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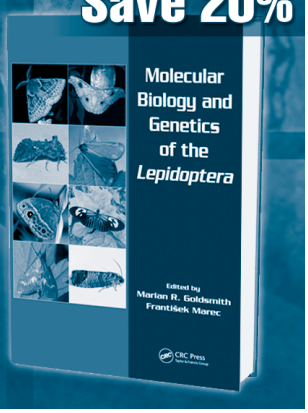

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A Need for Wedding Genetics, Biochemistry, and Molecular Biology

Fred Gould, Astrid T. Groot, Gissella M. Vasquez, and Coby Schal

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INTRODUCTION

Why study sexual communication in Lepidoptera?

In night-flying moths, highly specific, long distance, pheromonal communication is essential for mating success and reproductive isolation of species. Emission of two or more volatile compounds by females, in precise ratios, is typically required to attract conspecific males (e.g., Cardé and Haynes 2004). Although there are thousands of moth species with unique pheromone blends (e.g., Cork and Lobos 2003; Witzgall et al. 2004; El-Sayed 2008), the evolutionary processes that resulted in this diversity of sexual communication signals and species are not understood.

A rare female with a mutation leading to an alteration in pheromone blend is expected to have lower mating success than normal females unless males do not discriminate between the typical and altered blends (Butlin and Trickett 1997). And, in almost all published studies, normal males do discriminate against females with atypical pheromonal signals (e.g., Zhu et al. 1997). Similarly, a male with a mutation that results in response to an altered female pheromone blend is expected to be less efficient at finding typical females. Evidence of this lower efficiency comes from studies of moth genotypes that differ in pheromone responses (e.g., Linn et al. 1997). This selection against new male and female mating traits, when they are at low frequency, is expected to result in stabilizing selection that could constrain the evolutionary diversification of moth mating communication systems (Butlin and Trickett 1997; Phelan 1997).

Hypotheses to explain the evolution of new mating communication signals and responses have invoked the possibility that alteration of the signal and response are pleiotropically controlled by the same genes (Hoy, Hahn, and Paul 1977), or that males do not prefer normal females over those with altered blends. Published research to date does not support either of these assumptions (Butlin and Ritchie 1989; Butlin and Trickett 1997). Nevertheless, the impressive diversity of chemical mixtures used by moths for sexual communication stands as evidence that evolution of novel signal/response systems has not been stymied.

There is debate among evolutionary biologists who conclude that diversification is highly unlikely when there is stabilizing selection (e.g., Coyne, Barton, and Turelli 1997) and others who find evidence that stochastic events (i.e., genetic drift) could result in diversification, even in the face of stabilizing selection (Wade and Goodnight 1998). Both of these groups agree that the likelihood of such diversification would be influenced by (1) the number of genes involved in the initial divergence, (2) the magnitude of effect of each gene on fitness-related phenotypes, and (3) allelic interactions affecting fitness-related phenotypes (Coyne and Orr 1998; Wade and Goodnight 1998; Dieckmann and Doebeli 1999; Kondrashov and Kondrashov 1999; Whitlock and Phillips 2000).

The major premise of this chapter is that combining a detailed understanding of quantitative genetics, biochemistry, and molecular biology of the signals and responses used by moths will enable us to understand better how this system diversified, and could serve as a model for studying evolution of other traits that appear to be under stabilizing selection. Molecular and biochemical studies alone can tell us how many and which enzymes are in the biosynthesis pathways leading to production of a pheromone blend. They can also tell us which specific molecules are needed for males to perceive these blends. Quantitative genetic studies on their own could tell us a lot about how many genes affect variation in signals and responses within and between species. However, it is only by combining genetic and molecular studies that we will be able to understand just what kind of changes (e.g., single nucleotide polymorphisms in open reading frames, cis- or trans-regulatory changes) in which genes led to diversification of moth mating systems. These types of data can also inform the ongoing debate about whether changes in open reading frame sequences or in regulatory sequences have been more critical to the evolution of ecological adaptation and diversification (Carroll 2005; Hoekstra and Coyne 2007).

In this chapter we present an overview of what is and is not known about the genetics, biochemistry, and molecular biology of female moth pheromone production and male response. In doing so, we show that even though there are major gaps in our knowledge, overall, a great deal is known about these aspects of sexual communication in moths, and that by combining the knowledge in these areas we could make major steps forward in our understanding of evolution. At the end of this chapter we point out a few potential avenues for future research.

QUANTITATIVE GENETIC STUDIES

Female Pheromones

The state of knowledge regarding quantitative genetics of female pheromone blends has been reviewed in detail (e.g., Löfstedt 1990, 1993; Linn and Roelofs 1995; Butlin 1995; Phelan 1997; Roelofs and Rooney 2003; Cardé and Haynes 2004), so we will only present a selective overview.

Early genetic studies of sexual communication systems focused on determining whether the same genes that controlled signal production also controlled signal perception in the opposite sex through pleiotropic effects (sometimes referred to as genetic coupling). Although an early empirical study found evidence supporting the possibility of genetic coupling of acoustic mate communication (Hoy, Hahn, and Paul 1977), later studies of both acoustic and chemical sexual communication indicate that such coupling is very rare (see Butlin and Ritchie 1989). One study that did find a genetic correlation between male and female signal/response traits in offspring from field-collected insects determined that these correlations broke down after randomized mating in the laboratory (Gray and Cade 1999). This indicated that gametic disequilibrium and not pleiotropy (or strong physical gene linkage) had caused the correlation. Given the lack of evidence for genetic coupling, recent efforts have focused on understanding the genetic architecture of variation in signal production and response that would allow coevolution of male and female aspects of sexual signaling in insects (see Butlin and Trickett 1997; Phelan 1997).

Studies of the genetic architecture of differences in sexual communication between two races of *Ostrinia nubilalis* stand out as the most complete for any insect species. The ratios of the acetate pheromone components *E*11–14:OAc and *Z*11–14:OAc, produced by females of two races of *O. nubilalis*, differ dramatically (the E strain with a 97:3 ratio of E to Z acetates, and the Z strain with a 1:99 ratio), and males of each race prefer females of that race. The differences in pheromone blend between the races appear to be mostly controlled by a single autosomal gene (Klun 1975), although multiple genes with tight physical linkage cannot be ruled out. Other modifier genes have smaller impacts on the blend ratio (Löfstedt et al. 1989). The major gene that controls pheromone blend is not linked to the genetic region that controls male behavioral response to the pheromone (e.g., Linn et al. 1999). Recent genetic analysis of *Ostrinia scapulalis*, which also has a Z and an E race in Japan, demonstrates that the difference in this species' races also segregates as if it is mostly controlled by a single gene (Takanashi et al. 2005). The *O. scapulalis* system is especially interesting because it is one of the few in which the males have not been found to discriminate between the pheromone blends of the two races (Takanashi et al. 2005).

Studies of F_2 and backcross progeny from hybridization of other lepidopteran species have also uncovered evidence suggestive of single-gene control of production of specific pheromone component ratios. In most of these cases, as with *O. nubilalis*, the difference between the species is simply in the isomeric forms of the pheromone components, and both isomers are derived from the same precursor. A noteworthy exception, where one gene controls a change in ratios of less-related pheromone components, involves a laboratory-derived mutant of *Trichoplusia ni* (Haynes and Hunt 1990; Jurenka et al. 1994; Zhu et al. 1997). In this case, a genetic change in a chain-shortening enzyme is hypothesized to cause the altered component ratios, and as in other cases, the normal males were less attracted to the mutant female pheromone blend (Zhu et al. 1997). In the other cases mentioned, increases in one pheromone component result in a decline in only one other component.

Although the literature emphasizes examples of single-gene control, there are also cases where multiple genes may be involved in sexual communication differences between races and species (Cardé and Haynes 2004). Most studies supporting multiple-gene control involve simple segregation analyses that find no evidence for single-gene control, or find that single-gene explanations are not sufficient to account for all of the genetic variation observed (e.g., Teal and Tumlinson 1997). Unfortunately, these qualitative findings have little explanatory power.

