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Pheromone Reception

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Abstract Insects are analytical chemists *par excellence*. They perceive the world through semiochemicals with inordinate sensitivity. A male moth, for example, can detect a "scent of woman," i.e., a female-produced sex pheromone, even when the signal-to-noise ratio is very low. In a sense the antennae are "signal translators." The chemicals signals are "translated" into the language of the brain (nerve impulses or spikes) by an array of sensilla mainly located on the antennae. This information is conveyed to the brain for further processing. Chemical ecologists utilize insect antennae as biosensors for the identification of pheromones and other semiochemicals. The insect olfactory system is also highly selective, able to discriminate natural pheromones from molecules with minimal structural changes. In some cases, one stereoisomer functions as an attractant sex pheromone and its antipode is a behavioral antagonist (inhibitory signal). The specificity of the olfactory system seems to be achieved by two layers of filters. The first level of discrimination is determined by odorant-binding proteins (OBPs) that assist the hydrophobic pheromones to cross an aqueous barrier and reach their receptors. Both OBP and odorant receptor (OR) contribute to the specificity of the cell response and lead to the remarkable selectivity of the insect olfactory system. The members of the OBP-gene family, encoding the encapsulins, form a large group with olfactory and non-olfactory proteins. While the functions of many members of the family are yet to be determined, there is solid evidence for the mode of action of OBPs. Pheromones (and other semiochemicals) enter the sensillar lymph through pore tubules in the cuticle (sensillar wall), are solubilized upon being encapsulated by odorant-binding proteins, and transported to the olfactory receptors. Bound pheromone molecules are protected

from odorant-degrading enzymes. Upon interaction with negatively-charged sites at the dendritic membrane, the OBP-ligand complex undergoes a conformational change that leads to the ejection of pheromone. Direct activation of odorant receptors by odorant molecules initiates a cascade of events leading to the generation of spikes. Reverse chemical ecology is a new concept for the screening of attractants based on the binding ability of OBPs to test compounds.

Keywords Odorant-binding proteins · Odorant-degrading enzymes · Chiral discrimination · Encapsulins

1 Introduction

Insects perceive the world through small molecules which carry information (signature) for the recognition of potential mates, prey, and specific features of the environment, such as food sources, oviposition sites, etc. The information-carrying chemical compounds are referred to as semiochemicals, a generic term encompassing chemicals involved in intraspecific communications (pheromones) and interspecific interactions, such as kairomones (that give advantage to the receiver), and allomones (which benefit the sender). The entire olfactory process encompasses the perception of semiochemicals by a specialized apparatus in the periphery (normally the insect antennae; maxillary palpi in some cases), processing of signals in the antennal lobe, integration of these signals with other stimulus modalities in the protocerebrum, with ultimate translation into behavior (Fig. 1).

Because the chemical signals (semiochemicals) are normally produced in minute amounts and diluted in the environment with a complex mixture of chemical compounds derived from a myriad of sources, the olfactory system in insects evolved as a remarkably selective and sensitive system, which approaches the theoretical limit for a detector. For example, it has been estimated that the male silkworm moth is able to distinguish within 1 s 170 nerve impulses generated by the female silkworm moth's sex pheromone from 1700 spontaneous nervous impulses [1], thus, operating on a remarkably low S/N ratio!

In addition to sensitivity and selectivity, odor-oriented navigation in insects requires a dynamic process of signal deactivation (inactivation). While flying en route to a pheromone-emitting female (Fig. 2), males encounter pheromone molecules as intermittent signals comprised of short bursts of high flux separated by periods during which the flux is zero. The average duration of bursts of high flux is on the order of a millisecond and it decreases as the moth comes closer to the pheromone source [2]. Thus, a male moth has to detect rapidly and selectively minute amounts of pheromones buried in an "environmental mixture." Soon after the signal is detected, the pheromone detectors must be reset in a millisecond timescale so as to allow a sustained flight towards a pheromone source. In this chapter I provide a critical overview of our current under-

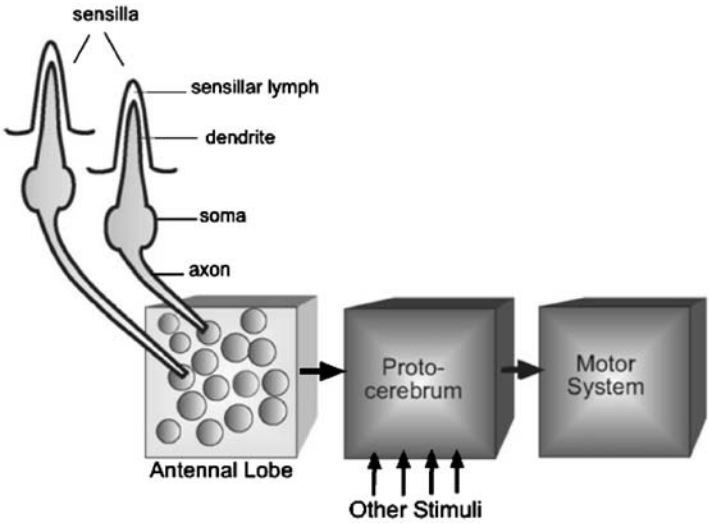

Fig. 1 Schematic view of the overall olfactory processing in insects. Pheromones and other semiochemicals are detected by specialized sensilla on the antennae, where the chemical signal is transduced into nervous activity. The olfactory receptor neurons in the semiochemical-detecting sensilla are connected directly to the antennal lobe. Here the semiochemical-derived electrical signals are processed and sent out (through projection neurons) to the protocerebrum. Olfactory information is then integrated with other stimulus modalities, a decision is made, and the motor system is told what to do

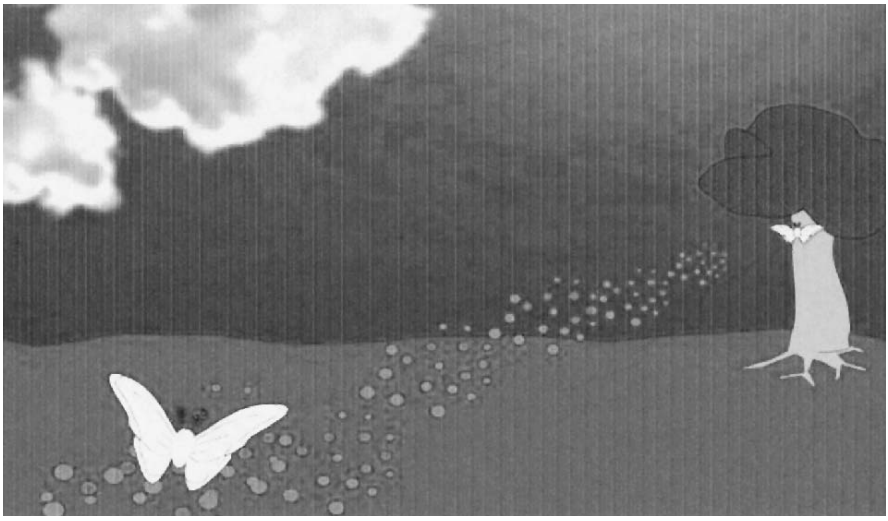

Fig. 2 Cartoon illustration of sex pheromone-mediated communication in insects. A female moth advertises her readiness to mate by emitting a chemical signal that permeates the air. Odorant-oriented navigation allows a male to pin-point the pheromone source

standing of olfactory mechanisms in insects, with emphasis on the molecular basis of pheromone reception.

2 Sensory Physiology

Largely, the insect detectors for pheromones and other semiochemicals are arrays of hair-like sensilla distributed over the surface of the antennae and palps. In some species, such as scarab beetles [3, 4] and the honeybee [5], semiochemicals are received by olfactory plates. The more ubiquitous hair-like sensilla typically consist of hollow cuticular hairs (10–400 µm long, 1–5 µm thick) innervated by one or several olfactory receptor cells (neurons) and three auxiliary cells [6].

The distal part of these receptor cells, the dendrites (0.1–0.5 µm in diameter), extend into the hair lumen (Fig. 3), whereas their axons are connected directly to the antennal lobes in the brain where they make the first synaptic contacts. In the giant silkmoth, *Antheraea polyphemus*, for example, each male

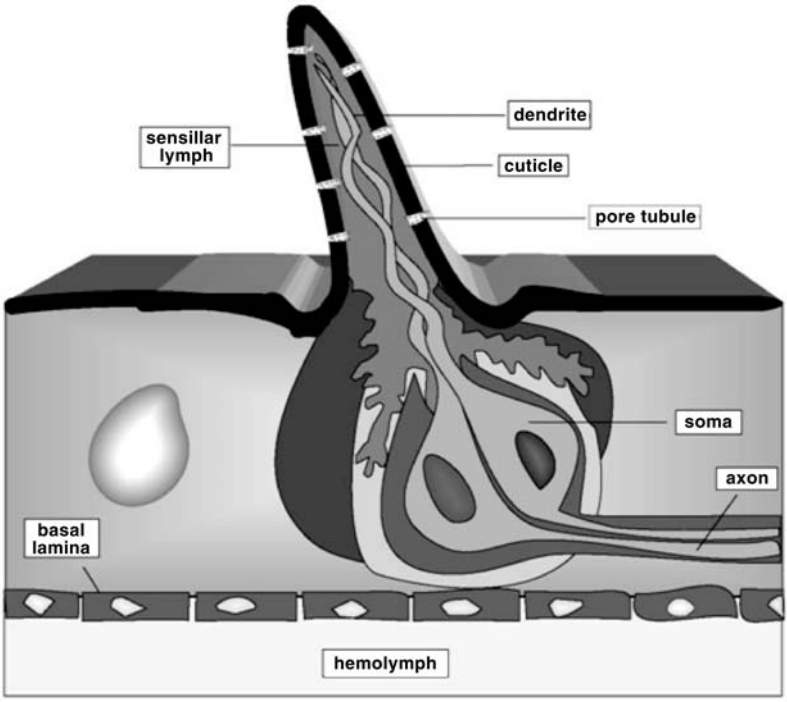

Fig. 3 Diagrammatic representation of a pheromone-detecting sensillum trichodeum of a moth antenna. Note the compartmentalization of the lymph and particularly its isolation from the hemolymph

antennae has ca. 60,000 pheromone-sensitive sensilla trichodea and 10,000 sensilla basiconica for the detection of other semiochemicals [7, 8]. On the other hand, females lack pheromone-detecting sensilla and have ca. 12,000 sensilla basiconica [9].

The first electrophysiological methods to study stimulus-response characteristics were developed along with the discovery of the first sex pheromones [10]. Upon interaction of pheromones and their receptors, the electrical conductance of the receptor cell membrane is modified producing a local depolarization, i.e., a receptor potential. Combined receptor potentials of many sensilla can be recorded in an electroantennogram (EAG). This is a simple approach to investigate stimulus-response characteristics, but requires pure chemicals. A powerful technique for the identification of pheromones, the gas chromatographic-electroanntenographic detection (GC-EAD) combines an EAG as a biological detector with a gas chromatograph (GC) for the separation of mixtures. The effluent from the GC column is split and sent towards a flame-ionization detector (FID) and an EAG, thus allowing the detection of stimuli "on the fly" from the GC. This "short-cut bioassay" allows the identification of minute chemical signals from highly contaminated samples (Fig. 4). This technique, widely applied in pheromone research, has also been utilized for the determination of the absolute configuration of pheromones, with stereoisomers being separated on a chiral phase capillary column [11].

