Cell. Mol. Life Sci. 63 (2006) 1658–1676
1420-682X/06/141658-19
DOI 10.1007/s00018-005-5607-0

Cellular and Molecular Life Sciences

Review

Soluble proteins in insect chemical communication

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Received 27 December 2005; received after revision 6 March 2006; accepted 13 April 2006
Online First 19 June 2006

Abstract. Our understanding of the biochemical mechanisms that mediate chemoreception in insects has been greatly improved after the discovery of olfactory and taste receptor proteins. However, the presence of soluble polypeptides in high concentration around the dendrites of sensory neurons still poses unanswered questions. More than 2 decades after their discovery and despite the wealth of structural information available, the physiological function of odorant-binding proteins is not well understood. More recently, members of a second family of soluble polypeptides, the chemosensory proteins, were also discovered in the lymph of chemosensilla. Here we review the structural properties of both classes of soluble proteins, their affinity to small ligands, and their expression in the different parts of the insect body and subcellular localisation. Finally, we discuss current ideas and models of the role of such proteins in insect chemoreception.

Keywords. Insects, odorant-binding proteins, chemosensory proteins, olfaction, pheromones, chemical communication.

Introduction

The study of olfaction has experienced an explosion of interest in recent times, turning the scattered observations of isolated chemists and perfumers into one of the most active fields of biochemistry and molecular biology. Odorant-binding proteins (OBPs), discovered at the beginning of the eighties in insects [1] and vertebrates [2, 3], represented the first products of biochemical investigation in this field. But it was the identification of olfactory receptors in vertebrates [4] 10 years later that gave the main impulse, providing defined targets for biochemical and physiological research. Olfactory receptors were soon after reported in nematodes [5], but it was not until the genome of the fruit fly was made available that olfactory receptors in insects were identified [6, 7]. These transmembrane olfactory receptors are believed to be responsible for recognising molecular structures of volatile molecules and starting the signal transduction cascade eventually leading to the electric signals that are processed and interpreted by the brain as olfactory and gustatory perceptions. This view is supported by recent experiments that have clearly and elegantly shown how olfactory receptor proteins, when expressed in heterologous systems, even in the absence of OBPs, can be activated by semiochemicals, starting the same chain of biochemical events observed in the natural host. Such results have been obtained with both vertebrate and insect olfactory receptors [8–11].

However, the high concentration of OBPs around the dendrites of olfactory neurons – the nasal mucus in vertebrates and the sensillar lymph in insects – poses unanswered questions about their physiological function, despite the large amount of structural information ac-

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cumulated in more than 2 decades of research on these proteins [12–22].

OBPs of vertebrates belong to the large superfamily of carrier proteins called lipocalins [23]. They generally consist of chains of about 150 amino acids, and are mainly structured in β-sheet domains and folded in the typical β-barrel structure. In native conditions, they are usually present as homodimers. Each subunit presents a binding pocket located inside the barrel that can accommodate ligands of medium size (10–20 carbon atoms) and hydrophobic nature. In insects, two different classes of polypeptides have been identified in the lymph of chemosensilla, OBPs and chemosensory proteins (CSPs). They both share with vertebrates OBPs a small size, a generally low isoelectric point, binding affinity to small ligands and very high concentrations in the proximity of chemosensory neurons. However, amino acid sequences and three-dimensional structures are completely different among lipocalins, insect OBPs and CSPs. A great amount of structural information has been accumulated on OBPs and CSPs in the attempt to elucidate their function, but physiological studies have so far been scarce and failed to provide convincing models for their action.

Certainly, as mentioned above, olfactory receptor proteins expressed in selected cell lines have been shown to respond to odorant stimuli in the absence of these soluble proteins. However, their responses are too slow (several seconds) compared with the fast behavioural reaction (milliseconds) of insects to olfactory stimuli. Moreover, the high amounts of energy used by the animal in the synthesis of these soluble polypeptides and the wide variety of types expressed in each species are clear indicators that OBPs as well as CSPs may perform important roles in insects.

Excellent reviews have recently been published on structural aspects of insect OBPs and CSPs, particularly on sequence analysis [20–22], three-dimensional folding [24] and phylogenetic aspects [17]. Therefore, this review, after briefly summarising the available structural information, will focus on binding data and differential expression of OBPs and CSPs, before discussing the current ideas on their mode of action in chemoreception.

Structural aspects

Odorant binding proteins

The first OBP of insects was discovered at the beginning of the eighties in the giant moth *Antheraea polyphemus* [1], using the tritium-labelled specific pheromone (E,Z)-6,11-hexadecadienyl acetate as a probe. This protein, named pheromone-binding protein (PBP), is 142 amino acids long with an isoelectric point of 4.7 [25]. A great number of proteins similar in their amino acid sequences to the PBP were later identified in many lepidopteran species (*Lymantria dispar* [26], *Manduca sexta* [27], *Bombyx mori* [28, 29]) and several others, reported in [20–22] and subdivided, on the basis of their amino acid sequences, into four classes: PBPs, two 'general odorant-binding proteins' (GOBP1 and GOBP2) [30–32] and 'antennal binding protein X' (ABPX) [29]. All of them are referred to as OBPs.