To understand better traits controlled by multiple genes, our laboratory has utilized a genetic approach called Quantitative Trait Locus analysis (QTL; Remington et al. 1999) for assessing the number of loci responsible for differences between *Heliothis virescens* and *H. subflexa* in their complex pheromone blends (Sheck et al. 2006). These two species differ in ratios or presence/absence of at least ten compounds and therefore present a rich system for analysis. In our first study, the two moth species were hybridized and then backcrossed to *H. subflexa*. Pheromone glands from female progeny of these backcrosses were analyzed, and DNA from each moth was subjected to AFLP marker analysis (Sheck et al. 2006) to construct a genetic map of all thirty-one chromosomes (Gahan, Gould, and Heckel 2001). Because the F_1 individual used in the cross was a female, there was no recombination (Heckel 1993), so each chromosome from *H. virescens* remained intact as a single LG. This crossing design enabled us to correlate the presence of specific chromosomes from the nonrecurrent parent (*H. virescens*), with the ratios of compounds in the pheromone glands of individual backcross progeny females. The basic results from these crosses are shown in Figure 10.1.

This study demonstrated that at least five chromosomes were involved in determining the difference between the two species in the composition of the blend in the pheromone gland, implying that at least five loci affect the difference between blends of the two species (Sheck et al. 2006). In two cases, a specific chromosome significantly impacted the relative amount of only a single compound, but other chromosomes affected the relative amount of two to four compounds; and in four cases a single compound was affected by more than one chromosome. Moreover, females that had both *H. virescens* chromosomes 4 and 22 had the least amount of the three acetate esters that are only found in *H. subflexa*. This coupling of genetic control of the amounts of all three acetates suggests that the same metabolic process affects the concentration of each.

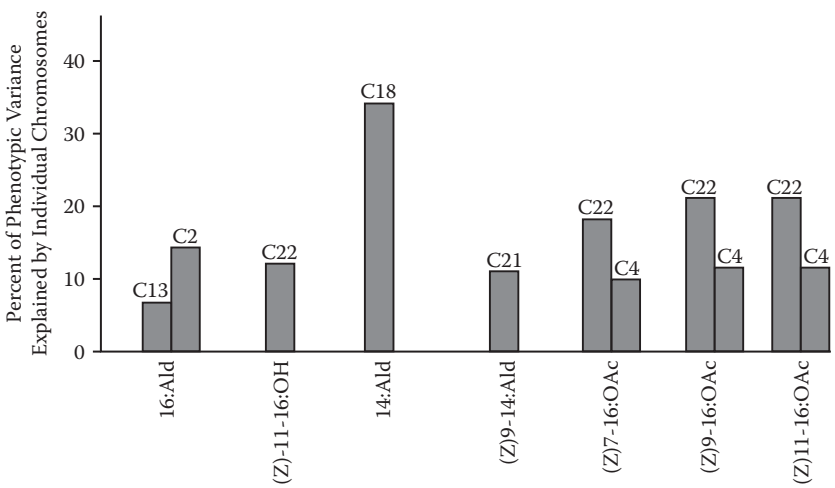

FIGURE 10.1 Results of QTL analysis of pheromone components in *Heliothis virescens* and *H. subflexa*. The percent of variation in the amount of specific *Heliothis* pheromone compounds in backcross females that can be explained by the presence/absence of specific chromosomes from *H. virescens* (out of thirty autosomes and one sex chromosome). Chromosomes are numbered with the prefix C. This backcross entailed mating of F_1 females (*H. virescens* X *H. subflexa*) to *H. subflexa* males. From data in Sheck et al. (2006).

We are conducting more QTL studies using backcrosses to both *H. virescens* and *H. subflexa* in an attempt to gain a better understanding of genes with smaller phenotypic effects that could impact evolutionary processes. Although the increased number of crosses and the higher sample size of backcross female offspring are expected to reveal more QTL that affect the pheromone blends, QTL studies are just one step toward the understanding of the evolutionary forces and genetic pathways that resulted in diversification of pheromone blends. Ultimately, it will be necessary to move from QTL analyses or other quantitative genetic methods to the molecular level to determine the types of genes and mutations that were involved in the diversification in moth mating systems.

Male Perception and Response

In comparison to what we know about the genetics of pheromone blends, very little is known about the genetics of male moth response to pheromones. The most detailed and fascinating published result in this area is a study by Cossé et al. (1995), who found that males from the two *O. nubilalis* pheromone races differed in signals transmitted by olfactory receptor neurons (ORNs) after stimulation with each of the two pheromone components, but that these differences in ORNs' responses to the pheromones mapped to a completely different genomic location than the males' actual behavioral response. This result emphasizes the point that studying the genetics or the receptors alone could lead to erroneous conclusions. Beyond the studies on genetics of male *Ostrinia* species and race response to pheromones, we could find published genetic experiments on only one other pair of moths. In crosses between *Ctenopseustis obliquana* and *C. herana*, male perception of pheromone blends was mostly sex linked (Hansson, Löfstedt, and Foster 1989). Studies of genetics of male response in more species groups is clearly needed to determine if this pattern is common.

MOLECULAR AND BIOCHEMICAL STUDIES OF PHEROMONE BLENDS

Biochemical Analyses of Pheromone Synthesis

The sex pheromones of many moths are even-numbered C_{10}–C_{18} straight-chain, unsaturated derivatives of fatty acids, with the carbonyl carbon modified to form an oxygen-containing functional group (alcohol, aldehyde, or acetate ester). Free saturated fatty acids are produced *de novo* and converted to their acyl-CoA thioesters before being incorporated into glycerolipids or converted to pheromone (Foster 2005). Pheromone precursor acids appear to be stored mostly in triacylglycerols, with lesser amounts associated with other glycerolipids and phospholipids (Foster 2005). During periods of high pheromone biosynthesis, triacylglycerols are hydrolyzed to release stored fatty acids, which can then be converted to pheromone (Foster 2005). The most common fatty acids produced in Lepidoptera pheromone glands are stearic acid (18:CoA), palmitic acid (16:CoA), and myristic acid (14:CoA; Jurenka 2003). These acids can subsequently be reduced to alcohols (OH) or aldehydes (Ald) via fatty acid reductase (Morse and Meighen 1987). Alcohols are converted to aldehydes by alcohol oxidase, and to acetate esters (OAc) by acetyltransferase. Conversely, aldehydes can be converted to alcohols by aldehyde reductase, while acetates can be converted (back) to alcohols by acetate esterase (Tumlinson and Teal 1987; Roelofs and Wolf 1988). The most commonly observed pathways of Lepidoptera sex pheromone biosynthesis are schematically depicted in Figure 10.2.

What We Know and Do Not Know About Enzymes and Genes Involved in Pheromone Biosynthesis

In contrast to the extensive biochemical studies to elucidate pheromone biosynthetic pathways, only a few studies have been conducted to identify molecularly the genes encoding the enzymes involved