The receptor potential, generated by interaction of pheromones and their receptors, spreads passively from the site of stimulation (somewhere in the

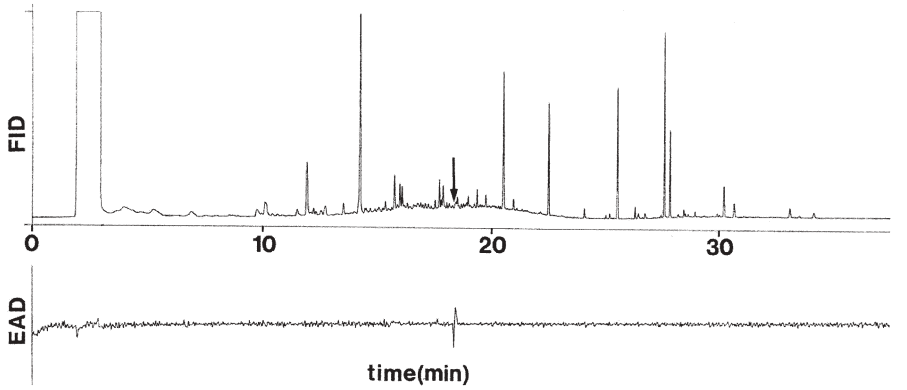

Fig. 4 Gas chromatographic traces of extracts from females of the pale brown chafer *Phyllopertha diversa* monitored by a conventional detector, flame-ionization detector (FID), and a biosensor, electroantennographic detector (EAD), using a male antenna as the sensing element. Although the peak of the sex pheromone (*arrow*) is hardly seen in the FID trace, its pheromonal activity was initially indicated by the strong EAD peak. Structural elucidation, followed by synthesis and behavioral studies lead to the identification of an unusual sex pheromone, 1,3-dimethyl-2,4-(1*H*,3*H*)-quinazolinedione [124]. It is unlikely that this minor compound would be fished out by a bioassay-oriented isolation procedure

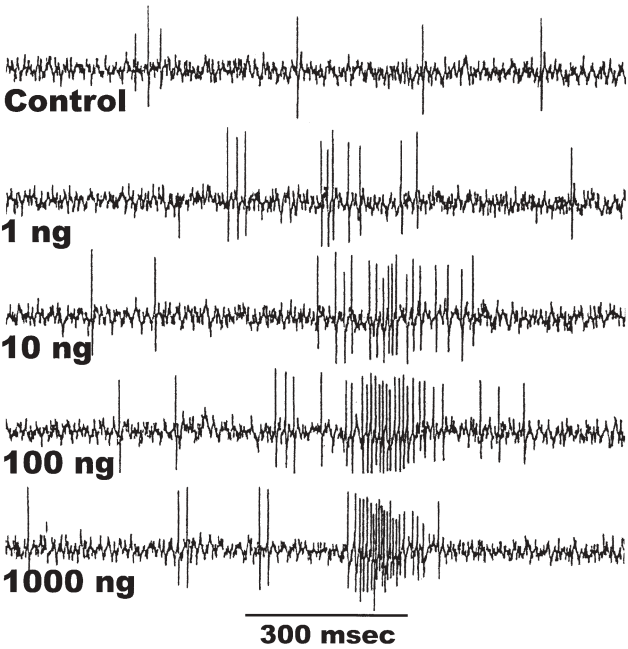

Fig. 5 Single sensillum recordings from the pheromone-detecting sensilla placodea on *P. diversa* male antennae. Note a dose-dependent increase in spike frequency after stimulus application for 300 ms (*bar*)

dendrite) towards an electrically-sensitive region (probably in the soma) where nerve impulses (spikes) are elicited [12] by the opening of voltage-dependent ion channels. Although intracellular recording of these nervous activities are technically difficult (if at all possible), olfactory sensilla allow extracellular recordings (Fig. 5), a technique called single sensillum recordings (SSR). As opposed to EAG, SSR represents the nervous activity generated by the neuron(s) innervating a single unit (sensillum) of the entire "compound nose." The number of olfactory receptor neurons (ORNs) in most olfactory sensilla ranges between two to five, but there are many exceptions, including sensilla placodea in wasps with as many as 140 ORNs [13]. Typically, multiple neurons in the same sensillum can be distinguished by different spike amplitudes, thus, allowing investigation of stimulus-response characteristics for each neuron.

Earlier experiments based on EAG and SSR highlighted the inordinate specificity and sensitivity of the insect olfactory system. While minimal structural modifications to pheromone molecules render them inactive [12], a single molecule of the native ligand is estimated to be sufficient to activate an olfactory neuron in male antennae [14]. The large number of detectors certainly contributes to the sensitivity of the olfactory system, but selectivity is a matter of

molecular recognition at the periphery. As described below, this remarkable selectivity of the insect olfactory system is likely to be achieved in two steps with odorant-binding proteins and odorant receptors participating as two "layers of filters."

3
Perireceptor Events in Insect Olfaction

Each sensillum in the insect antennae works as a "signal transducer" that responds to a specific chemical signal and "translates" it into the language of the brain, i.e., electrical signals. Interaction of pheromones and other chemical signals with their odorant receptors triggers a cascade of intracellular events called signal transduction (*sensu stricto*) which leads to nervous activity (spikes). Extracellular processes associated with the uptake, binding, transport, and release of the hydrophobic pheromones to their receptors as well as the post-interactive events related to inactivation of chemical signals are referred to as the "perireceptor events" [15] or early olfactory processing.

3.1
Odorant-Binding Proteins

In order to convey their message, pheromones and other semiochemicals must reach the dendritic surfaces of olfactory receptor neurons where the olfactory receptor proteins are located (Fig. 6). These odorant receptors are surrounded by an aqueous environment – the sensillar lymph. Although thin (1 µm), this aqueous layer is impenetrable for hydrophobic compounds per se. Thus, the transport through this barrier is assisted by odorant-binding proteins (OBPs). OBPs that are localized predominantly in pheromone-detecting sensilla with demonstrated ability to bind pheromones are referred to as pheromone-binding proteins (PBPs). Throughout this chapter the terms OBPs and PBPs are used as synonyms, although PBPs are OBPs which binds pheromones. PBPs are not only specific to antennae, but in some cases they occur mainly (if not only) in the sensillar lymph of male antennae. Strictly speaking, PBPs are not expressed in the sensillar cavity. They are expressed in auxiliary cells and secreted into the lumen; thus, the mature protein can be detected in the sensillar lymph. General odorant-binding proteins (GOBPs) are expressed in antennae of both sexes, or predominantly in female antennae, which are assumed to bind semiochemicals other than sex pheromones.

OBPs were initially identified in Lepidoptera and later isolated and/or cloned from various insect orders, namely, Coleoptera, Diptera, Hymenoptera, and Hemiptera ([16] and references therein). Recently, they have been identified from a primitive termite species [17], thus, suggesting that this gene family is distributed throughout the Neopteran orders. The three orders most

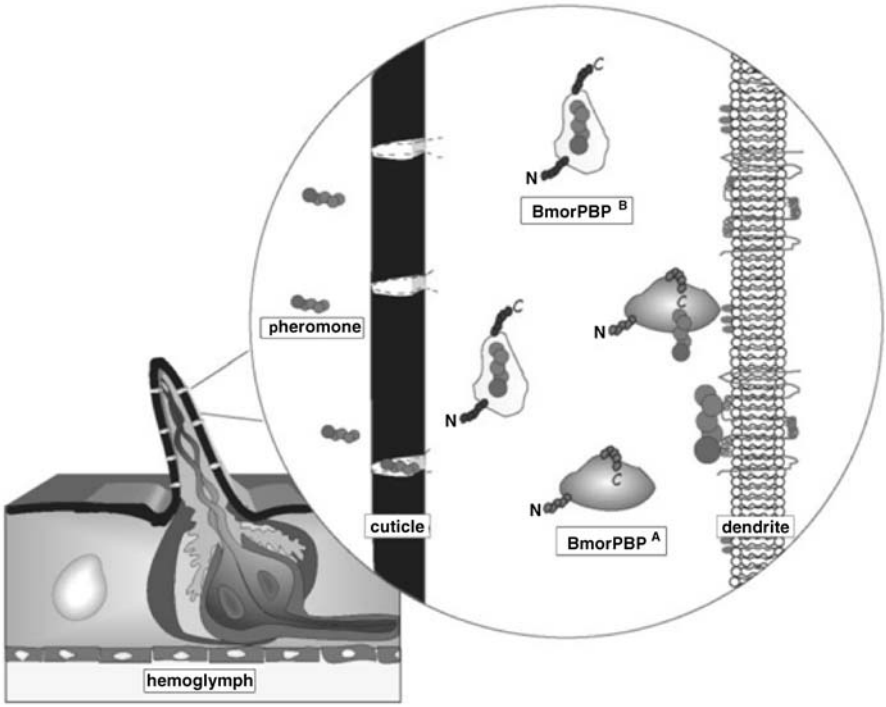

Fig. 6 Schematic representation of the proposed model for the mode of action of insect OBPs. Pheromones (and other semiochemicals) enter the sensillar lymph through pore tubules in the cuticle (sensillar wall), are solubilized upon being encapsulated by odorant-binding proteins, and transported to the olfactory receptors. Bound pheromone molecules are protected from odorant-degrading enzymes. Upon interaction with negatively-charged sites at the dendritic membrane, the OBP-ligand complex undergoes a conformational change that leads to the ejection of pheromone. In BmorPBP, this is achieved by the formation of a C-terminal α-helix in $BmorPBP^A$ that occupies the cavity that is the binding site in $BmorPBP^B$. In this model, the pheromone molecule (not the complex) activates the odorant receptor, thus, initiating a cascade of events leading to spike generation. As depicted in Fig. 1, the spikes travel through the axon to the antennal lobe

widely studied are the Lepidoptera (Saturniidae, Bombycidae, Sphingidae, Lymantridae, Tortricidae, and Pyralidae), Coleoptera mainly scarab beetles (Scarabaeidae), and Diptera (with the bulk of the literature focusing on *D. melanogaster*). In all rutelines (subfamily Rutelinae) investigated to date only one OBP has been found in each species, such as the Japanese beetle, *Popillia japonica*, the Osaka beetle, *Anomala osakana* [18], the Oriental beetle, *Exomala orientalis* [19], the cupreous chafer, *A. cuprea*, and *A. octiescostata* [20]. Binding data and homology suggest that the OBPs from these beetles are indeed PBPs. On the other hand, at least two OBPs have been identified in each melolonthine (subfamily Melolonthinae) species investigated, i.e., the pale

brown chafer, *Phyllopertha diversa* [21], the large black chafer, *Holotrichia parallela* and the yellowish elongate chafer, *Heptophylla picea* [22]. One of the two OBPs for each melolonthine species shows remarkable similarity to the pheromone-binding proteins from rutelines, whereas the second type of OBP forms a divergent group [20].

