that pioneer beetles are ready to begin pheromone production when they arrive at a host tree. Subsequent feeding-induced JH III production thus both activates existing components of the pathway and stimulates continued production of pheromone pathway enzymes. Such priming might avoid a potentially dangerous "waiting" period, during which the pheromone-biosynthetic pathway is being stimulated, which would delay the arrival of other beetles to assist colonizing the host tree.

JH appears to be necessary for pheromone production, but it is not always sufficient. Similar gene responses to topically applied JH in male and female *I. pini* belie very different metabolic responses (Tillman, Lu, Seybold, Keeling, Bearfield, Blomquist, and Tittiger, unpublished data), suggesting a control mechanism for *I. pini* that is more complex than having JH III simply stimulate expression of pheromone biosynthetic genes. Other factors may be involved. For example, while all mevalonate pathway genes are induced in male *I. pini*, only genes corresponding to early steps (e.g., thiolase, HMG-R, HMG-S, IPP isomerase) are induced by JH III in females, while those for latter steps (GPPS, farnesyl diphosphate synthase (FPPS)) are not (Keeling *et al*., 2004). Similarly, JH is clearly not sufficient to stimulate pheromone production in *I. paraconfusus* (Tillman, Lu, Tittiger, Blomquist, and Seybold, unpublished data). JH III-treated male *I. paraconfusus* have elevated HMG-R mRNA levels (the expression of other genes has not been studied in this insect), but neither HMG-R enzyme activity nor monoterpenoid pheromone component levels are significantly increased compared to controls. In contrast, male *I. paraconfusus* that have been allowed to feed on pine phloem have elevated HMG-R activity and, of course, elevated pheromone component levels. Decapitation studies confirm that an unknown ancillary hormone(s) is required in addition to JH III for pheromone biosynthesis (Lu, Blomquist, and Seybold, unpublished data). Presumably, one effect of this factor is in regulating the posttranscriptional activation of

HMG-R. Posttranscriptional regulation of pheromone biosynthetic activity has been demonstrated in other insect systems (e.g., PBAN-regulated activity of bombykol in silkmoths) (Moto *et al.*, 2003), and multilevel control of HMG-R activity is well documented (review: Hampton *et al.*, 1996). Future studies that concentrate on the effects of JH III on protein levels and enzyme activities should provide exciting new information of how JH III regulates the pheromone-biosynthetic machinery in bark beetles.

### 3.14.5.4. 20-Hydroxyecdysone Regulation of Pheromone Production in the Housefly

**3.14.5.4.1. Ecdysteroid regulation** Newly emerged female houseflies do not have detectable amounts of any $C_{23}$ sex pheromone components. Sex pheromone production correlates with ovarian development and vitellogenesis. The $C_{23}$ sex pheromone components first appear when ovaries mature to the early vitellogenic stages (**Figure 12**) and increase in amount until stages 9 and 10 (mature egg) (Dillwith *et al.*, 1983; Mpuru *et al.*, 2001). Females