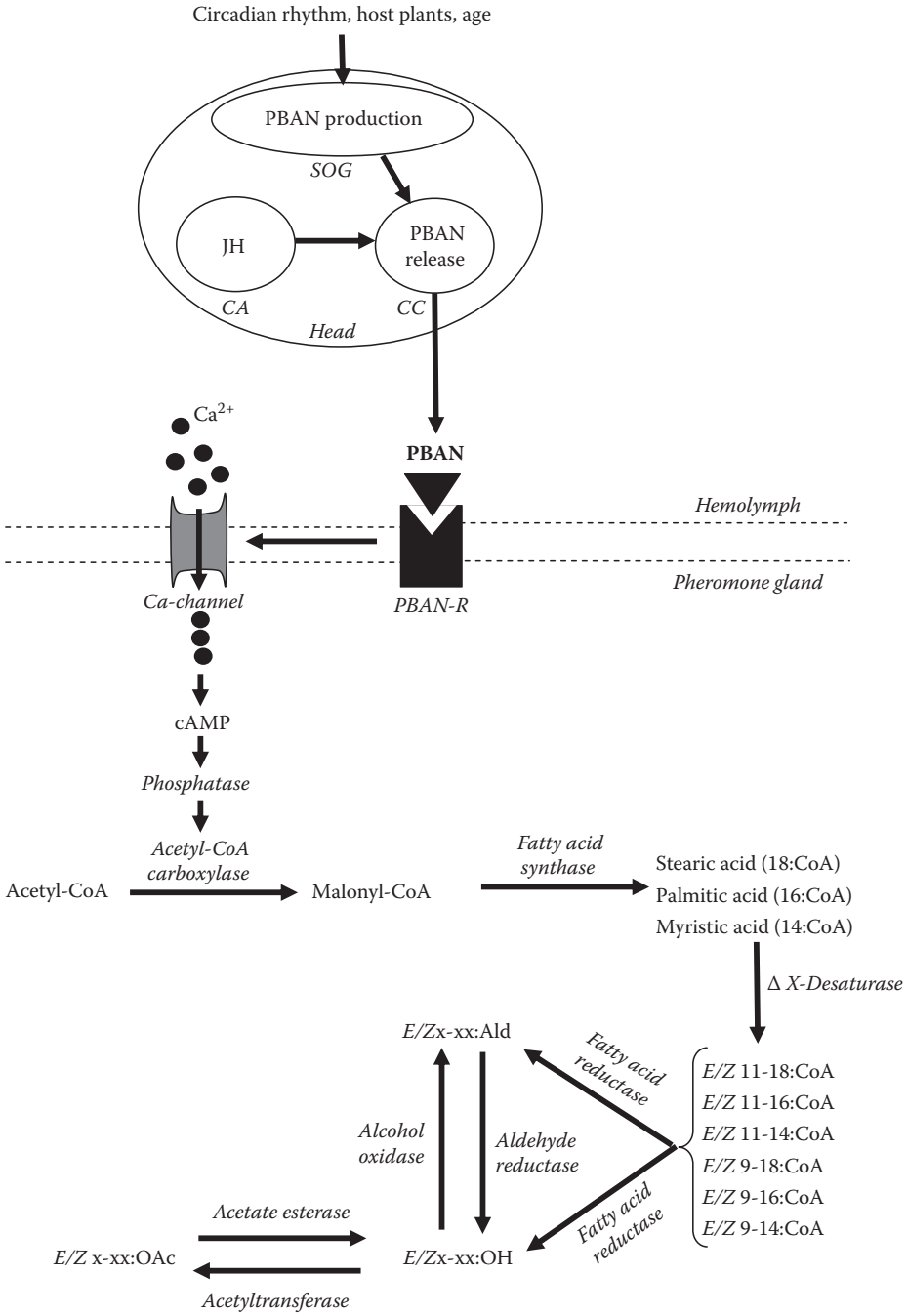

FIGURE 10.2 Schematic view of enzymes involved in the likely pathway of sex pheromone biosynthesis in most Lepidoptera. All possible enzymes are shown, although production of cAMP is not apparent in *B. mori* (Hull et al. 2007). Δx-desaturase may be Δ5, Δ9, Δ10, Δ11, Δ12, Δ13, and Δ14. PBAN, pheromone biosynthesis activating neuropeptide; SOG, suboesophagial ganglion; JH, juvenile hormone; CA, corpora allata; CC, corpora cardiaca; OH, alcohol; Ald, aldehyde. Adapted from Jurenka (2003), Rafaeli (2005), and Ohnishi, Hull, and Matsumoto (2006).

in these pathways. The desaturases stand out as the major exception, and most research has focused on identifying desaturase genes in a number of moth species (e.g., Knipple et al. 1998, 2002; Tsfadia et al. 2008). Only one additional enzyme has been molecularly characterized so far, a fatty acyl reductase in *Bombyx mori* (Moto et al. 2003). Below we give an overview of studies that have characterized enzymes and enzymatic reactions involved in pheromone biosynthesis in moths.

Acetyl-CoA Carboxylase

Acetyl-CoA carboxylase (ACCase) catalyzes the ATP-dependent carboxylation of acetyl-CoA to malonyl-CoA in the rate-limiting step of long-chain fatty acid biosynthesis (Pape, Lopez-Casillas, and Kim 1988). In *Helicoverpa armigera* and *Plodia interpunctella* females, when the activity of ACCase was inhibited, sex pheromone biosynthesis was inhibited as well, indicating that ACCase is a key regulatory enzyme in the pheromone biosynthetic pathway in these moth species (Eliyahu, Applebaum, and Rafaeli 2003; Tsfadia et al. 2008). Evidence from incorporation of labeled isotopes into sex pheromone components indicates that the activity of ACCase is influenced by pheromone biosynthesis activating neuropeptide (PBAN) in a number of moth species (e.g., Jurenka, Jacquin, and Roelofs 1991), but so far it has not been determined whether production of this enzyme is upregulated in response to PBAN treatment (Rafaeli 2005).

Fatty Acid Synthase

Animal fatty acid synthase (FAS) is the largest known multifunctional protein, having the most catalytic domains (Wakil, Stoops, and Joshi 1983). In insects, elongation reactions in integumental microsomal fractions have been studied in the housefly (Gu et al. 1997), German cockroach (Juárez 2004), and in triatomine bugs (Juárez and Fernández 2007), primarily in the context of production of long-chain methyl-branched fatty acids, alcohols, and hydrocarbons. The multifunctional enzyme FAS uses malonyl-CoA, acetyl-CoA, and NADPH to synthesize saturated fatty acids in two-carbon increments; methylmalonyl-CoA is used to insert a methyl branch in the aliphatic chain. The principal end-products of FAS in most lepidopteran systems, demonstrated with labeling studies with acetate, are palmitic acid (16:0) and stearic acid (18:0; e.g., Jurenka, Jacquin, and Roelofs 1991). FASs have been sequenced in a number of insect species (e.g., *Aedes aegypti*, accession XM_001658958 and XM_001654917; *Drosophila melanogaster*, accession number NM_134904). No FAS enzymes involved in sex pheromone biosynthesis of moths have been identified or characterized.

Chain-Shortening Enzymes

Changes in the substrate specificities of chain-shortening enzymes can lead to diversification of pheromone blends, as demonstrated in populations of *Zeiraphera diniana* (Baltensweiler and Priesner 1988), *Argyrotaenia velutinana* (Roelofs and Jurenka 1996), and *Agrotis segetum* (Wu et al. 1998). Of particular note is an in vitro enzyme assay study of a mutant line of *T. ni*, which produced elevated amounts of *Z*9–14:OAc, a minor component of the pheromone blend of normal *T. ni* females (Haynes and Hunt 1990). Jurenka et al. (1994) demonstrated that whereas pheromone glands of normal females mostly shorten *Z*11–16:CoA to *Z*7–12:CoA with two rounds of chain shortening, the pheromone glands of mutant females shorten *Z*11–16:CoA by only one round, to *Z*9–14:CoA. Chain-shortening enzymes have not been characterized or sequenced in insects, but they are presumably similar to vertebrate peroxisome enzymes (Bjostad and Roelofs 1983).

Desaturases

Integral membrane desaturases are ubiquitous in eukaryotic cells, where they play a primary role in the homeostatic regulation of physical properties of lipid membranes in response to cold (Tiku et al. 1996). In female moth pheromone biosynthesis, desaturases introduce a double bond into the saturated fatty acid chain or a second double bond into monounsaturated fatty acids. Moth pheromone desaturases, including Δ5, Δ9, Δ10, Δ11, Δ12, Δ13, and Δ14, have different regio- and

stereo-specificities. Several desaturases have been sequenced and characterized by expressing them in yeast cells lacking an endogenous desaturase in order to elucidate their specific role in the sex pheromone biosynthetic pathway (Knipple et al. 1998; Matoušková, Pichová, and Svatoš 2007).

Δ9-Acyl-CoA desaturases occur commonly in animal and fungal tissues (Liu et al. 1999), which suggests that these desaturases are ancestral and serve general functions in organisms. This may explain why the Δ9-desaturase sequences are highly conserved in animals (Rodriguez et al. 1992). Two Δ9-desaturase groups have been identified and characterized in pheromone glands of moth species: one with a substrate preference of $C_{16} > C_{18}$, and the other with a substrate preference of $C_{18} > C_{16}$ (Rosenfield et al. 2001). Thus, it seems that the integral membrane desaturase gene family has evolved in Lepidoptera to function not only in normal cellular lipid metabolism, but also in pheromone biosynthesis (Knipple et al. 2002).