The literature describing the number of OBPs in different species is controversial with numbers ranging from 1 to 51 OBPs per species, but these values seem to be inaccurate. Even if a single OBP is involved in the detection of multiple compounds (see below), one would expect that the insect antennae possess multiple OBPs considering that insects can detect a number of physiologically relevant compounds (pheromones, flower scents, green leaf volatiles, other plant-derived compounds, etc.), which vary largely in their chemical structures. However, it is not clear how many proteins function as OBPs in insects. The discrepancy in the literature may be related to the method of "identification" of OBPs. Protein-based approaches are aimed at the isolation and identification of OBPs, followed by the cloning of the genes (or cDNAs) encoding these proteins. On the other hand, the gene-based approaches give little emphasis to expressed and functional proteins. While minor OBPs may be expressed at levels below the detection limits of the protein-based methods, the gene-based approach may lead to putative proteins which may not even be expressed in the sensillar lymph (of insect antennae). Another complication is that an identifying feature of insect OBPs, the six cysteine residues, is sometimes misleadingly used. The pheromone-binding proteins identified to date have six well-conserved cysteine residues, but this is not exclusive to OBPs and PBPs; insect defensins, for example, also have six well-conserved cysteine residues too. The spacing pattern between cysteine residues may indicate that a putative OBP belongs to the same OBP-gene family, but some members of this family may not be involved in olfaction [16]. The cysteine spacing pattern shows some variation when comparing OBPs from different insect orders (or different groups of OBPs), but they all have three residues between the second and the third Cys and eight residues between the fifth and the sixth Cys. Considering that the six cysteine residues play a pivotal role in the folding of pheromone-binding proteins [23–25], it is unlikely that other OBPs deduced from *Drosophila* genome sequence and having as many as 12-Cys residues [26] (Obp58b, Obp58c, Obp58d, Obp83c, Obp93a) would bind, transport, and release ligands in the same way as pheromone-binding proteins (like BmorPBP) do.

Out of the 51 deduced *Drosophila* OBPs, expression data is known only for 28 putative OBPs. Galindo and Smith used an elegant molecular approach to study expression of deduced *Drosophila* OBPs [27]. They fused several kilobases of upstream regulatory sequence for each OBP gene to a reporter gene encoding a nuclear-localized β-galactosidase. The transgenic flies carrying reporter constructs fused to each OBP promoter were stained for β-galactosidase activity [27]. Surprisingly, most members of the OBP-gene family were detected in various taste organs and olfactory tissues and some of them were expressed exclusively in taste organs. A caveat to their method is that the expression of the

proteins was not confirmed by immunocytochemistry using anti-OBP antibodies, thus not excluding completely the possibility that the reporter gene only, not the OBP genes, were expressed in some cases. Although it has been suggested that the *Drosophila* OBP-gene family comprises as many as 51 putative OBPs [26], only seven of them have been demonstrated to be expressed specifically in olfactory organs of *Drosophila* adults (antennae only or antennae and maxillary palpi): Obp19a, Obp57a, Obp69a (formerly named PBPRP-1), Obp83a (PBPRP-3, OS-F), Obp83b (OS-E), Obp84a (PBPRP-4), and Obp99d. Two other putative OBPs – Obp28a (PBPRP-5) and Obp76a (LUSH) – were detected in the antennae of adults as well as in larval chemosensory organs [27].

That LUSH functions as an odorant-binding protein was inferred from olfactory trap assays comparing wild-type adults with transgenic flies [28]. For this bioassay [29], traps are made of microfuge tubes and two pipette tips for each tube, one with the narrow end inserted into the severed end of the microfuge tube and the other placed as a sleeve in the opposite direction. Flies that are attracted to the lure (which is placed inside the microfuge tube) can get through the small aperture, but are unlikely to find a way out of the trap. A trap is placed inside a Petri dish (100 mm×20 mm) where ten adults are tested. These tests (performed during a period of time not specified in the original publications [28, 30]) showed no difference between wild-type and a LUSH-deficient mutant when a panel of 60 compounds was tested at low concentrations. However, there was a significant increase in the number of mutant flies in traps containing high concentrations of ethanol, propanol, and butanol. The high trapping at high concentrations of these alcohols could be due to increased attraction or a defect in avoidance. The authors supported the latter hypothesis because wild-type flies are less likely to be trapped in baits with an attractant (yeast extract) spiked with 25% ethanol. In other words, the so-called "avoidance to ethanol" would decrease the catches in traps baited with an attractant. To me these bioassays do not demonstrate conclusively that the increased trapping of *lush* mutant flies is due to a defect in avoidance rather than for an increased attraction to high concentrations of ethanol. Indeed the results suggest a decrease in trapping of wild-type flies in the yeast+25% ethanol traps as compared to yeast traps. The same tests, however, showed that the number of LUSH-deficient mutant flies caught in the yeast+25% ethanol traps were twice as much the number of flies captured either in traps baited only with yeast extract or those baited with ethanol only (see Fig. 3C in [30]). If this is due to avoidance to ethanol (rather than an attraction) why did the trapping of the *lush* mutant flies increase in the yeast-25% ethanol baits as compared to the baits with yeast alone? If they do not avoid ethanol at high concentrations, what is the explanation for the synergistic effect of ethanol and yeast extract? Last but not least, if flies are not attracted to ethanol why do they get through the ingenious device and get trapped? The inconsistency of these results may be derived from the design of the bioassay in which flies are subjected to still air and the only quantified observation is the end-product of the behavior (trapping). Also, there are no controls tested under identical conditions. Indeed, when flies

were tested with two-choice assay, the T-maze assay [31, 32], the *lush* mutant responded normally to ethanol not only at low but also at high concentrations [33]. It was observed, however, that the LUSH-deficient mutant lost attraction towards low concentrations of benzaldehyde while being repulsed by high concentrations, whereas the wild-type mutants showed attraction and repellency at low and high concentrations, respectively [33]. On the basis of these experiments, one cannot conclude that LUSH is involved in the binding, release, and delivery of either ethanol or benzaldehyde to olfactory receptors. A caveat to all bioassays utilizing benzaldehyde is the possible effect of benzoic acid. Typically, benzaldehyde is purchased from commercial sources and utilized without purification. It is, therefore, a mixture of at least benzaldehyde and benzoic acid (Fig. 7). Particularly when high doses are tested the amount of benzoic acid may be physiologically relevant.

Using a specific antibody, Shanbhag and collaborators [34] demonstrated that LUSH is expressed in sensilla trichodea of the *Drosophila* antennae along with two other putative odorant-binding proteins Obp83a (PBPRP-3, OS-F) and Obp83b (OS-E). When antennal sections of the LUSH-deficient mutant were la-

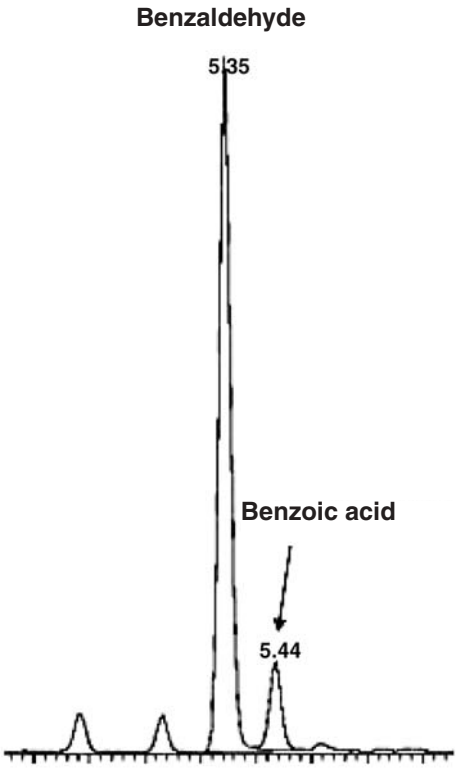

Fig. 7 GC-MS analysis data of a commercially available sample of benzaldehyde. Note the large peaks of impurities, particularly the considerable amount of benzoic acid

beled, they did not show any labeling with anti-LUSH, but showed normal staining with anti-Obp83a and anti-Obp83b [34]. Unfortunately, no electrophysiological data is available indicating that these sensilla are involved in the perception of benzaldehyde (or alcohol); it is known, however, that benzaldehyde and butanol are detected by sensilla coeloconica [35] and sensilla basiconica [36] and that the response for the whole antennae (EAG) recorded from *lush* mutant and wild-type flies were not different [28]. Also, there is no binding data supporting that LUSH binds benzaldehyde. Recently, the crystal structures of apo-LUSH was solved along with structures of LUSH bound to ethanol, propanol, and butanol [37], but there is no biochemical data indicating that LUSH binds to any ligand at physiologically relevant concentrations. Even if single sensillum recording experiments were to indicate that sensilla trichodea in *Drosophila* are involved in the detection of benzaldehyde or ethanol, one cannot make a clear-cut correlation between the defect of the *lush* mutant flies and the role of LUSH in olfaction. This is due to the co-expression of three putative odorant-binding proteins in these sensilla, namely, LUSH, OS-F, and OS-E [34].

In marked contrast to the ambiguous evidence for LUSH, there is growing evidence in the literature that other insect pheromone-binding proteins, such as, the PBP from the silkworm moth, *Bombyx mori* (BmorPBP), bind, solubilize, carry, and deliver pheromones to the pheromone receptors. (1) BmorPBP is predominantly expressed in the male antennae [38] and binds to bombykol, a cognate ligand [38] with some degree of specificity [39]. (2) BmorPBP is specifically localized in the long sensilla trichodea of males [40]. Females possess the same type of sensilla but rather than PBP they express a general odorant-binding protein. The long sensilla trichodea in male *B. mori* have been demonstrated to be the pheromone detectors [14], whereas in females they respond to benzoic acid and linalool [41]. (3) BmorPBP undergoes a pH-dependent conformational change [39, 42]. (4) The surfaces of dendrites are negatively-charged [43, 44], thus, generating localized low pH. (5) Evidence from structural biology (see below) demonstrates that the low pH (as expected near the surface of dendrites) triggers the formation of an additional C-terminal α-helix that fills the binding pocket thus leaving no room for pheromone in the binding cavity. (6) Binding assays showed that BmorPBP binds bombykol at the sensillar lymph pH but not at low pH as on the surface of dendrites [16].

3.1.1 Encapsulins, Members of the OBP-Gene Family

Insect OBPs are secretory proteins whose only posttranslational modification is the formation of three disulfide bridges [39, 45] from six cysteine residues. That six cysteine residues are well conserved in OBPs from species of the same order is a hallmark of these proteins. The disulfide links of OBPs in a few species have been determined by analytical methods, first in the OBPs from *B. mori* [45, 46]. As part of our attempt to get better insight into the structural biology of pheromone-binding proteins, we have determined the disulfide linkages

in recombinant and native BmorPBP [45]. The disulfide structures of the native PBP and GOBP-2 from *B. mori* were also identified by Scaloni and collaborators [46]. These OBPs showed the same cysteine pairing, i.e., Cys19-Cys54, Cys50-Cys108, and Cys97-Cys117. Similar disulfide structures were determined in the olfactory proteins from honeybee, *Apis mellifera*, ASP1 and ASP2 [47, 48] in the OBPs of the locust [49] and the paper wasp [50]. Therefore, the disulfide bridges of all OBPs analyzed to date show the profile of the first cysteine residue connected to the third one, the second linked to the fifth, and the fourth bound to the sixth, i.e., Cys(I)-Cys(III), Cys(II)-Cys(V), and Cys(IV)-Cys(VI). Another group of olfactory proteins, the chemosensory proteins (CSPs), differ from the six-cysteine-OBPs not only in the number of cysteine residues, but also in the function of the residues regarding the rigidity of their three-dimensional structures While in OBPs the three disulfide linkages play a pivotal role in the knitting together at least four of the helices (see below), the two disulfide bridges in CSPs close small loops involving residues 29 and 36 and 55 and 58 and, consequently, seem to have little rigidifying effect on the overall structure of CSPs [51].