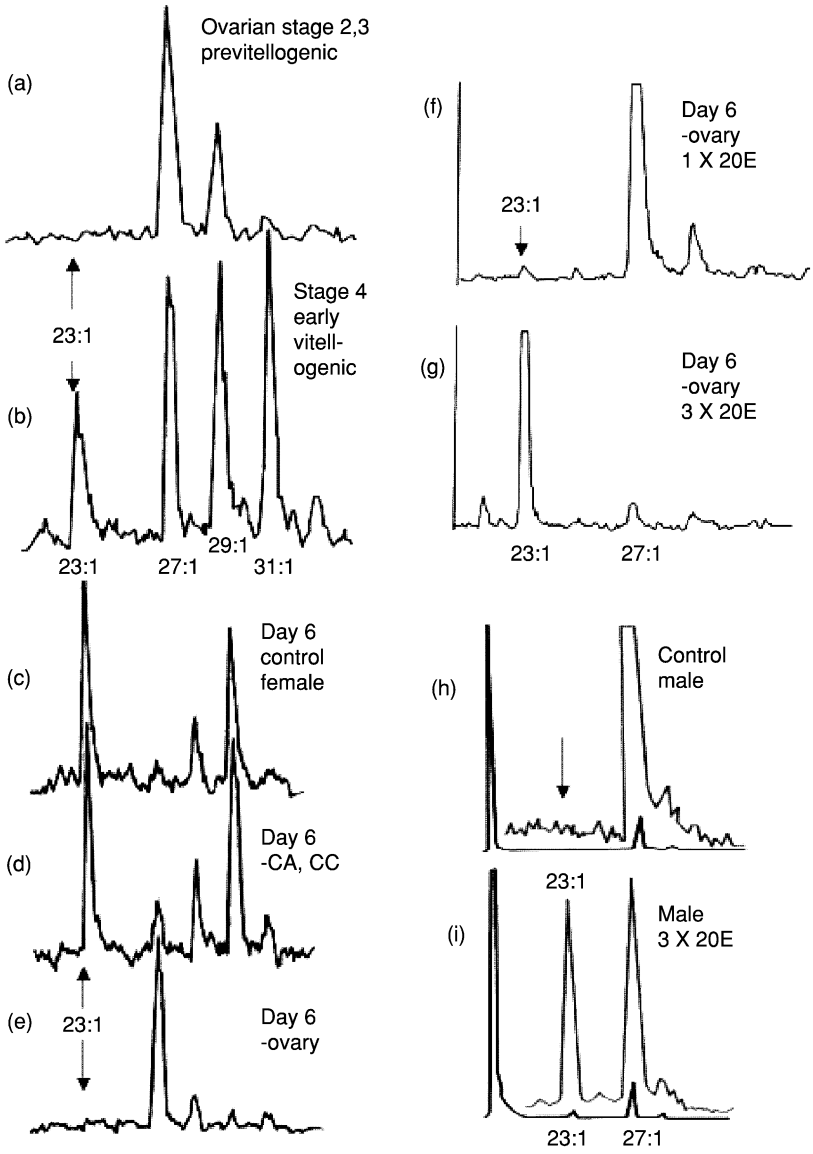


**Figure 12** Effect of ovarian development (a, b), ovariectomy and allatectomy (c, d, e), and treatment with 20-hydroxyecdysone (20E) on ovariectomized females (f, g) and males (h, i) on (*Z*)-9-tricosene production. The results show that ovariectomy abolishes pheromone production, whereas treatment with 20E induces pheromone production in ovariectomized females and in males. (Reprinted with permission from Blomquist, G.J., **2003**. Biosynthesis and ecdysteroid regulation of housefly sex pheromone production. In: Blomquist, G.J., Vogt, R.G. (Eds.), Insect Pheromone Biochemistry and Molecular Biology. Elsevier, New York, pp. 131–252; © Elsevier.)

ovariectomized within 6 h of adult emergence do not produce any of the $C_{23}$ sex pheromone components, whereas control and allatectomized females produced abundant amounts of (*Z*)-9-tricosene (**Figure 12c–e**). Ovariectomized insects that received ovary implants produced sex pheromone components in direct proportion to ovarian maturation. These data demonstrate that a hormone from the maturing ovary induced sex pheromone production (Blomquist *et al*., 1993, 1998).

Juvenile hormone regulates both vitellogenesis and pheromone production in some insect species (Tillman *et al*., 1999). In some Diptera, including the housefly, ovarian-produced ecdysteroids are involved in regulating vitellogenesis (Hagedorn, 1985; Adams *et al*., 1997) at the transcriptional level (Martin *et al*., 2001). Therefore, since ovariectomy abolished sex pheromone production whereas allatectomy (which abolishes JH production) had no effect on pheromone production (Blomquist *et al*., 1992), it was hypothesized that an ecdysteroid, and not JH, regulated sex pheromone production in the housefly. Injection of 20E at doses as low as 0.5 ng every 6 h induced sex pheromone production in ovariectomized houseflies in a time- and dose-dependent manner (**Figure 12f** and **g**) (Adams *et al*., 1984a, 1984b, 1995). Multiple injections of 20E into ovariectomized insects over several days resulted in as much 23 : 1 produced as in intact control females. Application of JH or JH analogs alone or in combination with ecdysteroids had no effect on pheromone production (Blomquist *et al*., 1992).