One phylogenetically related group of Δ11-desaturases that catalyzes the formation of Δ11 fatty acyl pheromone precursors is specifically expressed in lepidopteran sex pheromone glands (Knipple et al. 1998). Some amino acid positions in this desaturase group are hypervariable among species (Knipple et al. 2002). No function has been determined for the other three desaturase types which are also regularly found in sex pheromone glands (Knipple et al. 2002).

Some desaturase genes are transcribed in pheromone gland cells but are not translated to proteins (see Roelofs and Rooney 2003; Xue et al. 2007). For example, in *O. nubilalis* three Δ14 gene sequences and ten Δ11 desaturase genes have been found; but only one transcript, for a Δ11 desaturase, appears to be functional in this species, which uses *Z*11– and *E*11–14:OAc pheromone components. *Ostrinia furnacalis*, which uses *Z*12– and *E*12–14:OAc pheromone components, has two Δ14 desaturase genes and five Δ11 genes (Xue et al. 2007). However, in *O. furnacalis* only protein products of a Δ14 desaturase gene were found in the pheromone gland (Roelofs and Rooney 2003).

Fatty Acid Reductase

There are two routes for aldehyde pheromone biosynthesis in moths. The fatty acyl CoA pheromone precursor can be reduced to the corresponding alcohol by certain fatty acid reductases (FARs) and then oxidized to the corresponding aldehyde through an alcohol oxidase (e.g., Rafaeli 2005). Alternatively, aldehydes can be formed by direct action of a specific FAR on fatty acyl CoA. In the two races of the European corn borer (*O. nubilalis*), the distinct pheromonal blends appear to be determined by differences in the specificity of their respective fatty acyl reductase (Zhu et al. 1996): The FAR in the Z strain shows greater selectivity for *Z*11–14:Acyl, whereas in the E-strain there is greater selectivity for *E*11–14:Acyl. Unfortunately, the actual enzymes have not been identified or isolated.

Evidence for FAR activity was found in homogenates of *B. mori* pheromone glands by reduction of palmitoyl-CoA to the corresponding hexadecanol without the release of the aldehyde intermediate (Ozawa and Matsumoto 1996). Subsequently, Moto et al. (2003) identified an alcohol-generating FAR in *B. mori*. The sequence of this FAR showed homology with that of a plant FAR (jojoba), which converts seed wax fatty acids to their corresponding fatty alcohols (Metz et al. 2000). Ohnishi, Hull, and Matsumoto (2006) used dsRNA injections into pupae to silence the pheromone gland FAR in *B. mori*. Suppression of FAR expression reduced bombykol (alcohol pheromone) production to basal levels, confirming that FAR plays an important role in pheromone production *in vivo* (see Matsumoto et al. 2007). No other FARs have been identified from moth pheromone glands.

Aldehyde Reductase

Activity of aldehyde reductase has been detected in gland extracts of *Choristoneura fumiferana* (Morse and Meighen 1986). It is very difficult to prove that these enzymes first produce aldehydes that are then converted to alcohols because aldehyde reductases are also present that catalyze the reduction of the fatty aldehyde to the alcohol, so alcohols and not aldehydes are the major products (e.g., Fang, Teal, and Tumlinson 1995). The reverse reaction is catalyzed through alcohol oxidases. Both enzymes are more generally called alcohol dehydrogenases.

Alcohol Oxidase

Fatty alcohols are pheromone intermediates as well as pheromone components in the pheromone glands of many moth species, and alcohol oxidases catalyze the formation of aldehyde pheromones from these alcohols. Fang, Teal, and Tumlinson (1995) demonstrated that the oxidase in the cuticle of the pheromone gland of *Manduca sexta* converts alcohols of different chain length (C_{14}–C_{17}). Hoskovec et al. (2002) showed that the oxidase in *M. sexta* glands can also oxidize other primary alcohols, including aromatic, allylic, or heterocyclic compounds, although there is a strong preference for primary alcohols of benzylic, saturated, and allylic types (Luxová and Svatoš 2006). The overall substrate specificity closely resembled yeast alcohol dehydrogenase, but so far the enzyme has not been successfully isolated (Luxová and Svatoš 2006).

Acetyltransferase

This functional class of enzyme converts fatty alcohols to acetate esters in pheromone glands; it has been biochemically characterized in *C. fumiferana* (Morse and Meighen 1987) and *A. velutinana* (Jurenka and Roelofs 1989). In both species acetyltransferases were found only in the pheromone gland. Substrate preference assays conducted in vitro indicated specificity for the Z isomer in *A. velutinana* as well as in other tortricid moths, but not in *T. ni* (Noctuidae) or *O. nubilalis* (Pyralidae; Jurenka and Roelofs 1989). Remarkably, although acetate esters are common pheromone components in moths, no acetyltransferase genes have been cloned.

Acetate Esterase

Hydrolysis of esters occurs during pheromone synthesis as well as degradation (Ding and Prestwich 1986; Prestwich, Vogt, and Riddiford 1986). Acetate esterase activity in pheromone glands has been shown in *C. fumiferana* (Morse and Meighen 1987), *Hydraecia micacea*, *H. virescens*, and *H. subflexa* (Teal and Tumlinson 1987). In *H. subflexa* acetate esters are components of the pheromone blend (e.g., Groot et al. 2007), but in *H. virescens* acetates have never been found in the gland and they strongly antagonize attraction in an otherwise attractive blend (e.g., Groot et al. 2006). Teal and Tumlinson (1987) suggested that acetate esterase in *H. virescens* glands converts the acetates into alcohols as rapidly as the acetate esters are produced.

MOLECULAR AND BIOCHEMICAL ANALYSIS OF PHEROMONE PERCEPTION

Male moth navigation toward receptive females is achieved through intermittently emitted trace quantities of female sex pheromones (Roelofs and Cardé 1977). Male moths intercept these chemical signals by means of trichoid sensilla (Kaissling and Priesner 1970). These specialized antennal cuticular hairs contain one to three specialized ORNs narrowly tuned to distinct pheromone compounds (e.g., Baker et al. 2004). The pheromone molecule enters the trichoid sensillum lumen through a cuticular pore tubule and is typically encapsulated by a pheromone-binding protein (PBP) that transports the hydrophobic molecule through the sensillum lymph and toward the ORN dendrite (reviewed in Leal 2005; Rützler and Zwiebel 2005; Vogt 2005). The PBP ejects the pheromone upon interaction with negatively charged sites at the dendritic membrane, allowing it to bind with pheromone receptor proteins (PRPs) located on the ORN dendritic surface (Leal 2005; Rützler and Zwiebel 2005). Coupling of the pheromone molecule with its receptor results in a local depolarization that spreads to an electrically sensitive region of the neuron where nerve impulses are elicited. The electrical signal travels through the ORN axon to the brain, where axons of pheromone-responsive ORNs converge into the macroglomerular complex in the antennal lobe for further processing (Mustaparta 1996). Resetting of the ORN is possible through degradation of the pheromone molecule upon release from PRPs by pheromone-degrading enzymes (PDEs). This signal inactivation is essential for pheromone plume resolution (Vickers 2006).

While little is known about the genetics of differences among species and populations in male responses to pheromones, recent breakthroughs in molecular biology have led to a much better understanding of the amino acid sequences and biochemical properties of PRPs and pheromone-processing proteins [i.e., PBPs, PDEs, and chemosensory proteins (CSPs)] involved in pheromone perception (Jurenka 2003; Knipple and Roelofs 2003; Leal 2005; Rützler and Zwiebel 2005; Vogt 2005; Gohl and Krieger 2006; Hallem, Dahanukar, and Carlson 2006; Sato et al. 2008; Wicher et al. 2008). A model for the role of these molecular components in pheromone signal processing and proposed transduction mechanisms is depicted in Figure 10.3.