Although the occurrence of six conserved cysteine residues, the spacing patterns of these residues, and possibly the pattern of disulfide structures are hallmarks of OBPs, the six-cysteine criterion alone is not sufficient to classify a certain protein as an olfactory protein [16]. It is important to demonstrate that an OBP is expressed only (or predominantly) in olfactory tissues. Evidence for their ability to bind odorants is also desirable, but *not sine qua non*. One of these criteria alone would not be enough to define a given protein as an OBP. For example, bovine serum albumin (BSA) binds to insect pheromones (Leal, unpublished data) and yet it is not an OBP because it not expressed in insect olfactory tissues. Conversely, a protein specific to antennae is not necessarily an OBP. There are other proteins that may be expressed in antennae but not in control tissues. Non-OBPs specifically accumulated in insect antennae have been previously detected (Ishida and Leal, unpublished data). Also, a glutathione-*S*-transferase has been reported to be expressed specifically in antennae of *M. sexta* [52].

The six conserved cysteine residues in a protein exhibiting the same pattern of cysteine spacing along with significant sequence similarity suggest that the protein may belong to the same structural (folding) family as PBPs and, consequently, infer that it may function in the same fashion. The assumption that such a protein is involved in olfaction, however, would be compromised if the protein was identified in non-olfactory tissues. Even if a non-olfactory protein has the same function as an OBP (carrier, for example), one has to keep in mind that the requirements for transport of hydrophobic ligands in non-olfactory tissues may not necessarily be as stringent as those for the fast delivery and inactivation of chemical signals.

Unfortunately, the term OBP has been rather imprecisely used in the literature. It sometimes refers to the olfactory function played by proteins, such as the pheromone-binding protein from BmorPBP. However, quite often OBP

refers to members of a gene-family, which may not be involved in olfaction. For example, a number of proteins with four conserved cysteine residues isolated from hemolymph of insects [53–55] are referred to as OBPs because of their sequence similarities and their conserved cysteine residues. Of particular note is the fact that the sensillar lymph (where OBPs assist in the transport of semiochemicals) is compartmentalized in olfactory tissues and completely isolated from the hemolymph by the epithelial cells, septate junctions between them, and basal membrane (Fig. 3). Indeed, the composition of the sensillar lymph is remarkably different from that of the hemolymph [56], particularly the unusual ion concentration (200 mmol/l K^+, 40 mmol/l Na^+), thus generating a transepithelial potential of +40 mV [6, 12, 56]. This compartmentalization is, therefore, the raison d'être for signal transduction. It is conceivable that these hemolymph proteins are part of a large family of carrier proteins that perform diverse functions in insects [55], but they are unlikely to be involved in any of the olfactory processes, particularly the perireceptor events.

The lack of a better term to separate the gene-family from the olfactory function performed by a few members of the family may be misleading. For example, Krieger and Ross [57] isolated two isoforms of a protein (GP-9) from the thorax of queens of the red imported fire ant, *Solenopsis invicta*, which has the same spacing pattern of six cysteine residues as observed in moth pheromone-binding proteins. Because the monogyne social form (colony having a single queen) and polygyne (multiple queens) form had only one (GP-9B) and two isoforms (GP-9B and GP-9b) of the protein, respectively, they suggest that these proteins may cause differences in worker's ability to recognize queens [57]. This work is widely referred to as "the first evidence for the direct involvement of PBPs in olfaction" [58]. Another citation is: "the two PBP alleles governing social behaviors suggest that different receptors might be activated by a specific PBP allele-social pheromone complex" [58]. The work by Krieger and Ross [57] lacks evidence that GP-9 either functions as a pheromone-binding protein or has any olfactory function. First, the protein was isolated from the thorax of queens; the existence of the protein in the sensillar lymph (where PBP functions) has never been demonstrated. Second, it is believed that workers detect a specific chemical signature related to by *Gp-9*^{b} gene in polygyne queens and thereby accept them, whereas all sexually mature queens lacking the same chemical signal are attacked and killed [59]. In other words, queens send off the signal that workers detect. If one is interested in "detection" of these semiochemicals, the olfactory system of workers (receivers) is to be investigated, not queens who are the emitters. Is it the lack of a "PBP" that makes them perceive a certain chemical signal? An elegant work [60] demonstrated that the monogyne queen emits a primer pheromone that makes the workers aggressive, i.e., the behavior is elicited because the workers can detect a certain primer pheromone, not because the monogyne workers are genetically impaired (anosmic) to some smell. In conclusion, Krieger and Ross work showed the existence of proteins from the OBP-gene family in the thorax of the red import fire ants, but there is no evidence for any chemosensory function, much less to explain differences in social behavior.

The field of insect olfaction could be devoid of such dogmas by the use of adequate terminology. Previously, I proposed that proteins of the *PBP*-gene family in general be named "encapsulins" [16]. As indicated by the structures of a hemolymph protein (GSP), THP12 [61], a pheromone-binding protein, BmorPBP [23–25], a chemosensory protein, MbraCSP6 [51], and a cockroach PBP [58], members of the *OBP*-gene family belong to the same structural family of helical proteins. In addition, their structures suggest that the olfactory and non-olfactory members of the *OBP*-gene family encapsulate hydrophobic ligands, with the ability to transport them in aqueous environments. The term "encapsulins" implies the common role of encapsulating small ligands. The encapsulin family would, therefore, encompass odorant-binding proteins (OBPs and PBPs), CSPs, and other non-olfactory proteins. The proposed terminology is not a replacement for pheromone-binding proteins, but rather would avoid mixing up function and gene family. Thus, all members of the PBP-gene family with no evidence for olfactory function (tissue specificity, binding ability and the like; see above) should be referred to as "encapsulins," not odorant-binding proteins.

3.1.2 Mechanism of Pheromone Binding and Release

3.1.2.1 The Pheromone-PBP Complex Model

In one of the earliest modes of action proposed for OBPs, Pelosi [62] hypothesized that – in analogy to a model of bacterial chemotaxis – OBPs not only solubilize specific pheromones, but trigger the olfactory receptors when bound to odorant molecules [62]. In a later version of the pheromone-PBP complex model, it was suggested that electrostatic and hydrophobic interactions from both the bound ligand and ligated protein are necessary and sufficient for receptor activation [63]. The notion that olfactory receptors are activated by interactions with pheromone-PBP complexes is not supported by recent findings. The structure of the BmorPBP-bombykol complex [23] showed the pheromone is completely buried inside the protein, thus, indicating that in the bound form it is highly unlikely that the ligand (pheromone) interacts directly with the pheromone receptor. Based solely on the structural biology of the BmorPBP-bombykol complex, one cannot refute Pelosi's model. However, recent electrophysiological evaluation of odorant receptors in a heterologous system suggest that ligand per se, not the complex, activates the odorant receptors. A putative odorant receptor from *Drosophila*, Or43a [64, 65], expressed in *Xenopus laevis* oocytes [66], was activated by four odorants, i.e., cyclohexanone, cyclohexanol, benzaldehyde, and benzyl alcohol [66] in the absence of *Drosophila* OBPs. This is in agreement with an earlier work showing that PBP was not necessary to obtain pheromone-dependent responses in cultured olfactory receptor neurons of *Manduca sexta* [67]. In the earlier case, however, the possibility that OBPs

have been produced in vitro and were present in cultured ORNs could not be excluded. The expression of a *Drosophila* odorant receptor in a heterologous system is very likely devoid of OBPs. In conclusion, the evidence that *Drosophila* receptors expressed in *Xenopus* oocytes responded to odorants in the absence of OBPs speak against the OBP-odorant complex model. However, OBPs are essential for the kinetics and sensitivity of the insect olfactory system (see below).

3.1.2.2 Conformational Changes of OBPs

My collaboration with structural biologists led to the serendipitous discovery of a pH-dependent conformational change in pheromone-binding proteins [39]. When Kurt Wüthrich and his co-workers analyzed by NMR our highly purified samples of ^{15}N- and ^{15}N,^{13}C-labeled BmorPBP, they were surprised with the number of "extra" peaks indicating inhomogeneity of the sample, possibly due to degradation or contamination. We were also surprised because, before sending the first samples to Zurich, we first analyzed the effect of lyophilization by chromatography, gel electrophoresis, mass spectrometry, circular dichroism (CD), etc. We found no evidence for degradation or any other changes in the samples before and after lyophilization, thus suggesting the samples were pure. The same was observed with the samples returned from Zurich; they showed "extra" peaks by NMR, but they were pure! A thorough investigation of the stability of the protein by various spectroscopic methods led to the conclusion that, although very stable, BmorPBP showed a pH-dependent conformational change. While the secondary structure of the protein was affected only slightly by changes in pH (as demonstrated by far-UV-CD), the tertiary structure (analyzed by near-UV-CD) exhibited a conformational transition between pH 6 and pH 5 [39]. It was somewhat intriguing that the protein kept its secondary structure but changed its tertiary structure at low pH. It became evident later that one helix is unfolded at low pH, whereas another flexibly disordered part of the molecule folds into an α-helix, thus maintaining the overall content of secondary structure (see below). pH titration using NMR showed that at pH below 4.9 there was a single form, whereas another form of the protein existed at pH above 6 [42]. We named these forms the "A" (BmorPBP^{A}) and "B" (BmorPBP^{B}) forms, respectively for "acid" and "basic" form. Note that strictly speaking at the bulk pH of the sensillar lymph (6.5) [56] the "B" form is not basic, but this was a rather simplified nomenclature. At the intermediate pH in the first NMR analysis the sample was a mixture of BmorPBP^{A} and BmorPBP^{B}, thus, explaining the "extra" peaks.

Conformational changes in BmorPBP were also studied in the presence of model membranes using CD spectroscopy. Conformational changes more pronounced than those observed at low pH were detected in the presence of anionic vesicles of dimyristoylphosphatidylglycerol (DMPG), whereas the effect of neutral phospholipids vesicles, dimyristoylphosphatidylcholine (DMPC) was

marginal [39]. The presence of a physiological concentration of KCl reduced the effect, but the interaction with negatively-charged membrane in the presence of KCl was still comparable to the effect of lowering the pH. The negatively-charged head groups of lipids in cell membranes give rise to an electrical surface potential, which in turn decreases the surface pH [68].