In the last few years, OBPs have been isolated and cloned from more than 40 insect species, belonging to eight different orders. Often what all these proteins have in common is little more than a pattern of six cysteines, whose relative positions are well conserved across all insect orders. Such pattern has become a 'signature' for insect OBPs. For some of these polypeptides it has been experimentally demonstrated that the six cysteines are paired in three disulphide bridges in an interlocked fashion (1–3, 2–5 and 4–6) [33–37]. This arrangement confers great stability to the protein and seems to represent another conserved feature of OBPs. Figure 1 reports some representative examples of insect OBPs. The recent availability of several genomes has allowed the identification of all the putative OBP genes, although their involvement in olfaction can be questioned in the absence of biochemical data. While lepidopteran OBPs represent a group of closely related proteins, they appear to be very divergent when compared with those of different insect orders. As an example, the OBPs of *Drosophila* share no more than 15% of their residues with those of Lepidoptera [38, 39]. In *D. melanogaster* there are 51 genes [40–44], 39 encoding classical OBPs with the typical six-cysteine motif, while the remaining 12 exhibit specific characteristics in addition to this signature. It is worth observing that the 39 classical OBPs represent a family of divergent proteins, with the percentage of identical amino acids around 10–15%, reduced in some cases to as little as 4% (corresponding to the sole six-cysteine motif) and in only one case reaching the unusual value of 60%. The genome of *Anopheles gambiae* also contains a great number of genes encoding putative OBPs: 37 classical sequences and 35 with additional motifs [44–47].

Other OBP-like proteins, both in *Drosophila* and *Anopheles*, have more conserved cysteine residues in addition to the six of the classical motif and have been named 'Plus-C' OBPs [41, 44]. Independent experimental work discovered and cloned a similar sequence in the cockroach *Leucophaea maderae* [48]. Moreover, searching through the expressed sequence tag (EST) sequences deposited in the GenBank, we have found members of Plus-C OBPs in the moths *Manduca sexta* and *Bombyx mori*, thus suggesting that such polypeptides could be present also in other insect orders. Finally, a specific group of mosquito OBPs were annotated from the genome of *A. gambiae* and named Atypical OBPs [47]. We still do not know how the cysteines are connected in these longer polypeptides, nor even if such proteins are involved at all in chemical communication. In Figure 2 representative examples of Pluc-C OBPs are aligned.

Figure 1. Representative examples of OBPs from different orders of insects. *Lepidoptera*: *Bombyx mori* (Bmor); *Diptera*: *Drosophila melanogaster* (Dmel), *Anopheles gambiae* (Agam); *Hymenoptera*: *Apis mellifera* (Amel), *Polistes dominulus* (Pdom), *Solenopsis invicta* (Sinv); *Coleoptera*: *Anomala osakana* (Aosa), *Phylloperta diversa* (Pdiv), *Popilia japonica* (Pjap); *Hemiptera*: *Lygus lineolaris* (Llin); *Orthoptera*: *Locusta migratoria* (Lmig); *Dictyoptera: Leucophaea maderae* (Lmad). Only sequences of mature proteins are reported. The six conserved cysteines are marked with asterisks.

Figure 2. Sequences of OBPs containing four to six cysteines in addition to the six of the conserved motif. Eleven sequences of this class have been annotated in the *Drosophila* genome and are reported as OBPs, followed by a code. The alignment includes also some representative sequences of *A. gambiae* (Agam), one identified in the cockroach *L. maderae* (Lmad) and two partial sequences found in the EST database, from the two lepidopteran species *Manduca sexta* (Msex) and *B. mori* (Bmor). Conserved cysteines are highlighted.

Chemosensory proteins

CSPs are another class of small soluble polypeptides in insects. Several members of CSPs are highly expressed in the lymph of chemosensilla and exhibit binding activity towards odorants and pheromones. It is worth emphasizing, however, that not all the proteins classified as CSPs on the basis of their amino acid sequences are found in chemoreception organs. Some of them, in fact, might be involved in storing and releasing pheromone molecules (see later), while at least in one case a protein of this class was reported to promote the regeneration of legs in the cockroach. Actually, this was the first member of CSPs to be isolated [49], but its similarity with CSPs was only recognised after its sequence was determined [50]. A similar protein, OS-D (or A10), was cloned from the antennae of *D. melanogaster* in two independent studies [38, 39]. Later, proteins bearing sequence similarity to OS-D were isolated from the sensory organs of some phasmids [51–53] and locusts [54]. The immunocytochemical localisation of these proteins in the lymph of chemosensilla suggested that at least some members of these soluble proteins could be involved in insect chemoreception. The general name of chemosensory proteins (CSPs) was proposed for this class of polypeptides to include roles in both olfaction and taste [54]. CSPs have since been isolated and cloned in several insect species, belonging to different orders, such as Lepidoptera [55–60], Hymenoptera [37, 61–65], Blattoidea [50, 66, 67], Orthoptera [54, 68–70] and Hemiptera [71]. CSPs are smaller than OBPs, with 100–120 residues and bear no sequence similarity to OBPs. They present a motif of four conserved cysteines linked by disulphide bridges between neighbouring residues [54]. Unlike OBPs, such an arrangement does not contribute to the stability of the protein, but only results in the presence of two small loops, of eight and four residues, respectively, whose function is still unknown. In general, CSPs are better conserved than OBPs, with often 40–50% of identical residues even between species from different orders. Interestingly, the conserved residues seem to cluster in defined parts of the sequence, while other regions are more variable. However, sequence similarity does not necessarily imply similar function, leaving open the possibility that some CSPs, and probably also some OBPs, may perform roles not related to chemoreception, as in the above-mentioned case of the cockroach p10 involved in leg regeneration. Other sequences encoding putative CSPs have been obtained from the analysis of the recently completed genomes. The review of Wanner *et al.* [22] represents an updated classification of these proteins with a comparative analysis of 70 genes in different insect species.