**3.14.5.4.2. Induction of female sex pheromone production in male houseflies** Male houseflies normally produce no detectable $C_{23}$ sex pheromone components, but do produce the same $C_{27}$ and longer alkenes as previtellogenic females. Implantation of ovaries into male houseflies resulted in a change in the chain length specificity of the alkenes such that (*Z*)-9-tricosene became a major component (Blomquist *et al*., 1984a, 1987). Likewise, injection of 20E into males induces sex pheromone production in a dose-dependent manner (**Figure 12h** and **i**). Thus, males possess the biosynthetic capability to produce sex pheromone, but normally do not produce the 20E necessary to induce sex pheromone production. This makes male houseflies a very convenient model to study the regulation of sex pheromone production, circumventing the need to ovariectomize a large number of female insects.

**3.14.5.4.3. Ecdysteroids affect fatty acyl-CoA elongation enzymes** There are two likely possibilities to account for the change in the chain length of the alkenes synthesized by the female housefly in the production of (*Z*)-9-trecosene. They are: (1) the chain-length specificity of the reductive conversion of acyl-CoAs to alkenes is altered such that 24:1-CoA becomes an efficient substrate, or (2) there is a change in the chain-length specificity of the fatty acyl-CoA elongation enzymes such that 24:1-CoA is not efficiently elongated, resulting in an accumulation of 24:1-CoA. To determine which enzyme activities are affected by 20E to regulate the chain length of the alkenes, experiments were performed to examine the chain-length specificity of the fatty acyl-CoA reductive conversion of acyl-CoAs to alkenes and elongation. Microsomal preparations from both males and females of all ages examined readily converted 24:1-CoA and the 24:1 aldehyde to (*Z*)-9-tricosene, indicating that 20E was not acting on this activity (Tillman-Wall *et al*., 1992; Reed *et al*., 1995). In contrast, microsomes from day-4 females (high ecdysteroid titer and production of (*Z*)-9 tricosene) did not elongate either 18:1-CoA or 24:1-CoA beyond 24 carbons, while microsomes from day-4 males or day-1 females (both of which produce alkenes of 27:1 and longer) readily elongated both 18:1-CoA and 24:1-CoA to 28:1-CoA (Tillman-Wall *et al*., 1992; Blomquist *et al*., 1995). Thus, 20E appears to regulate the fatty acyl-CoA elongases and not the enzymatic steps in the conversion of acyl-CoA to hydrocarbon.

**3.14.5.4.4. Transport of pheromone** The role of hemolymph in transporting hydrocarbons and hydrocarbon pheromones has only recently become fully appreciated. Older models of hydrocarbon formation showed epidermal-related cells (oenocytes) synthesizing and transporting hydrocarbons directly to the surface of the insect (Hadley, 1984). In the housefly, the role of hemolymph is most clearly seen when (*Z*)-9-tricosene production is initiated. (*Z*)-9-Tricosene first accumulates in the hemolymph, and then, after a number of hours, is observed on the surface of the insect. Modeling of the process (Mpuru *et al*., 2001) showed that the delay is surprisingly long; a period of more than 24 h is necessary for transport from site of synthesis to deposition on the surface of the insect.

In sexually mature females, (*Z*)-9-tricosene comprised a relatively large fraction of the hydrocarbon of the epicuticle and the hemolymph, but much smaller percentages of the hydrocabons in other tissues, including the ovaries. It appears that certain hydrocarbons were selectively partitioned to certain tissues such as the ovaries, from which pheromone was relatively excluded (Schal *et al*., 2001). Both KBr gradient ultracentrifugation and specific

immunoprecipitation showed that over 90% of the hemolymph hydrocarbon was associated with a high-density lipophorin (see **Chapter 4.6**). Lipophorin was composed of two aproproteins under denaturing conditions: apolipophorin I (≈240 kDa) and apolipophorin II (≈85 kDa) (Schal *et al*., 2001). The data suggest that lipophorin may play an important role in an active mechanism that selectively delivers specific hydrocarbons to specific sites.