There is growing evidence in the literature that the pH-dependent conformational change in BmorPBP (and other PBPs) is physiologically relevant. Negatively-charged surface coats have been demonstrated on the pore tubules and dendritic membranes of olfactory hairs of male *A. polyphemus* by application of cation markers, such as lanthanum, ruthenium red, and cationized ferritin [43, 44]. As I pointed out earlier [69], as far as pheromone-binding proteins are concerned, the physiologically relevant pH is likely to be not only that of the sensillar lymph [56] (the bulk pH), but also the pH at the surface of dendrites (localized pH). It is yet to be determined whether the negatively-charged surface that may interact with odorant-binding proteins and promote conformational changes is a moiety from a glycoprotein, amino acid residues from membrane proteins like SNMPs [52, 70–72], or even an external site of olfactory receptors.

The kinetics of conformational changes is consistent with the fast kinetics of neuronal activities. Stopped-flow measurements of the pH-dependent conformational change in BmorPBP monitored by fluorescence showed that it has characteristics of first-order kinetics, with a rate constant, $k=184\pm6\ s^{-1}$ [16]. Thus, the time required for half of the conformation at the bulk pH to change into the conformation at lower pH (equivalent to the pH of a dendritic surface) is 3.8 msec. This half-time fits to a model of perireceptor events [73]. Also, the fast conformational change is consistent with the millisecond timescale for the dynamics of the olfactory system [14]. For example, males of *B. mori* respond to bombykol with wing vibration 100–500 ms after the onset of stimulation [74]. Moreover, the binding ability of odorant-binding protein is lost at low pH as demonstrated by fluorescence [39] and mass spectrometry [75] for BmorPBP and by calorimetric titration for an odorant-binding protein from the honeybee [47]. That BmorPBP binds bombykol at the bulk pH but not at the membrane-localized pH has been further demonstrated by a cold binding assay [16]. In addition, this binding assay showed that the loss of binding ability at low pH is not affected by the high salt concentrations, i.e., there is no binding of bombykol to BmorPBP at pH 5 either with 0, 170, or even 500 mmol/l of KCl [16]. The notion that the pH-dependent conformational change is a physiologically relevant mechanism for pheromone delivery (to olfactory receptors) is further substantiated by striking evidence from structural biology for an intramolecular mechanism of "occupation" of the binding site at low pH (see below).

3.1.2.3
Structures of OBPs and Encapsulins

In collaboration with Dr. Jon Clardy and Dr. Kurt Wüthrich we have studied the crystal and solution structures of BmorPBP bound to bombykol, unliganded at high and low pH. The crystal structure of the BmorPBP-bombykol complex (Fig. 8a) shows a roughly conical arrangement of six α-helices [23] remarkably similar to the NMR structure of the protein devoid of ligand (Fig. 8d) [25].

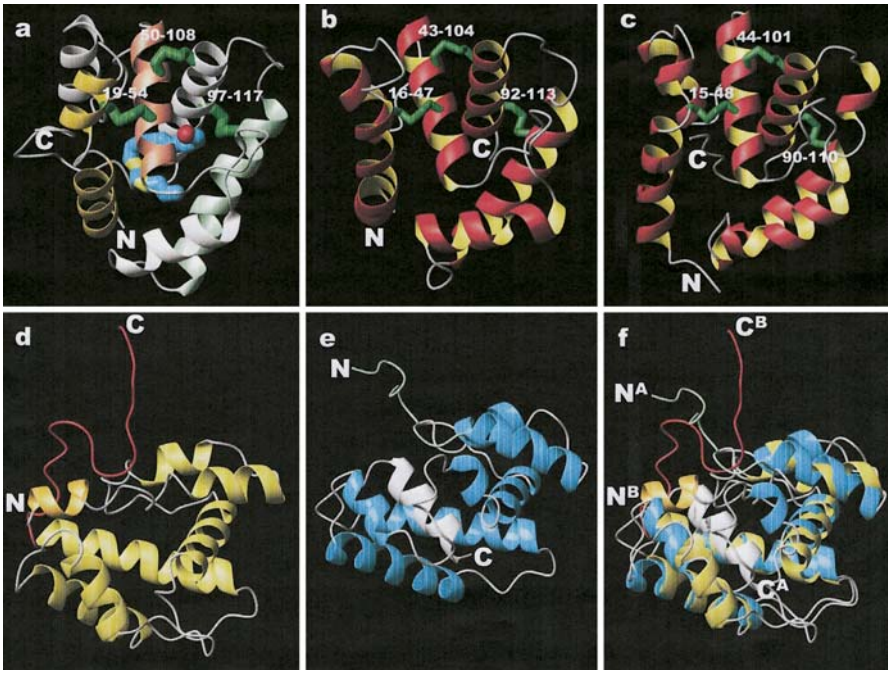

Fig. 8a–f Structures of proteins of the OBP-gene family prepared by using the program MOLMOL [125]. The N and C termini are denoted by N and C, respectively: **a** pheromone-binding protein of *B. mori* (BmorPBP) bound to bombykol, 10,12-(*E,Z*)-hexadecadienol. The polar end of the ligand is highlighted with the oxygen atom in *red*, whereas the double bonds are shown in *yellow*. Disulfide bridges (19–54, 50–108, and 97–117) are shown in *green*. Note the unstructured C-terminal as an extended conformation of the protein; **b** pheromone-binding protein of the cockroach *L. maderae*. Note the C-terminal α-helix; **c** LUSH, a putative odorant-binding protein from *D. melanogaster*. Note the unstructured C-terminus; **d**, **e** structures of BmorPBP (d) at the bulk high pH of the sensillar lymph ($BmorPBP^B$) and (e) at the localized low pH in the vicinity of the dendritic membrane ($BmorPBP^A$). At high pH, the C-terminal dodecapeptide (*red*) is unstructured, whereas the N-terminal segment (*gold*) forms a α-helix (d). At low pH, a new helix is formed (*gray*) and occupies the binding site, whereas the N-terminus (*green*) is unstructured (e); **f** superposition of the structures of $BmorPBP^B$ and $BmorPBP^A$

We kept the same nomenclature used in the initial work where the two segments of the interrupted N-terminal helix were named α1a and α1b and the loops linking the helices were named after the helix preceding them. For example, the loop following α1a is L1a, whereas the loop connecting helices α2 and α3 is L2. The most striking feature of the solution structure of $BmorPBP^B$ devoid of ligand is a hydrophobic cavity (occupied by bombykol in the solid state structure) with a volume of 272±17 $Å^3$, which is suitable to accommodate bombykol [25]. The preservation of the cavity in the absence of ligand is primarily due to the inherent rigidity of the disulfide structure linking a scaffold of four helices, namely α1b, α3a, α5, and α6 (Fig. 8a). Ab initio calculations indicated that reorganization of the binding cavity can be energetically expensive [76].

Utilizing recombinant protein expressed by another group, Oldham and colleagues [75] observed a possible noncovalent adduct generating an "extra" peak in the mass spectral analysis of BmorPBP. Later, they identified the contaminant as (*Z*)-11-octadecenoic acid (*cis*-vaccenic acid) and described a delipidation protocol [77]. They also suggest that the lipid is derived from *E. coli* and acquired by the protein during expression. Despite several attempts, we never found any contaminants in the BmorPBP samples prepared in my lab. Most likely the discrepancy between the findings of different labs is due to the different expression and purification protocols. In our case, NMR showed that the binding cavity is preserved in the absence of ligand [25].

The binding cavity of BmorPBP is formed by four antiparallel helices (α1, α4, α5, and α6) that converge to form the narrow end of the pocket, whereas the opposite end is capped by α3 (Fig. 8a). Bound bombykol has a roughly planar hook-shaped conformation and the outside (convex) part of bombykol interacts with numerous protein residues, whereas the inside (concave) part has fewer contacts. Interestingly, residues from all parts of the protein contribute to the binding cavity [23] that protects bombykol from the aqueous solvent. The solution structure showed that the binding cavity is lined with 21 hydrophobic side chains, namely, Met5 and Leu8 from the helix α1a, Phe12 from the loop L1a, Phe33, Tyr34, and Phe36 from L2, Ile52 from α3, Met61, Leu62 and Leu68 from L3, Ala73, Phe76, Ala77 from α4, Ala87, Leu90, Ile91, and Val94 from α5, and Trp110, Val114, Ala115, and Phe118 from α6 [25]. The cavity contained also four polar side chains of Asp32 from α2, Thr48 and Ser56 from α3, and Glu98 from α5. In the BmorPBP-bombykol complex, the hydroxyl group of bombykol forms a hydrogen bond with the side chain of Ser56 [23]. Ab initio calculations suggested that another hydrogen bond with Met61 may result in slightly stronger interaction [76]. The conjugated double bonds of bombykol are sandwiched by Phe12 and Phe118 with the aromatic rings parallel to the molecular plane of bombykol [23]. Bound bombykol is completely engulfed in BmorPBP, and the structure does not clearly indicate how the ligand enters or exits the binding cavity. The only part of the pheromone that is not surrounded by α helices is the hydroxyl end, which is covered by loop L3 [23]. As noted in the solution structure of $BmorPBP^B$, except for loop L2, the loops connecting the he-

lices contain numerous hydrogen bonds that help in the formation of well-ordered structures [25]. L3 is held together in an approximate antiparallel β-strand conformation by three hydrogen bonds (Gly66N-Asp63O, Asp63N-Asn67O, and His69N-Met61O), with additional interaction between the side chain of Asp63 and the backbone NH of Asn67. This loop is held in place by an interaction between the side chain NH of Leu68 and the side chain of Glu98. If this loop were not in place, the resulting opening would be adequate for bombykol to enter and egress [23]. Testing of this hypothesis is still underway.

The unliganded solution structure of BmorPBP at pH 4.5 ($BmorPBP^A$) showed remarkable conformational differences to the crystal structure of the BmorPBP-bombykol complex (Fig. 8e,f) [24]. The most pronounced differences are in the region of helix α1, which is N-terminally elongated in $BmorPBP^A$ (helix α1a in the BmorPBP-bombykol complex [23] and $BmorPBP^B$ [25]) and in the C-terminal helix α7, which is not present in $BmorPBP^B$ [25] and the complex [23]. The helices forming the bombykol-binding cavity in the complex and in $BmorPBP^B$ occur in close similar positions in $BmorPBP^A$ [24]. The most significant difference between the structures of BmorPBP-complex or $BmorPBP^B$ and the acidic form is the C-terminal helix (α7) in $BmorPBP^A$ which occupies a position that corresponds to the hydrophobic binding cavity in the crystal structure. The C-terminal dodecapeptide segment, which is an extended conformation and located on the protein surface at high pH forms a α-helix at low pH. This is one of the most remarkable conformational changes yet observed in receptor-ligand or enzyme-substrate binding, and leads to occupation of the binding site by an intramolecular mechanism triggered at low pH. The three histidine residues (His69, His70 and His95), forming a cluster at the end of loop L3 in $BmPBP^B$, are more widely separated in $BmorPBP^A$ [24]. This would reduce the charge repulsion resulting from histidine protonation at slightly acidic pH values and could thus destabilize the structure of the complex in favor of $BmorPBP^A$.

Recently, the structure of a pheromone-binding protein from the cockroach *Leucophaea maderae*, LmadPBP (Fig. 8b) has been solved by X-ray crystallography [58]. Despite the fact that LmadPBP and BmorPBP shared low amino acid identity (15%; similarity 22%) (Fig. 9), the two proteins present similar folds.