Pheromone Receptor Proteins

PRPs are members of a divergent family of insect ORs that contain seven-transmembrane domains (Mombaerts 1999). These PRPs lack any sequence similarity to vertebrate GPCRs, and exhibit an atypical membrane topology with the amino terminus located intracellularly (Benton et al. 2006). Insect ORs typically form a heteromeric complex composed of two subunits, a conventional and variable OR coupled with a highly conserved, ubiquitously expressed, Or83b coreceptor (for receptor phylogenetic relationships see Chapter 9). Lepidopteran PRPs also appear to dimerize with an Or83b ortholog chaperone protein as suggested by *in situ* hybridization and heterologous expression of *B. mori* PRPs in *Xenopus* oocytes (Nakagawa et al. 2005). However, in another *in situ* hybridization study (Krieger et al. 2005), there was no clear coexpression of *B. mori* PRPs with the chaperone protein. Furthermore, in Flp-In T-REx293/G_15 cells (Große-Wilde, Svatoš, and Krieger 2006) and *Drosophila* ab3A neurons (Syed et al. 2006), PRP was activated by the pheromone alone without expression of the chaperone protein. Differences in labeling techniques and PRP processing by the different heterologous host cells used may explain these conflicting results. Recent electrophysiological and fluorescent optical experiments on heterologously expressed insect OR heteromeric complexes, including a *B. mori* PRP-Or83b ortholog complex, showed that they form a cation nonselective ion channel, directly gated by odor or pheromone binding to the OR (Sato et al. 2008). In addition to this ionotropic signal transduction pathway, a metabotropic pathway involving a cyclic-nucleotide-activated channel in the Or83b coreceptor has also been shown (Wicher et al. 2008). These recent findings indicate that functional PRPs require the presence of the "helper" protein for chemical signal transduction.

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ORNs typically express only one conventional OR gene (Vosshall et al. 1999; Mombaerts 2004) that determines the ORN odorant response profile (Hallem, Ho, and Carlson 2004), and PRPs generally follow this one receptor–one ORN organization. However, unlike general insect ORs that typically bind to more than one ligand (e.g., Hallem, Ho, and Carlson 2004), PRPs are narrowly tuned to specific ligands (e.g., Große-Wilde et al. 2007).

Lepidopteran PRPs share little sequence similarity with general insect ORs, and possibly form a single lineage of proteins sharing a high degree of sequence similarity and exhibiting conserved functions as indicated by phylogenetic analyses of *B. mori* and *H. virescens* ORs (Krieger et al. 2005; Nakagawa et al. 2005; Wanner et al. 2007). These analyses also suggest that PRPs form two main lineages: one that expanded in the bombycids and the other in the noctuids (Figure 10.4). The higher degree of sequence identity within lineages suggests that these clusters may have arisen from ancestral pheromone receptor gene duplication events. As PRPs of more moth lineages are sequenced, this pattern may become more complex (for additional details of insect OR lineages see Chapter 9).

Candidate genes for *H. virescens* PRPs have been examined by J. Krieger and his colleagues. *H. virescens* olfactory receptors (HRs) were first identified by screening an antennal cDNA library with probes generated by an analysis of an *H. virescens* genomic database based on similarity to *Drosophila melanogaster* OR sequences (Krieger at al. 2002). Further screening of the antennal cDNA library with probes encoding HRs and other insect OR short regions allowed the identification

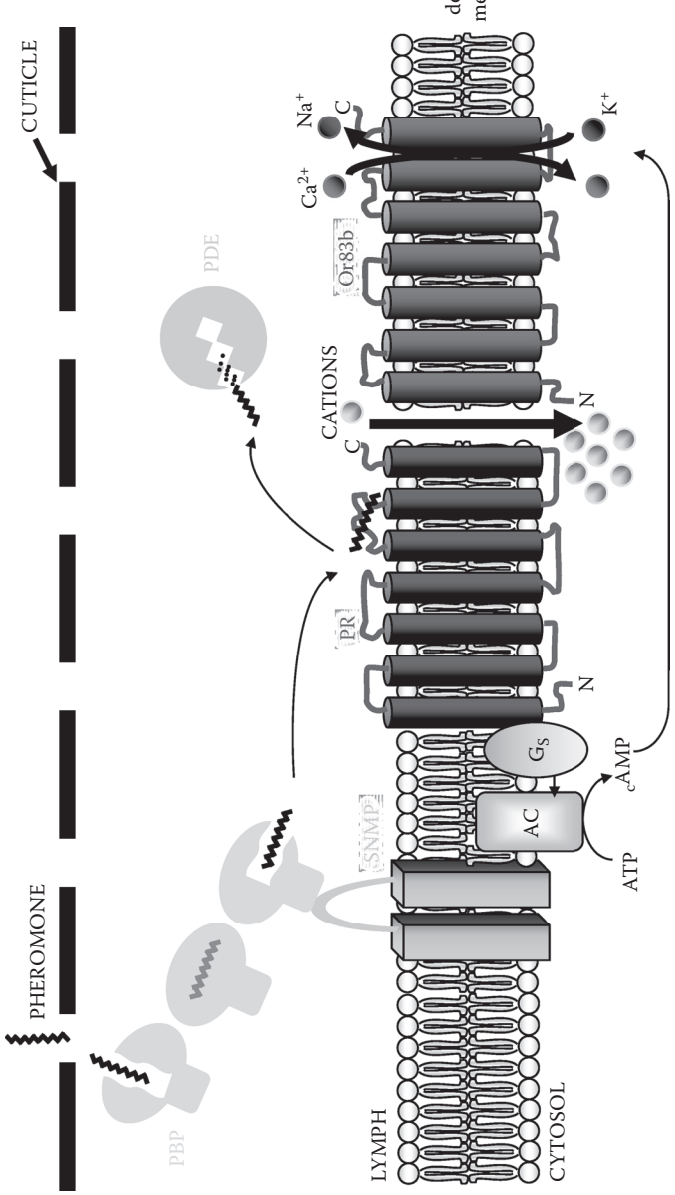

FIGURE 10.3 ***A color version of this figure follows page XXX.*** Schematic model of pheromone perception in moths. A pheromone molecule entering the lumen of a trichoid sensillum through a cuticular pore is bound to a pheromone binding protein (PBP), which transports the pheromone to the dendritic membrane of the olfactory receptor neuron (ORN). A sensory neuron membrane protein (SNMP) binds to the PBP-pheromone complex, or the pheromone only, and directs the pheromone to the nearby pheromone receptor (PR). The pheromone binds to the PR-Or83b heteromeric complex resulting in either very rapid recognition by means of an ionotropic pathway, or slower yet prolonged detection via a metabotropic G protein–mediated signal amplification. Pheromone degrading enzymes (PDEs) inactivate unbound pheromones.

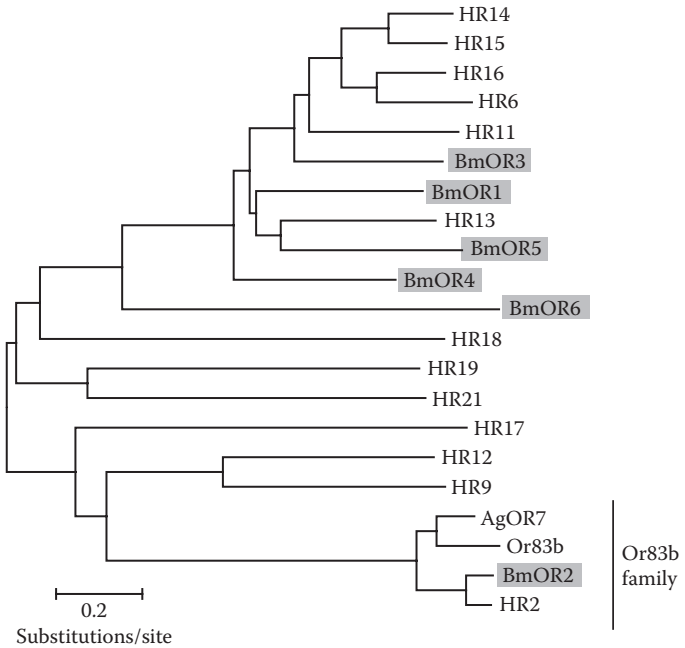

FIGURE 10.4 Phylogenetic tree of Ors for *Heliothis virescens* (HR)*, Bombyx mori* (BmOR), and *Anopheles gambiae* (AgOR). From Nakagawa et al. (2005).

of four candidate PRPs (HR13, HR14, HR15, and HR16) that are exclusively expressed beneath pheromone-responsive sensilla trichoidea of male antennae, and share at least 40 percent amino acid identity (Krieger et al. 2004). HR13 has been shown to be expressed in neurons of sensilla trichoidea type A (Gohl and Krieger 2006). Moreover, immunohistochemical studies combined with functional analysis in a heterologous expression system clearly indicate that HR13 specifically interacts with *Z*11–16:Ald, the major pheromone blend component of *H. virescens* (Gohl and Krieger 2006; Große-Wilde et al. 2007).