### 3.14.5.5. Regulation of Pheromone Production in Cockroaches

**3.14.5.5.1. Development and cellular plasticity of pheromone glands** In cockroaches, in striking contrast to many moths, pheromone glands acquire functional competence during an imaginal maturation period, and developmental regulation involves factors that also control adult reproductive readiness. Also, because reproduction in cockroaches is interrupted by periods of sexual inactivity (i.e., gestation), developmental regulation of the sex pheromone gland can result in alternating cycles of acquisition and subsequent waning of competence through maturation and retrogression, respectively, of cellular machinery. Consequently, in female cockroaches pheromone production is controlled by cyclic maturational changes in the gland in relation to the ovarian cycle.

Best exemplifying this phenomenon are the tergal and sternal glands of *N. cinerea* and *B. germanica* males. Both species possess class-3 glandular units, composed of two cells – a secretory cell and a duct cell (Quennedey, 1998). But after apolysis and before the imaginal molt the immature gland contains four concentric cells, including in addition to the two adult cells an enveloping cell and a ciliary cell (Sreng and Quennedey, 1976; Sreng, 1998). During several days after the adult molt the gland matures, in part 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). In concert, before day 5 the immature sternal glands of *N. cinerea* males produce little pheromone, but after day 5 their pheromone content increases significantly (Sreng *et al*., 1999). Decapitation or allatectomy of *N. cinerea* males completely blocked the apoptotic process, while JH III treatment restored apoptosis (Sreng *et al*., 1999). Brain extracts or synthetic moth PBAN failed to restore gland differentiation or stimulate pheromone production.

Female *B. germanica* employ similar class-3 glands to produce a volatile sex pheromone that is yet to be identified. Ultrastructural, behavioral, and electrophysiological studies have shown that, as in males, pheromone gland cells mature as the female sexually matures (Abed *et al*., 1993b; Liang and Schal, 1993; Tokro *et al*., 1993; Schal *et al*., 1996). The secretory cells of newly formed glands in the imaginal female are small and they contain little pheromone (determined by behavioral and EAG assays). As the female sexually matures, the size of pheromone-secreting cells increases, as does its pheromone content (Liang and Schal, 1993). The mature pheromone gland then undergoes cycles of cellular hypertrophy and retrogression in relation to the JH III titer in successive reproductive cycles. The gland becomes atrophied and its pheromone content declines during gestation, but as a new vitellogenic cycle begins after the egg case is deposited, the pheromone gland undergoes rapid regrowth and proliferation of cellular organelles and an increase in its pheromone content. Although this pattern corresponds well with the JH III titer in the hemolymph (Liang and Schal, 1993; Schal *et al*., 1996), no experimental manipulations of hormone titers have been conducted to verify the hypothesis that JH III controls the cellular plasticity of the pheromone gland.

**3.14.5.5.2. Pheromone production regulated by juvenile hormone** Barth and Lester (1973) and Schal and Smith (1990) reviewed the early literature on hormone involvement in pheromone production in cockroaches. With the exception of *Nauphoeta* (detailed above), no studies on the regulation of volatile pheromone production are available that use analytical or biochemical approaches. The most detailed studies, with *B. germanica* and *S. longipalpa*, have employed behavioral and EAG responses of males to estimate the relative amount of pheromone in females or their pheromone glands. In both species, virgin females initiate pheromone production 4 days after the imaginal molt, in relation to increasing titers of JH III (Smith and Schal, 1990a; Liang and Schal, 1993). Ablation of the corpus allatum (CA) of newly emerged adult females prevents pheromone production in both species, and pheromone production is restored after reimplantation of active CA or by treatment with JH III or JH analogs. Interestingly, although growth of the vitellogenic oocytes is controlled by and highly correlated with JH III titers, direct or even intermediary involvement of the ovaries in regulating pheromone production and calling behavior in both species was excluded by ovariectomies (Smith and Schal, 1990a).