When compared to the structure of the BmorPBP-bombykol complex, the six helices have similar orientations; the three disulfide linkages knit together four of the helices in a similar pattern (Fig. 8b). The binding cavity is much smaller than the bombykol-binding cavity in $BmorPBP^B$ and in the complex structure; in LmadPBP the binding pocket is wide open to the bulk solvent. The conformations of LmadPBP unbound and bound to its pheromone (3-hydroxybutan-2-one) are very close [58], but these comparisons were made only at high pH values (>7) (for experimental details see [78]). That the bound and unbound structures are remarkably similar is also true for BmorPBP at high pH, but the acidic form is quite different from the basic form (see above). The major difference between BmorPBP and LmadPBP is that the cockroach

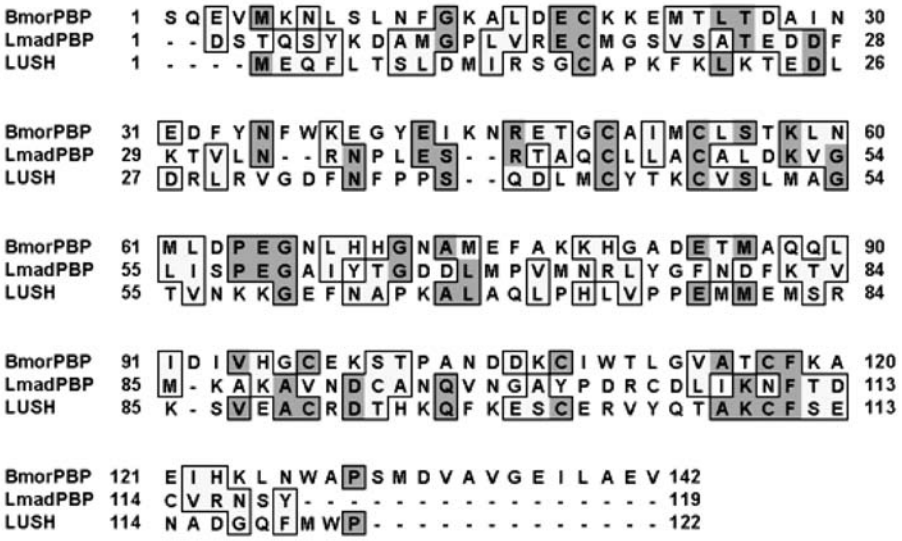

Fig. 9 Alignment of the amino acid sequences of pheromone-binding proteins from the silkworm moth *B. mori* and the cockroach *L. maderae*, BmorPBP and LmadPBP, respectively and a putative odorant-binding protein from *D. melanogaster*, LUSH. In LmadPBP and LUSH the N-terminal sequence of the mature proteins were predicted by cleaving signal peptides in silico [28, 79], whereas in BmorPBP this was confirmed by the sequence of the isolated protein [38]

protein is shorter by four residues at the N-terminus and 15 residues at the C-terminus. The authors suggest that due to the shorter C-terminus, LmadPBP would not undergo a pH-dependent conformational change [58] as observed for BmorPBP. It may be true that PBPs from insects of different orders have different "modes of action," but the evidence for the lack of a pH-dependent conformational change in LmadPBP is still missing. Although the shorter C-terminus indicates that a new helix may not be formed, this hypothesis can be tested only when the structure at low pH is determined. In their work, Lartigue and collaborators obtained solid state structures only at high pH [58]; thus, one cannot conclude what happens at low pH without experimental data.

One drawback of the cockroach PBP structure is that the recombinant protein was composed of 129 amino acids, with 11 of them (Met-Asp-Ile-Gly-Ile-Asn-Ser-Asp-Pro-Asn-Ser) not belonging to the native structure [79]. In their recombinant vector, pET-LmadPBP, the cockroach cDNA encoding the target protein was inserted away from the pelB leader sequence using an *Eco*RI recognition site. Thus, a long non-natural peptide at the N-terminus of the recombinant protein was added to the native sequence [79]. The possible effect of this extended N-terminal segment – although not discussed when the structure was reported [58] – may influence the folding of the protein. Note that in BmorPBP

the α1a helix unfolds at low pH. It is very unlikely that the conformational change in the native conformations of BmorPBP would be "visualized" if we had studied recombinant BmorPBPs having an addition N-terminal sequence. Therefore, we never use fusion tags such as His tags, GST tags, etc. in our recombinant proteins for structural studies. These non-native proteins may speed up the purification process, but may slow down our understanding of the physiology and molecular basis of insect olfaction.

The structure of LUSH, a putative odorant binding protein from *Drosophila* (see above), has just been solved [37]. In contrast to the recombinant proteins from *B. mori* and *L. maderae*, which were obtained by periplasmic expression, LUSH was obtained by cytosolic expression and refolding using a cysteine-cystine redox reaction [37]. In addition, recombinant LUSH had three additional amino acids at the N-terminus, Gly, Ser, and His, which were leftover after the removal of a His tag [37]. In LUSH (Fig. 8c), the C-terminus forms part of the alcohol-binding pocket, whereas helix α1 packs on the outside of the protein [37] and does not participate in the ligand-binding cavity as in BmorPBP-bombykol complex [23]. Based on these differences, it was suggested that the OBP family has at least two distinct structural isoforms [37]. Interestingly, crystal structures of LUSH at high and low pH values (6.5 and 4.6, respectively) showed alcohol in the binding pocket. Given the contradictory information regarding detection of alcohol by *lush* mutants, the lack of binding assays (see above), and the fact that the protein was incubated with extremely high concentration of alcohol (1%), it is difficult to interpret the physiological relevance of the occurrence of alcohol in the binding pocket at low pH. Artifacts may lead to compounds of little physiological significance being trapped in a binding pocket. The cavity of LmadPBP, for example, contains a ubiquitous glycerol molecule [58], which derives from the considerable amount of glycerol brought into the crystal for cryocooling. If LUSH functions as an odorant-binding protein, it may have a different mode of action. Of particular notice is the fact that LUSH is the only putative odorant-binding protein reported to date that is basic at the sensillar lymph pH. LUSH has a calculated pI of 8.28; thus, it is positively charged at the sensillar lymph pH (ca. 7). All other OBPs identified to date are acidic and they are considered to contribute to the anions in the sensillar lymph of which a minor fraction is covered by Cl^- [6]. In conclusion, the physiological function of LUSH is not yet clarified, despite the elegant structural biology studies [37]. It is clear, however, that despite the low sequence similarities (Fig. 9), BmorPBP, LmadPBP, and LUSH belong to the same structural family. Another insect protein of known structure is THP12 [61], a protein isolated from the hemolymph of the beetle *Tenebrio molitor* [55]. The overall folds of OBPs and THP12 are similar, but the latter is missing the N-terminal α1a helix. Similar to OBPs (above), four helixes are knitted together by two disulfide bridges. Because hemolymph is completely isolated from the sensillar lymph (see above), it is very unlikely that THP 12 has any olfactory function and, as such, it should be referred to as encapsulin rather than odorant-binding protein.

3.2 Mode of Action of OBPs

The following evidence based mainly on the pheromone-binding protein from *B. mori* strongly supports that OBPs uptake compounds entering the sensillar lymph through pore tubules, bind physiologically relevant ligands, encapsulate them, ferry these semiochemicals to the olfactory receptor, and deliver the chemical signal by a conformational change upon interaction with negatively charged sites in the dendrites; this model is depicted in Fig. 6. BmorPBP undergoes a pH-dependent conformational change [39, 42], binds bombykol at the sensillar lymph pH, but not at lower pH [16, 39]. Negatively-charged groups in cell membranes give rise to an electrical surface potential, which in turn decreases the surface pH [68]. In other words, a negatively-charged surface is equivalent to a low pH region (localized pH). The pH-dependent conformational change leads to a remarkable intramolecular "re-arrangement" in BmorPBP. At the bulk pH of the sensillar lymph, the C-terminus in BmorPBP (either bound to bombykol or unbound) is an extended conformation located on the protein surface [23, 25], whereas at low pH this C-terminal dodecapeptide segment forms a α helix that occupies the pheromone-binding cavity in the core of the protein [24]. The growing evidence from structural biology studies suggests that upon interaction with negatively-charged membrane (regions of low pH), the C-terminal helix takes over the binding pocket, thus, ejecting the pheromone out of the protein. Stopped-flow fluorescence measurements showed that this rapid conformational change is in the timescale of milliseconds [16]. Functional expression of an odorant receptor from *Drosophila* in *Xenopus laevis* oocytes [66], devoid of odorant-binding proteins, suggests that an odorant per se (not an OBP-odorant complex) can activate the receptor. The same experiments indicate that odorant-binding proteins are essential for the kinetics (and likely the specificity) of the olfactory system.

In this model, OBPs participate in the selective transport of pheromone and other semiochemicals to their olfactory receptors. The selectivity of the system is likely to be achieved by "layers of filters" [16], i.e., by the participation of compartmentalized OBPs and olfactory receptors. It seems that OBPs transport only a subset of compounds that reach the pore tubules. Some of these compounds may not bind to the receptors compartmentalized in the particular sensilla. The odorant receptors, on the other hand, are activated by a subset of compounds, as indicated by studies in *Drosophila*, showing that a single OR is activated by multiple compounds [66]. If some potential receptor ligand reaches the pore tubules but are not transported by OBPs, receptor firing is prevented because the receptors are "protected" by the sensillar lymph. In other words, even if neither OBPs nor odorant receptors (ORs) are extremely specific, the detectors (olfactory system) can show remarkable selectivity if they function in a two-step filter.

While engulfed in the binding cavity of an OBP, a pheromone (or other semiochemical) is not only solubilized, but also protected from odorant-degrading

enzymes (see below). Assisted by a protein, the pheromone is now transported through the sensillar lymph until it reaches certain negatively-charged sites on the surface of dendrites. The low pH at these sites triggers a conformational change of the OBP-odorant leading to the release of the ligand to the receptors. After stimulating the odorant receptor, the pheromone is inactivated or deactivated. Note that in this model OBPs are not merely carrier proteins, but they contribute to the specificity of the olfactory system. Also, they have evolved the ability to undergo a rapid pH-dependent conformational change for the fast delivery of ligands to the olfactory receptors, which contributes to the dynamics of the olfactory system.

3.3 Specificity of the Insect Olfactory System

The inordinate specificity of the insect olfactory system was highlighted in electrophysiological studies of pheromone perception. There is a body of evidence in the literature indicating that minimal structural modifications of pheromone molecules render them inactive, as demonstrated initially in the pheromone detectors in *B. mori* antennae [12]. Even the olfactory receptor neurons (detectors) for plant compounds in insect antennae, once called "generalists", have now been demonstrated to have remarkable specificity [20, 80–84]. In some cases, these specific detectors may respond when challenged with extremely high concentrations of other compounds. These responses may not be physiologically significant because insects will never encounter such high concentrations in the natural environment. When electrophysiological studies precede the discovery of physiological relevant semiochemicals (say pheromones), one tends to try high concentrations of test compounds and this may lead to the identification "non-specific" ORNs. Some ORNs in scarab beetle antennae were initially considered generalists, but are now known to be specific detectors for (*Z*)-3-hexenyl acetate [20, 80, 82, 83]. On the other hand, behavioral evidence that a certain compound has a physiological function (like a sex pheromone, for example) facilitates the discovery of specific ORNs. For that reason most of the evidence for the specificity of the olfactory system comes from studies on species of known pheromones. From an anthropomorphic perspective, stereochemical discrimination may be considered the ultimate refinement in the insect olfactory system. Scarab beetles, for example, can discriminate stereoisomers of a lactone pheromone and perceive one antipode as a sex pheromone and the other as a behavioral antagonist [18, 84, 85]. Interestingly, they perceive the two stereoisomers with two ORNs co-localized in the same sensilla [18] and respond differently if the stereoisomers are perceived either simultaneously or isolated by a few milliseconds [84].