Male-specific *B. mori* OR genes were isolated by differential screening of a male antennae cDNA library, and the first *B. mori* PRP, BmOR-1, was identified based on sequence similarity to other insect ORs (Sakurai et al. 2004). BmOR-1 is exclusively expressed in cells located beneath the long trichoid sensilla and has high homology to some *H. virescens* receptors. Ectopic expression of this receptor in female antennae and in *Xenopus* oocytes demonstrated its specificity for bombykol, the silk moth sex pheromone (Sakurai et al. 2004). Subsequent studies using different heterologous expression systems further corroborated this finding (Große-Wilde, Svatoš, and Krieger 2006; Syed et al. 2006). Similar *in situ* hybridization and heterologous expression studies identified BmOR-2 as the receptor for bombykal, an oxidized form of bombykol that does not elicit male-orientating behavior (Nakagawa et al. 2005).

Heterologous expression systems, including *Xenopus laevis* oocytes (Sakurai et al. 2004), modified HEK 293 cells (Große-Wilde, Svatoš, and Krieger 2006), and the *D. melanogaster* Δ*halo* mutant with an empty ab3A neuron (Dobritsa et al. 2003) and *Or67d-GAL4* mutant (Kurtovic, Widmer, and Dickson 2007), have been used successfully for the functional characterization of candidate moth PRPs *in vivo*. Additionally, these systems could be used in the future for comparative functional analyses between wild and mutated PRPs to determine if specific changes in pheromone receptor gene sequences affect ligand specificity.

Pheromone-Binding Proteins

PBPs are members of the encapsulin family, proteins that solubilize hydrophobic compounds in aqueous environments (Vogt 2005). PBPs are ⟨-helical proteins characterized by the presence of a major hydrophobic domain, a signal peptide, and six well-conserved cysteine residues forming three disulfide bridges (e.g., Sandler et al. 2000). Unlike other members of the OBP gene family, PBPs are expressed exclusively or predominantly in long sensilla trichoidea (e.g., Laue and Steinbrecht 1997), where they are produced by support cells and found at high concentration in the lumen (Steinbrecht, Ozaki, and Ziegelberger 1992). PBPs bind, encapsulate, and ferry pheromones to the external PRP loops on the ORN dendritic membrane, and protect them from PDEs as well (Krieger and Breer 1999; Leal 2005). Contact with dendritic membrane negatively charged sites leads to the formation of an additional C-terminal ⟨-helix that fills the pheromone binding site and ejects the pheromone out of the PBP (Leal 2005, and references therein).

PBPs, first identified in *Antheraea polyphemus* (Vogt and Riddiford 1981), have been characterized from several moth species, allowing identification of multiple PBP subtypes displaying considerable diversity (32–92 percent amino acid identity; e.g., Abraham, Löfstedt, and Picimbon 2005). Phylogenetic analyses have shown that several duplication events appear to have given rise to specific subtypes (e.g., Robertson et al. 1999; Xiu, Zhou, and Dong 2008). Lepidoptera PBPs divide into three main groups, each comprising PBPs from various species, with noctuid PBPs forming three distinct groups (Figure 10.5) that may have arisen through two duplication events (Xiu and Dong 2007).

Cloning of PBP genes predates work on pheromone receptor genes; Krieger et al. (1993) cloned an *H. virescens* PBP over a decade ago. Functional assays using modified HEK 293 cells showed that an *H. virescens* PBP, HVIRPBP2, increased HR13 sensitivity and specificity to *Z*11–16:Ald (Große-Wilde et al. 2007). However, the specificity of two other heterologously expressed PRPs, HR14 and HR16, was not increased in the presence of HVIRPBP1 or HVIRPBP2. Interestingly, heterologous expression of *BmOR1* in HEK 293 cells showed that BMORPBP increased specificity to bombykol (Große-Wilde, Svatoš, and Krieger 2006), whereas *BmOR1* expression in *Xenopus* oocytes and Δ*halo* mutants indicated that BMORPBP was not necessary for response to bombykol (e.g., Syed et al. 2006). The latter findings correspond with early studies in *M. sexta*–cultured ORN, suggesting that PBPs are not necessary for PRP response to pheromones (Stengl et al. 1992). Hence, the pheromone alone rather than a PBP-pheromone complex appears to activate the PRP. However, PBPs play a role in pheromone perception kinetics and sensitivity: (1) PBP pH-dependent conformational change is consistent with the millisecond time scale of pheromone peripheral perception events essential during male moth oriented navigation (Leal et al. 2005); (2) PBPs facilitate the diffusion of pheromones into the sensillar lymph and transport them selectively (Leal 2005, Syed et al. 2006); (iii) PBPs screen out a subset of odorants and concentrate pheromones in the sensillum lymph (Pelosi 1996). By increasing the uptake of pheromone molecules, PBPs could lower the threshold for pheromone response (van den Berg and Zielgelberger 1991). Ligand binding ranges from very specific to very broad in PBPs (e.g., Rivière et al. 2003), thus only PBPs with high-binding specificity may be involved in pheromone component discrimination (Bette, Breer, and Krieger 2002; Maida, Ziegelberger, and Kaissling 2003). PBPs appear to be required for olfactory system sensitivity, and to some extent, specificity. However, the specific mechanisms involved remain to be determined.

Pheromone Degrading Enzymes

PDEs are thought to inactivate pheromones before they reach the PRPs if they are not bound to PBPs, and to degrade pheromone molecules that have already stimulated PRPs. PDEs can modify pheromone chemistry (Rybczynski, Reagan, and Lerner 1989), and degrade pheromone molecules on a millisecond timescale in vitro (Vogt, Riddiford, and Prestwich 1985), although degradation