It is not known whether JH exerts its pheromonotropic effects directly on mature secretory cells of

the pheromone gland or if it acts indirectly by stimulating the synthesis and/or release of pheromonotropic neuropeptides. Although cockroach brain extracts induce PBAN-like pheromonotropic activity in moth pheromone glands (Raina *et al*., 1989), they fail to do so in allatectomized *Nauphoeta* males (Sreng *et al*., 1999) or *Supella* females (Schal, unpublished data). Moreover, lack of pheromone production in mated females that periodically produce large amounts of JH III suggests that JH plays a "permissive" role (Smith and Schal, 1990b; Schal *et al*., 1996). 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.

*Blattella germanica* has served as a useful model for delineating endocrine regulation of nonvolatile cuticular pheromones, namely (3*S*,11*S*)-dimethylnonacosan-2-one. Both the amount of pheromone on the cuticular surface and *in vivo* incorporation of radiolabel from [$^{14}C_1$]propionate into the sex pheromone coincide with active stages of vitellogenesis, suggesting the involvement of JH (Schal *et al*., 1990a, 1994; Sevala *et al*., 1999). Indeed, females treated so as to reduce their JH III titer (allatectomy, antiallatal drugs such as precocene, starvation, or implantation of an artificial egg case into the genital vestibulum, which inhibits JH biosynthesis) produce less pheromone (Schal *et al*., 1990a, 1994; Chase *et al*., 1992). Furthermore, pheromone production is 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 11**) (Chase *et al*., 1992).

Normally, adult male cockroaches have a much lower titer of JH III in the hemolymph (Piulachs *et al*., 1992; review: Wyatt and Davey, 1996). Because males also produce 3,11-dimethylnonacosane, metabolism of the alkane to contact pheromone may be contingent upon high JH titers in the adult female. In response to exposure to the JH analog hydroprene, female pheromone increased sixfold in treated males (Schal, 1988), showing some capacity to express the putative female-specific polysubstrate monooxigenase. The parallels are striking with estrogen induction of vitellogenin synthesis in the male liver of oviparous vertebrates, JH induction of vitellogenin synthesis in male cockroaches (Mundall *et al*., 1983), and ecdysteroid induction of female pheromone production in houseflies (see Section 3.14.5.3.2) (Blomquist *et al*., 1984b, 1987).

Contact pheromone production in *B. germanica* is also regulated through the regulated production of its precursor, 3,11-dimethylnonacosane. Biosynthesis of this alkane drops dramatically when food intake declines at the end of each vitellogenic phase (Schal *et al*., 1994, 1996), suggesting that hydrocarbon biosynthesis is linked to food intake, as in nymphs (Young *et al*., 1999), and not directly to either the ecdysteroid or JH titers. Dietary intake also stimulates the production of JH III (Schal *et al*., 1993; Osorio *et al*., 1998), which in turn stimulates the conversion of the alkane to contact pheromone. In allatectomized females large amounts of hydrocarbons accumulate in the hemolymph because food intake is not suppressed (i.e., no gestation) and hydrocarbons are not provisioned into oocytes (i.e., no vitellogenesis) (Schal *et al*., 1994; Fan *et al*., 2002). As hydrocarbons accumulate in the hemolymph, the amount of cuticular pheromone also increases, suggesting that excess 3,11-dimethylnonacosane is 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 JH-mediated metabolism of 3,11-dimethylnonacosane to 3,11-dimethylnonacosan-2-one.

**3.14.5.5.3. Transport and emission of 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. 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. Recent studies in *L. 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*., 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* (Cornette *et al*., 2001). These exciting findings suggest that carrier proteins might be involved in the transport of volatile pheromones to the cuticle, but functional studies will be needed to verify this hypothesis.

Attractant sex pheromones are usually emitted while the female or male cockroach performs a species-specific calling behavior (Gemeno and Schal, 2004; Gemeno *et al*., 2003). As in most lepidopterans, JH III regulates calling behavior in both species *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 (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 analog (Liang and Schal, 1994). In this species, JH is also required for females to become sexually receptive and accept courting males (Schal and Chiang, 1995).