Of notice is the case of *D. melanogaster*, a highly suitable model system for olfactory research given that it is an insect amenable to genetic manipulations, the complete genome has been sequenced, and the olfactory system is relatively simple, thus, allowing precise physiological measurements. *D. melanogaster*

possesses two olfactory organs, the antennae with ca. 1,200 ORNs and the maxillary palpi containing ca. 120 ORNs. These ORNs are compartmentalized in olfactory sensilla, which divide into morphologically distinct classes, including large basiconica, small basiconica, trichodea sensilla, and coeloconic sensilla [86]. The ORNs both in the antennae [87] and in the maxillary palpi [88] showed unique response spectra to a panel of tested compounds, ORN raising the question whether these sensilla are "generalists" or if the "key stimulus" for each has not yet been discovered. Recently, Stensmyr and collaborators [36] screened a large number of potential semiochemicals for *Drosophila* from food sources and conclude that "key stimuli" are detected by the fruitfly with high specificity at low concentration, but when the concentrations are increased the specificity decreases. One of the tested ORNs responded to ethyl hexanoate and methyl hexanoate with similar dose-response curves and threshold of 100 pg, whereas ethyl butyrate and butyl butyrate required 100-fold and 10,000-fold increase, respectively, in dose to produce any response [36]. That the *Drosophila* olfactory system is indeed specific to a physiological relevant "key stimulus" has been previously demonstrated [35]. Sensilla trichodea in the antennae responded in a dose-dependent manner to an aggregation pheromone, *cis*-vaccenyl acetate, but were not activated by 16 other compound tested, thus suggesting they are narrowly tuned to the pheromone [35]. In conclusion, the specificity (also the sensitivity and dynamics) of insect olfactory system may be a common feature, with the apparent exception of *Drosophila* where the "key stimuli" have yet to be discovered.

3.4 Odorant-Degrading Enzymes

In addition to sensitivity and discrimination, odor-oriented navigation requires a dynamic process of signal inactivation. While flying en route to a pheromone-emitting female (Fig. 2), males encounter pheromone molecules as intermittent signals comprised of short bursts of high flux separated by periods during which the flux is zero. The average duration of bursts of high flux of pheromones is on the millisecond scale and it decreases as the moth comes closer to the pheromone source [2]. Thus, a male moth has to detect selectively minute amounts of pheromones and reset the pheromone detectors (cells) on a millisecond timescale. The literature on the inactivation of chemical signals is dichotomous. One school favors the hypothesis that rapid inactivation of chemical signals is an enzymatic process regulated by pheromone-degrading enzymes, whereas the other school favors that preceding the "slow process of degradation" there is some molecular interaction of pheromones and other olfactory proteins. Based on an estimation of pheromone degradation in vitro, it has been hypothesized that fast inactivation of pheromones is achieved by pheromone-degrading enzymes [89]. However, the enzymatic degradation in vivo has been considered too slow (on a minute timescale) [90] to account for the fall of the receptor potential [73]. It has been suggested that the discrepancy

between data from in vivo and in vitro experiments is due to the involvement of PBPs that protect the pheromone from degradation [73].

If these pheromone-degrading enzymes are indeed involved in the fast inactivation of pheromone signals, they have a potential application in agriculture as their inhibitors could be used in insect pest management [91–94]. However, a rational approach for their design of environmentally-safe inhibitors requires full knowledge of the biological system. Specificity and selectivity of inhibitors can be dramatically improved upon design of new compounds, which fit not only into the binding pocket of pheromone-binding proteins, but also in the active site of pheromone-degrading enzymes. These compounds could then penetrate the sensillar lymph and inhibit the fast degradation of pheromone, thus disrupting chemical communication. Recent structural biology studies on pheromone-binding proteins already shed some light on specificity binding determinants [23], which may lay the foundation for the design of parapheromones developed based not on trial-and-error strategies, but rather on rational structure-activity relationships. Nevertheless, the complete lack of knowledge on the molecular structures of these pheromone-degrading enzymes prevents further progress in the rational design of inhibitors, parapheromones, and other semiochemical-based pest control strategies.

Hitherto, no pheromone-degrading enzymes has been isolated, identified and cloned. As with odorant receptors, the amount of protein is so low that isolation for protein identification is technically very difficult. In marked contrast to PBPs, which are expressed in the sensillar lymph in concentrations as high as 10 mmol/l [95], odorant-degrading enzymes are estimated to occur in concentrations at least four-order of magnitude below that of PBPs [96]. Thus, it has not been possible to date to generate large enough amounts of odorant-degrading enzymes (ODEs) for protein sequencing (by Edman degradation and/or mass spectrometry). It is possible, however, to isolate enough material for identification of olfactory enzymes involved in pheromone degradation. These studies require lower amounts of proteins and samples enriched in the enzymes, but not necessarily pure. For example, a sensillar esterase [89], partially isolated from *A. polyphemus*, was demonstrated to degrade the pheromone, 6,11-(*E*,*Z*)-hexadecadienyl acetate, by attacking the acetate group. Using a bioinformatics approach, we have recently cloned a cDNA encoding a male antennae-specific esterase in the same moth species [97]. It is yet to be demonstrated if the enzyme degrading the pheromone is the same as that encoded by the cDNA we have cloned. Similarly, Maibeche-Coisne and co-workers [98] have cloned the cDNA encoding a cytochrome P450 enzyme from *Mamestra brassicae*. On the other hand, we have demonstrated that the sex pheromone of the pale chafer, *Phyllopertha diversa*, 1,3-dimethyl-2,4-(1*H*,3*H*)-quinazolinedione is rapidly degraded in vitro by a membrane-bound P450 [99]. Interestingly, the ability to degrade this unusual sex pheromone was not detected in 12 other species of scarab beetles. In addition, in *P. diversa* the enzymatic activity was restricted to male antennae, with no degradation observed in extracts from female antennae or control tissues.

The sex pheromone of the Japanese beetle, *Popillia japonica,* is a chiral compound, (*R*,*Z*)-5-(dec-1-enyl)oxacyclopentan-2-one ((*R*)-japonilure), whereas the other enantiomer ((*S*)-japonilure) is a behavioral antagonist that shuts down male response [100]. It seems that this chiral discrimination has evolved as part of the isolation mechanism between the Japanese beetle and the Osaka beetle (*A. osakana*) that share the same habitats in Japan [85]. Previously, it has been demonstrated that this chiral discrimination is not achieved by pheromone-binding proteins as the Japanese beetle possesses only one PBP (that binds to (*R*)- and (*S*)-japonilure) [18]. Studies on the degradation of radiolabeled enantiomers of japonilure by the Japanese beetle antennal enzyme(s) shed new light on chiral discrimination. Crude extracts of the Japanese beetle antennae showed a significant preference for the pheromone, (*R*)-japonilure, over the behavioral antagonist, (*S*)-japonilure (Fig. 10), whereas enzymes from non-sensory tissues (legs) showed no substrate specificity. These findings indicate that integumental esterases in leg tissues are not specific, but sensillar esterases may have evolved for the specific degradation of pheromones. Thus, I hypothesized that one stage of chiral specificity is

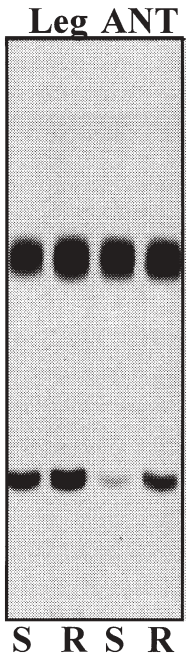

Fig. 10 TLC plate showing degradation of (*R*)- and (*S*)-japonilure (*upper spots*) by esterases from the legs (Leg) and antennae (Ant) of the Japanese beetle. The corresponding hydroxy-acids appear as *lower bands*. Note the slower degradation of the behavioral antagonist, (*S*)-japonilure, by sensillar esterase(s) from the antennae. Neither (*R*)- nor (*S*)-japonilure is degraded in control experiments (data not shown) under the same conditions, i.e., with the compounds incubated in buffer without Japanese beetle tissue extracts

achieved in the perireceptor events (early olfactory processing) by the pheromone-degrading enzymes as a mechanism of pheromone inactivation. Work is now in progress in my lab to test this hypothesis. Pheromone-degrading enzymes will be isolated, the cDNAs encoding these proteins will be cloned, and kinetics of degradation (of pheromone and behavioral antagonist) will be studied in a cell-free system with native and recombinant PDEs. Because the pheromone may be protected from degradation while bound to PBP [73], kinetics will be studied in the presence of the Japanese beetle PBP ([18] in a cell-free system mimicking the in vivo conditions. If these pheromone-degrading enzyme(s) is (are) fast enough (in the millisecond timescale) and isolated enzyme(s) show substrate-specificity the hypothesis will be supported.

4 Olfactory Receptors

That olfactory receptors in vertebrates are G-protein-coupled receptors (GPCRs) was suggested by early evidence that odorant receptors are membrane proteins [101, 102] and that cell-free preparations of rat olfactory cilia contain odorant-sensitive adenylyl cyclase, whose sensitivity depends on activation of a G protein [103]. The evidence was further substantiated by the cloning of a multigene family of vertebrate GPCRs [104]. Given the large body of evidence indicating that pheromone-dependent effects of secondary messengers, such as IP_3, cAMP, and cGMP (reviewed in [105]) have been observed in intact antennae and antennal homogenates, and that odorant receptors are also GPCRs [106], the cloning of vertebrate olfactory receptors prompted various groups to "fish" out insect pheromone receptor "homologs." Various approaches, including photoaffinity labeling, genetic mutants, radioligand bioassays, and PCR with primers designed on the basis of vertebrate GPCR sequences, were unrewarding [107]. With the sequence of the *Drosophila* genome about to be completed, two approaches led to the identification of the first insect odorant receptors. A bioinformatics approach that examines DNA databases for proteins that have a particular structure like the seven-transmembrane-domain of GPCRs led to several genes that could encode seven-transmembrane-domain proteins [64]. RT-PCR experiments showed that two of the genes were expressed specifically in *Drosophila* antennae. BLAST searches identified homologs of these genes, which were used to search for further homologs; a total of 16 genes were identified by this bioinformatics approach [64]. On the other hand, Vosshall and collaborators found a putative odorant receptor by a strategy designed to detect cDNA copies of mRNA present at extremely low frequencies in an mRNA population [65]. In situ hybridization revealed that the cDNA encoding the putative olfactory receptor anneals to ca. 15% of the 120 olfactory receptor neurons within the maxillary palpi but does not anneal with neurons in either the brain or antennae [65]. Searches of the then incomplete *Drosophila* sequence database led to 229 candidate genes, 11 of which encode

putative GPCRs with sequences similar to those obtained by the rare mRNA strategy. Completion of the genome sequence allowed extension of the odorant receptor family to 60 receptors, which is now predicted to consist of 62 odorant receptors [108].