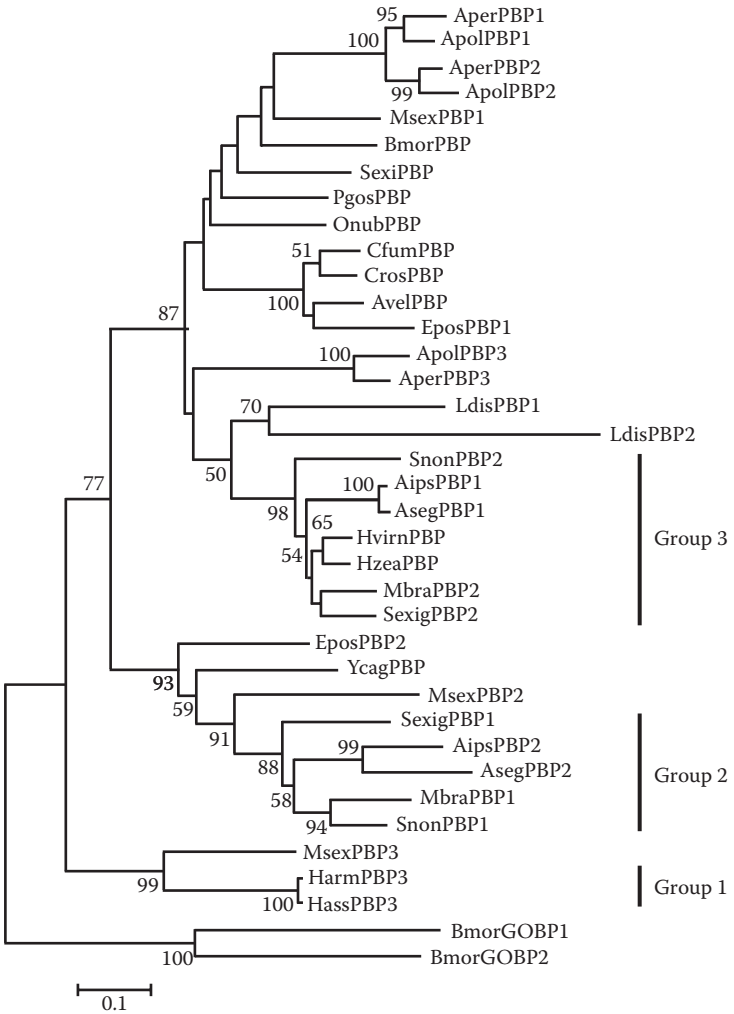

FIGURE 10.5 Phylogenetic analysis of amino acid PBP sequences. General odorant binding protein (GoBP) sequences are the outgroup. *Agrotis ipsilon* (Aips), *A. segetum* (Aseg), *Anthreraea pernyi* (Aper), *A. polyphemus* (Apol), *Argyrotaenia velutinana* (Avel), *Bombyx mori* (Bmor), *Choristoneura fumiferana* (Cfum), *C. rosaceana* (Cros), *Epiphyas postvittana* (Epos), *Helicoverpa armigera* (Harm), *H. assulta* (Hass), *Heliothis virescens* (Hvir), *H. zea* (Hzea), *Lymantria dispar* (Ldis), *Mamestra brassicae* (Mbra), *Manduca sexta* (Msex), *Ostrinia nubilalis* (Onub), *Pectinophora gossypiella* (Pgos), *Spodoptera exigua* (Sexig), *Sesamia nonagrioides* (Snon), *Yponomeuta cagnagellus* (Ycag). From Xiu and Dong (2007).

in vivo is slower, probably due to pheromone protection by PBPs (Kaissling 2001). Despite their important role in signal inactivation, knowledge on the molecular structures of PDEs is limited, and only a few genes have been identified and characterized (Vogt 2005).

A male antenna-specific esterase and a cytochrome P450 enzyme cDNA have been cloned in *A. polyphemus* (Ishida and Leal 2002) and *Mamestra brassicae* (Maïbèche-Coisne et al. 2002), respectively, but whether they function as PDEs is yet unclear. A previously characterized *A. polyphemus* sensillar esterase (Vogt, Riddiford, and Prestwich 1985), *ApolPDE*, has been isolated, cloned, and expressed in a baculovirus vector, allowing estimation of sensillar lymph concentration (approximately 20,000-fold lower than a PBP) and the study of pheromone inactivation kinetics (Ishida and Leal 2005). Hence, the *ApolPDE* sequence could be used to identify PDEs in other moth species.

In *B. mori*, an aldehyde oxidase preferentially expressed in male antennae catabolizes bombykal (Rybczynski, Vogt, and Lerner 1990). Recently, a partially sequenced *M. brassicae* aldehyde oxidase expressed exclusively in olfactory sensilla (Merlin et al. 2005) was used to identify putative aldehyde oxidase genes in *B mori* (Pelletier et al. 2007). A single gene selectively expressed in male *B. mori* antennae, *BmAOX2*, may code for the PDE involved in bombykal degradation; however, functional characterization is needed. Extended neural activity in ab3A sensilla heterologously expressing *BmorOR1* has been hypothesized to result from a lack of bombykol inactivation due to the absence of PDE in this system (Syed et al. 2006). This expression system could be used to test the role of *BmAOX2* in bombykal degradation.

Interactions Among PRP, PBP, and PDE

It is likely that PBPs, PDEs, PRPs, and the central nervous system all play roles in specificity within species and the differences among species in male response to pheromones. The importance of each is likely to differ by compound and taxonomic lineage (Leal 2005; Rützler and Zwiebel 2005; Vogt 2005; Hallem, Dahanukar, and Carlson 2006). Two "layers of filters," PBPs and PRPs, are thought to be involved in male response specificity through a combinatorial process (Leal 2003, 2005). For example, heterologously expressed *BmOR1* responds to both bombykol and bombykal; however, *BmorPBP* increases specificity, possibly by selective delivery of bombykol to this receptor (Große-Wilde, Svatoš, and Krieger 2006). Moreover, higher *BmOR1* response has been observed in the presence of BmorPBP in ab3A empty cells, possibly through pheromone solubilization (Syed et al. 2006). Heterologous coexpression of heterospecific PBPs or OBPs with *BmOR1* would ultimately corroborate BmorPBP function.

As previously mentioned, *Or83b* homologues appear also to play a role in pheromone molecular recognition through interaction with PRPs. *Or83b* is involved in Or localization to ORN dendrites and heterodimer formation, which is essential for receptor responsiveness and signal transduction (Rützler and Zwiebel 2005; Wicher et al. 2008; Sato et al. 2008). Similarly, other molecules such as sensory neuron membrane proteins could interact with ligands (Vogt 2003; Benton, Vannice, and Vosshall 2007) or act as PBP receptors (Rogers et al. 1997; Rogers, Krieger, and Vogt 2001; Jacquin-Joly and Merlin 2004).

Processing of Signals

In male moths, pheromone response is governed by pheromonal excitation of peripheral olfactory pathways that activate behavioral circuits in the brain. Pheromone-induced electrical signals spread from the ORN dendrites to axons that project to enlarged glomeruli in the macroglomerular complex (MGC) of the antennal lobes, where signals are further processed and sent out through projection neurons to the protocerebrum (Vickers, Poole, and Linn 2005). It is known that each pheromone component of a blend is represented in a single MGC glomerulus and that the combinatorial pattern of activity across several glomeruli represents the pheromonal blend (Vickers and Christensen 2003). Moreover, ORNs expressing the same pheromone receptor (PR) gene are expected to converge onto one glomerulus (e.g., Datta et al. 2008). Unlike mammals, where ORN axons' convergence in the olfactory bulb is receptor dependent, insect ORs, and possibly PRPs, are not involved in guiding axon convergence into their cognate glomeruli (Dobritsa et al. 2003).

In *D. melanogaster* males, activation of the sex pheromone cis-vaccenyl acetate (cVA) receptor inhibits male-male courtship, whereas in females it promotes receptivity to males. Using an approach combining genetics and optical neural tracing, Datta et al. (2008) found that cVA activates a single glomerulus, which is innervated by post-synaptic projection neurons (PNs) exhibiting sexually dimorphic projections in the protocerebrum lateral horn. A male-specific transcription factor, Fruitless (Fru^M) in the glomerulus PNs and other Fru^M-expressing cells, controlled the formation

of the male-specific axonal arbor in the lateral horn. Behavioral dimorphism could have resulted from third-order neurons receiving greater input from male PNs or restricting their synapses to the male-specific region of the glomerulus axon arbor. A similar sexually dimorphic neural circuit in the protocerebrum may occur in moth species. Moreover, between-species differences in male response to the same pheromone compound may be explained by a comparable interspecific anatomical dimorphism that may lead to either positive or antagonistic responses in closely related species perceiving the compound. Thus, it is feasible that central nervous system processes dictate these opposite responses.

FUTURE DIRECTIONS

In comparison with most other ecological traits of animals, we know quite a bit about the molecular and biochemical underpinnings of pheromone production and perception. Less is known about the genetic architecture of variation in sexual communication systems, but that knowledge base is increasing. Each of these areas of research is of interest on its own, but it is a synthesis of these three areas of knowledge that will provide more insight into the processes that gave rise to evolutionary diversification of pheromone-based sexual communication systems. A few researchers have begun this synthesis, but much more is needed. Below, we summarize a number of testable hypotheses and research approaches that could aid in this synthesis, and we describe some of the pioneering work in this area.

"Candidate Gene" versus "Genomic Network" Hypotheses

Based on what we know, it is possible that most of the genetic changes involved in diversification of pheromone-based systems were changes in the amino acid sequences of enzymes in pheromone production pathways, and in sequences of receptors and other proteins involved in pheromone detection. This could be considered the "candidate gene hypothesis." At the other extreme, it is possible that most of the evolutionarily important genetic changes that led to diversification were due to alterations in complex trans-acting genetic factors that regulate expression of these candidate genes or modify their effects on the phenotype. This could be considered the "genomic network hypothesis." Although one can find examples of other traits in which one of the two hypotheses hold, there has been considerable discussion about which of these two mechanisms is most important to evolution in general; however, the data base is limited (Wittkopp, Haerum, and Clark 2004, 2008; Carroll 2005; Sambandan et al. 2006; Hoekstra and Coyne 2007). Because candidate genes for male perception of pheromones are well described, and those for pheromone biosynthesis should soon be in hand, testing these hypotheses in the moth sexual communication system should be feasible and promises to help resolve this more general debate.