In *B. germanica*, the transfer of contact pheromones to the epicuticular surface is mediated by lipophorin, a high-density hemolymph lipoprotein (reviews: Schal *et al*., 1998b, 2003). As in *M. domestica* (see Section 3.14.5.4.4), newly biosynthesized pheromone appears first in the hemolymph before it is noted on the epicuticle (Gu *et al*., 1995). That hemolymph is required to transport the pheromone to the cuticular surface was demonstrated by severing the veins that enter the forewings. Subsequently, the amount of hydrocarbons and pheromone that appeared on the wings was significantly lower than on the intact forewings of the same insects.

In the cockroaches *P. americana* and *B. germanica*, virtually all newly synthesized hydrocarbons that enter the hemolymph are bound to lipophorin (Chino, 1985; Gu *et al*., 1995). Moreover, in both species newly synthesized hydrocarbons can only be transferred from the integument to an incubation medium if lipophorin is present, and other hemolymph lipoproteins, such as vitellogenin, cannot mediate this transfer (Katase and Chino, 1982, 1984; Fan *et al*., 2002). The mechanisms by which hydrocarbons and pheromones are taken up by lipophorin are poorly understood. Takeuchi and Chino (1993) demonstrated clearly in the American cockroach that a very-high-density lipid transfer particle (LTP) catalyzes the transfer of hydrocarbons between lipophorin particles. However, *in vitro* experiments with purified lipophorin of *P. americana* and *B. germanica* have shown that lipophorin accepts hydrocarbons from oenocytes, apparently without the involvement of LTP (Katase and Chino, 1982, 1984; Fan *et al*., 2002). It remains to be determined whether this is because sufficient LTP remains bound to dissected tissues, if LTP is produced by the dissected tissues, or whether it plays no significant role in hydrocarbon and pheromone uptake by lipophorin.

Interestingly, while the uptake of hydrocarbons and pheromones by lipophorin *in vitro* appears to lack molecular specificity (Katase and Chino, 1984; Fan and Schal, unpublished data), their delivery to pheromone gland cells is highly specific (Schal *et al*., 1998a, 1998b). Uptake of lipophorin and its ligands might involve receptor-mediated endocytosis, as demonstrated in mosquito oocytes (Cheon *et al*., 2001; see **Chapter 3.9**). This might explain why some 3,11-dimethylnonacosan-2-one has been isolated from mature ovaries of the German cockroach (Gu *et al*., 1995) and (*Z*)-9-tricosene is found in housefly ovaries (Schal *et al*., 2001). However, an endocytic process would fail to discriminate various ligands, suggesting that alternative mechanisms need to be investigated.

## 3.14.6. Concluding Remarks and Future Directions

Our increased understanding of the biochemistry and regulation of pheromone production over the last two decades has been most impressive. A 1983 review of the biochemistry and endocrine regulation of insect pheromone production (Blomquist and Dillwith, 1983) was 16 pages long. It was limited to early work on pheromone biosynthesis in moths and the housefly, and recognized that JH and ecdysteroids may play a role in the regulation of pheromone production. Since that time the discovery of PBAN and its role in the regulation of lepidopteran pheromone production have been elucidated, along with determining which enzymes are affected by JH and ecdysteroids to regulate pheromone production in model cockroaches/beetles and flies, respectively. The work in Lepidoptera has moved from simply demonstrating that pheromone components were synthesized *de novo* to the molecular characterization of the unique Δ11-desaturase and other desaturases that are involved in many female moths and their interplay with specific chain shortening steps. While it is still true that in no system do we have a complete understanding of both the biochemical pathways and their endocrine regulation, we do have a much better understanding of how pheromones are made and in some systems are developing an understanding of their regulation at the molecular level. The continued application of the powerful tools of molecular biology along with studies using genomics and proteomics will only increase the rate at which we increase our understanding of pheromone production. Ultimately, just as behavioral chemicals themselves have been extended into pest

control, research on pheromone production will be directed toward practical applications in insect control.

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## Relevant Website

http://www.nysaes.cornell.edu – The Phenolist, a listing of lepidopteran phenomones compiled at Cornell University.