Direct demonstration of the function of one member of the *Or* gene family (*Or43a*) was obtained by overexpression of the gene in the fly antennae [109], as well as by expression in a heterologous system, *Xenopus* oocytes [66]. The GAL4/UAS system was used to overexpress *Or43a* in the *Drosophila* antennae. In wild-type flies, Or43a expression is restricted to ca. 15 ORN at the distal edge of the third antennal segment, but in the transformed flies Or43a expression was drastically increased. In addition to the cells at the distal edge of the antennae, there were Or43a overexpressing cells in the transformed lines in a more proximal region that is covered mainly by large sensilla basiconica [109]. Electroantennogram experiments showed that the transformed flies showed (dose-dependent) increased responses to benzaldehyde as compared to wild-type flies, whereas ethyl acetate evoked similar responses in control and transformed lines at all concentrations [109]. In addition to benzaldehyde, EAG showed increased responses to cyclohexanol, cyclohexanone, and benzyl alcohol, thus, suggesting that Or43a is a "generalist" type of odorant receptor. That Or43a is a *Drosophila* odorant receptor was also suggested by two-electrode voltage-clamp recordings from *Xenopus* oocytes injected with Or43a (and Ga15) cRNA [66]. Again, benzaldehyde, cyclohexanol, cyclohexanone, and benzyl alcohol elicited responses, with current being developed at low micromolar concentrations, whereas eight other test compounds failed to activate Or43a [66]. Moreover, oocytes not injected with Or43a failed to respond to the four odorants (benzaldehyde, cyclohexanol, cyclohexanone, and benzyl alcohol) even at millimolar concentrations [66]. These two lines of evidence were the first demonstration that a *Drosophila Or* gene indeed functions as an olfactory receptor. Of particular note is the fact that in the heterologous system – devoid of odorant-binding proteins and odorant-degrading enzymes – the response to odorant was extremely slow compared to the dynamics of the *Drosophila* olfactory system. When *Xenopus* oocytes were stimulated (with cyclohexanol, for example) for as long as 15 s, it took as long as 2–5 s to develop inward currents [66]. By contrast, ORNs in *Drosophila* antennae when stimulated for 0.3–0.5 s generate slow potential and nerves impulses in less than 100 ms [87]. The slower response may be explained by the lack of other olfactory proteins, such as odorant-binding proteins. As previously discussed (see above), these proteins are essential for the detection of semiochemicals as they help in the transport of chemical signals through an aqueous environment while protecting the ligands from "deactivation." In the absence of OBPs in the *Xenopus* oocytes, the ligands were less soluble in water (as compared in the natural system), thus requiring a longer time to generate a threshold concentration at the receptor. In the natural insect system, stray semiochemicals (unbound odorants) in the sensillar lymph may never evoke neural activity as they are likely to be "deactivated" by aggressive odorant-degrading enzymes before reaching the receptors.

It has been a matter of considerable debate whether the remarkable selectivity of the insect olfactory system [12] is achieved by the specificity of pheromone-binding proteins or the odorant receptors. The expression of Or43a in transformed lines and a heterologous system suggests that this odorant receptor is "sloppy." OBPs, on the other hand, have been demonstrated to bind specifically when tested with a limited number of candidate ligands [39, 110–113], but lack specificity in various other cases [114]. I suggest that neither OBP nor OR specificity per se can account for the selectivity of the insect olfactory system, which is likely achieved by "layers of filters" (see above) [16]. The notion of a "dual layer of filters" is supported by the number of genes encoding OBPs and ORs. Even in *Drosophila*, with large numbers of putative OBPs [26] and ORs [108], the number of these olfactory proteins is much lower than the number of compounds insects can smell. Thus, it is not entirely surprising that neither OBPs nor ORs are specific. The specificity of the detectors must be achieved by a combinatorial process.

Putative odorant receptors were fished out from the sequenced genome of the malaria vector mosquito *Anopheles gambiae* by analyzing sequences similar to *Drosophila* ORs. Initially, five putative odorant receptors, AgamOR1-5, were identified [115]; RT-PCR analyses suggest that they are all expressed exclusively in olfactory tissues (antennae and maxillary palpi). Interestingly, one of the putative ORs, AgamOR1, was detected only in female antennae. Intriguingly, RT-PCR showed that AgamOR1 is down-regulated 12 h after a blood meal [115]. By contrast, levels of most OBP mRNAs in the same species remained the same 24 h after a blood meal [116]. Later, a bioinformatics-based approach to identify genes encoding putative transmembrane proteins led to the characterization of 79 candidate odorant receptors in *A. gambiae* [117]. As will be published in Nature, the Carlson's group demonstrated recently that AgamOR1 and AgamOR2 expressed in *D. melanogaster* respond to human odorants (John Carlson, personal communication), thus "de-orphanizing" two of the putative receptors.

The first putative odorant receptors in moths were identified by assessing a genome database of *Heliothis virescens* [118]. Following BLAST searches to identify sequences with significant similarity to *Drosophila* ORs, exon-specific probes of promising sequences were employed to screen antennal cDNA library [118]. RT-PCR results indicate that all nine HvirORs were mainly expressed in the antennae, with two of them (HvirOR7 and HvirOR9) being restricted to antennae [118]. Because they are not sex specific, it is unlikely that any of these ORs is a sex pheromone receptor. The search for pheromone receptors in *H. virescens* is somewhat limited by not having a complete genome given that the database was generated by a shot gun cloning strategy. Thus, one is limited to finding only genes that share significant sequence similarity to *Drosophila* ORs (Jürgen Krieger, personal communication). However, the use of low stringency screening may lead to other genes; this is the case of HvirOR9, which was obtained from HvirOR7.

Immunoelectron microscopy revealed localization of two *Drosophila* ORs, OR22a and OR22b, to the membranes of outer dendritic segments of ORNs. These neurons are housed in a subset of the large basiconic sensilla (LB-I) in the dorso-medial region of the antennae [119]. There are three types of basiconic sensilla in *Drosophila* antennae: ab1 housing four ORNs and ab2 and ab3 each with two ORNs. These sensilla can be distinguished by their response profiles to a panel of odorants tested by single sensillum recordings. To pinpoint the type of basiconic sensilla, strains of transgenic flies were generated in which the presumed promoters for OR22a/OR22b were used to drive expression of GAL4, which in turn drives expression of green fluorescence protein (GFP). Physiological recordings from the GFP-labeled sensilla led to the conclusion that both *22a-GAL4* and *22b-GAL4* drive expression in the ab3 sensillum. To pinpoint further the neuron in ab3 sensilla expressing OR22a/b, the *Or* promoter-*GAL4* constructs were used to drive the cell death gene *reaper* (*rpr*). Recordings from ab3 sensilla in flies engineered to lack *OR22a* (*OR22a-rpr*) did not show the large spike characteristic of ab3A neuron, whereas the small spikes of ab3B were present [119]. Interestingly, the ab3A neuron is also "silent" in the other genotype (*OR22b-rpr*), whereas the ab3B neuron in both genotypes responded to all of the odorants that elicit a response from a control line (*OR22a-GFP*). In conclusion, both *Or22a* and *Or22b* drivers direct expression in the ab3A neuron. Moreover, deletion of *Or22a* and *Or22b* (*Δhalo* mutant) showed an effect on the ab3A neuron similar to that observed in *rpr*-ablation experiments. Transformation rescue experiments demonstrate that rescue is provided only by those constructs containing an intact *Or22a* gene, suggesting that *Or22a* is necessary for rescue, whereas no rescue was provided by *Or22b* [119]. These results indicate that only *Or22a* is necessary for the electrophysiological responses obtained from ab3A with a panel of test compounds.

The *Δhalo* mutant with an empty neuron (ab3A) is an invaluable resource to test putative odorant receptors from flies and possibly other insect species. Indeed, a line designed to express another odorant receptor, *Or47a*, in ab3A neurons gave a different response spectrum as compared to the control lines. The response pattern of this transformed line was similar to that of the ab5B neurons, thus, suggesting that the *Drosophila* receptor *Or47a* is expressed in ab5B neurons [119]. It will be interesting to test the response of putative odorant receptors from other species and different orders to determine if/when the lack of odorant binding proteins from the same species would impair the olfactory function (for physiologically relevant odorants). It will be particularly exciting to test candidate pheromone receptors from moths when they become available. Note that in moths, scarab beetles, and other species of insects pheromone-detectors are narrowly tuned, whereas in *Drosophila* most of the detectors respond (to a panel of test compounds) with a broad spectrum.

5
Reverse Chemical Ecology

As discussed above, EAG and GC-EAD are invaluable tools in pheromone research. Characterization of pheromones from a mixture of compounds is tremendously simplified by using insect antennae as the sensing element either in EAD or in GC-EAD experiments. Although a compound eliciting electrophysiological response is not necessarily behaviorally active, the identification of EAD-active peaks expedites the process by leading to a few candidate compounds (whose biological function is confirmed by behavioral studies). The "molecular" equivalent of these electrophysiology-based approaches is the screening of potential attractants, pheromones, and repellents based on binding affinity to odorant-binding proteins. As with activity indicated by GC-EAD (and EAG) measurements, binding per se does not necessary imply a physiological function. Some test compound may be EAG-active without showing any pheromonal activity, i.e., there is a possibility of "false positives". However, compounds that do not bind (or are EAD inactive) can be eliminated from further behavioral tests. The protein-based screening of semiochemicals requires the full identification of odorant-binding proteins, cloning of the cDNAs (genes) encoding these OBPs, and expression of functional OBPs for binding assays. This "reverse chemical ecology" process is justified for cases in which semiochemicals are sorely needed, but bioassay-oriented approaches have failed. Conventional trial-and-error screenings in the field are too expensive and time-consuming [120]. Three years ago, I proposed the concept of OBP-based screening of mosquito attractants and repellents. Work is now in progress in my lab towards these goals; we have isolated OBPs from *Culex* species [121, 122], the principal vectors of West Nile Virus, and generated recombinant proteins for binding studies. The development of binding assays for throughput screening of candidate semiochemicals is underway. The concept of reverse chemical ecology is also aimed at the development of better lures for the Navel Orangeworm moth, *Amyelois transitella*. Hitherto, only one constituent of the sex pheromone (11,13-(*Z*,*Z*)-hexadecadienal) of this important agricultural pest has been identified [123] and better lures are highly desired for monitoring populations and applications in integrated pest management.

Protein-based assays are routinely used by the pharmaceutical industries for the development of new drugs, but their approach is largely based on receptor-drug interactions. Theoretically, screening of potential semiochemicals could be made by studying odorant receptor-ligand interactions. However, odorant receptors and putative odorant receptors are only known for species whose genome has been sequenced. Even for known ORs, such as *Drosophila* odorant receptors (see above), functional expression is technically very difficult. Thus, screening based on in vitro binding studies with receptors is as yet not technically feasible.

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