Molecular Analysis of Past Selection on Pheromone Production and Response

There are direct and indirect ways to test the two hypotheses defined above. One approach is to build databases of the genomic DNA sequences that code for the production of each of the candidate genes in many species from a genus or family of moths that have diversified in the components of their pheromone blends. This information can then be used to examine patterns of change in homologous genes of these species and to search for signatures of stabilizing/directional selection, drift, and gene duplication/loss. This was an onerous task a few years ago, but recent technological breakthroughs in sequencing make this more feasible today. As discussed above, Knipple et al. (2002) made a major breakthrough by using cDNA coding sequences for desaturases in a number of subfamilies of Lepidoptera as a means to assess such patterns. Their findings were complex, and patterns differed for the six grouping of desaturases examined. Overall, there was no signature of directional selection, but some amino acid substitutionsin one group of desaturases are in positions

that could alter the catalytic site of the enzyme. More detailed structure-function relationships are needed to examine this possibility. Comparisons of the desaturases of Lepidoptera and *D. melanogaster* indicate that gene duplication resulting in at least three of the desaturase groups occurred around 280 million years ago (Knipple et al. 2002).

The general pattern of coding sequence conservation found by Knipple et al. (2002) at least hints at the possibility that differences in expression of desaturase genes between species may have a more important role in species-to-species pheromone differences than do changes in coding sequence. The approach taken by D.C. Knipple and his colleagues could be applied to the reductases and to other enzymes in the pheromone biosynthetic pathway once they are identified.

In terms of male response, cDNA sequences coding for PRPs and OBPs in a variety of species have been analyzed for simple phylogenetic relatedness (Figures 10.4 and 10.5). Willett (2000) took this one step further and found evidence for directional selection on PBPs from *Choristoneura* species. Curiously, there was no relationship among species in the extent of directional change in the PBPs and changes in the pheromone blends. Willett (2000) postulates that a selection force unrelated to pheromone blend could have been selecting on the protein sequences.

We feel that these few studies point toward a useful direction for future research. At least at the peripheral sensory level, we have many good candidate genes involved in male moth pheromone perception, so the raw material is available to conduct more detailed phylogenetic analyses. A caveat is that these phylogenetic studies are clearly informative, but typically they are not definitive. Without careful analyses such as done by Willett (2000), it is easy to draw erroneous conclusions about selective factors.

W.L. Roelofs and his collaborators and colleagues have conducted pioneering work that used the *Ostrinia* genus to couple more fully phylogeny, gene transcription, and translation. The pheromone composition of eight *Ostrinia* species has been determined. The major components in all of the species are unsaturated acetates except for *O. latipennis*, which only uses *Z*11–14:OH (see Roelofs and Rooney 2003; Xue et al. 2007). As described in this chapter, the surprising result was that for each of the two species examined in detail, many more desaturase genes are transcribed than are translated. In the distantly related moth *B. mori*, it was also found that not all destaturase genes that were transcribed in pheromone gland cells produced active proteins (Moto et al. 2004). Although it is clear that desaturase production differences have been involved in diversification of *Ostrinia* pheromones, many of the differences appear to be due to posttranscriptional processes, so a simple candidate gene analysis could miss the important factors.

At the evolutionary and mechanistic levels, it becomes important to determine what type of changes in what genomic DNA sequences determine which desaturase mRNA transcripts are translated into functional proteins. At this point we cannot answer the question of how many genetic changes or genetic networks control differences among the species in enzymes involved in pheromone biosynthesis. There may be a single, small, critical gene sequence within the candidate gene, or a key cis regulatory element that controls most of the variation in the concentration and activity of a single enzyme; on the other hand, unrelated and unlinked genes could also regulate which mRNAs result in the production of active enzymes.

At the level of male response to pheromones, W.L. Roelofs' group again led the way in revealing the problem with assuming that changes in or around a candidate gene would be responsible for differences among males that respond to different pheromone blends. Their early work with *O. nubilalis* clearly showed that males of the E and Z races differed in the amplitude of the neuronal response spikes formed when exposed to the E and Z isomers of 11–14:OAc; the E strain had a higher amplitude spike when exposed to *E*11–14:OAc, and the Z strain had a higher amplitude spike to *Z*11–14:OAc (Roelofs et al. 1987). F_1 hybrids had spikes of intermediate amplitude, and F_2 male spikes indicated that the difference was inherited on a single autosome (Roelofs et al 1987). The simplest hypothesis was that the differences in spike amplitude were responsible for, or at least would be correlated with, the differences in behavioral response. Wind tunnel analysis of F_2 offspring found that males who

inherited the autosome that coded for the E race spike amplitude were no more likely to respond to the E strain pheromone blend than males who inherited the homologous chromosome coding for the Z race spike amplitudes. Further genetic analysis indicated that genes encoding the differential behavioral response were sex-linked (Roelofs et al. 1987). We still do not know what those sequences are, but it is clear that they are not in cis with genes that determine spike amplitude.

When W.L. Roelofs and his collaborators were doing this early genetic work, they were probably pleased that they were dealing with one autosome and one sex chromosome, because it made the genetic analysis feasible. Today, AFLP and microsatellite markers have made it much easier to localize loci that control pheromone responses in *O. nubilalis* (e.g., Dopman et al. 2005), and in the next few years it is reasonable to expect the entire nuclear genome of *O. nubilalis* to be sequenced, providing even more detailed information on the sequences differentiating the two *O. nubilalis* races. With greater information at the genomic level, we should certainly be able to identify alleles on the sex chromosome responsible for the differential male response. Similarly, we should be able to identify the enzyme-coding genes and regulatory sequences in the two races that code for changes in Z and E isomer ratios. Once the overall sequence differences are identified, it may be possible to determine which of the nucleotide differences are most important in altering the phenotypes. This kind of information will bring us much closer to understanding the evolutionary genetics of diversification in *Ostrinia* sexual communication.

Is the Genetic Architecture of Differences in Pheromone Blends and Responses the Same Within and Among Species?

A general question in evolutionary biology is whether the genes responsible for microevolution (changes within species) are the same as those that result in macroevolutionary changes (differences among species and higher taxa). Weber et al. (2008) reviewed a variety of studies in which a number of populations were selected equally for change in a trait. They conclude that for some traits, the genes responding to selection can differ completely between populations, but for other traits they always seem to be similar. In cases where the genetics of response is always similar, there is a higher likelihood that macroevolutionary changes will utilize the same genes as microevolutionary ones. If this is the case with moth sexual communication traits, then detailed studies on the origin of among population differences will be extremely helpful in understanding the macroevolution of diversity in moth sexual communication. On a cautionary note, however, in a study by Gleason and Ritchie (2004) the genetic regions found to affect differences in courtship song between two *Drosophila* species were not the same as the genetic regions associated with differences within *D. melanogaster.* If this is the case in other sexual communication systems, studies at the population level may not offer strong inference about macroevolutionary processes.

Coupling Molecular and Genetic Analyses With Lab and Field Studies of Behavior

Identifying which DNA sequence changes impact moth phenotypes is very important. However, if we are to understand how specific sequences have spread by natural selection, we must couple studies of sequence identification with tests of how they impact mating fitness of males and females. The ideal approach would be to examine effects of single genetic alterations on fitness of field-released individuals. While this may be possible in some species, it is not feasible with most moths. To get around this problem, Groot et al. (2006) estimated the fitness effect of genes that alter the amount of acetates in the pheromone blend of *H. subflexa* by using a combination of field and cage studies. The field studies estimated effects on long-distance attraction, while the cage studies measured probability of mating and sperm transfer. Similar kinds of approaches could be used to link phenotype to mating fitness in other moth systems. With these data in hand it would finally be possible to

determine the intensity of selection for or against rare females and males with single mutations in genes coding for sexual communication traits.

CONCLUSION

Moth sexual communication systems are intriguing to molecular biologists, biochemists, and evolutionary biologists because of their precision, efficiency, and incredible diversity. Although much could be learned by researchers in each of these disciplines working independently, the time seems to have arrived when more interdisciplinary work could result in breakthroughs that would be important for the understanding of moth sexual communication, and more generally for our understanding of the evolutionary process as a whole.

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