CSPs are more widely distributed in insect species; they have been identified in 10 insect orders. As in the case of OBPs, the number of CSPs reported in each species is quite variable, although only the availability of the complete genome can provide definite information on the maximum number that can be expressed in a given species. The fragmentary information available in the literature has been organised more than once [17, 22], but the whole picture is continuously changing with the addition of new experimental data. In Diptera, the number of OBPs is much higher than the number of CSPs (51 OBPs vs. 4 CSPs in *D. melanogaster*, and 57 OBPs vs. 7 CSPs in *A. gambiae*). In Lepidoptera, 10 different CSPs have been cloned from tissue samples of *Mamestra brassicae* [59], and the same number of genes is present in the genome of *B. mori* [22, 57]. Five CSP ESTs have been found in *M. sexta* [58], but only one CSP has been reported in *Cactoblastis cactorum* [55]. In locusts, at least three subclasses of CSPs can be distinguished, each represented by several isoforms, with a total of at least 20 different sequences [54, 68–70]. In the genome of the honeybee, in contrast, only 6 genes encoding CSPs can be identified [22, 62, 64, 72], while in each of the hymenopteran *Polistes dominulus* [37] and *Vespa crabro* [64] a single CSP has been reported. Figure 3 lists the amino acid sequences of some representative members of this class of proteins.

The patterns of relatedness of peptide sequences of the two classes of soluble proteins, OBPs and CSPs, are visualised in the cladograms of Figure 4, where all the members of the proteins so far annotated in insects are included. We can observe a certain degree of segregation of the sequences according to the order. However, sequences in some species of the same order (e.g. in Hymenoptera), or even in the same species (e.g. *D. melanogaster* and *A. gambiae*), can be found in different branches of the tree. This indicates the existence of several subgroups of OBPs, defined only on the basis of sequence similarity. Figure 4a clearly shows that lepidoteran OBPs are grouped into three clusters, PBP, GOBP and ABPX, with the PBPs further subdivided into two clusters (cycled in Fig. 4a). Two OBPs of the fruit fly *D. melanogaster* and the PBP of the cockroach of *L. maderae* are closely related to Lepidoptera PBPs. In the order Hymenoptera OBPs from 18 species of *Solenopsis* genus are clustered together [76], while the other OBPs are scattered in different branches. Another order-specific cluster is formed by OBPs from 6 species of Coleoptera. On the other hand, the dipteran OBPs are much more divergent. We have also identified – and here report for the first time – CSP sequences in EST database of the brine shrimp *Artemia franciscana,* a crustacean species and the millipede *Julida* sp.*,* a myriapodan species. Figure 4b shows that the crustacean CSP (AfraCSP1) and the myriapodan CSP (JulidaCSP1) are closely related to other CSPs. Both sequences fall right among the CSPs of insects, suggesting that these soluble proteins could be present across all arthropods, and their origin therefore could be predated before the divergence of subphyla Crustacea, Uniramia and

Myriapoda, much earlier than previously reported from the separation of the class Neoptera [22].

Three-dimensional structures

So far the structures of six insect OBPs have been resolved. A recent excellent review provides most of the information available on such aspects [24]. The PBP of *B. mori* was the first to be studied, both in its crystallised form by X-ray diffraction spectroscopy [77] and in solution using nuclear magnetic resonance (NMR) techniques [78, 79].

Figure 5a shows the structure of the *B. mori* PBP complexed with a molecule of bombykol, the sex pheromone for this species. The protein, mainly folded into α-helical domains, is very compact due to the presence of three interlocked disulphide bridges. The molecule of bombykol is bound in a pocket formed by helices α1, α4, α5 and α6. More recently, calculations of the energies involved in the formation of the complex have indicated that the binding of the pheromone to the protein is due to specific forces, not the result of general hydrophobic interactions [80]. The compact structure of the PBP seems to require a conformational change to accommodate the molecule of bombykol inside the binding pocket. However, at pH 6.5 the structure of the protein appears the same both in the presence and in the absence of bombykol. Therefore, any conformational change allowing the ligand to enter the binding cavity would not be detectable once the complex was formed [81]. A major conformational change, on the other hand, has been observed when the pH changes from 6.5 to 4.5. In acidic conditions the C-terminus of the protein folds into an α-helical domain and enters the bombykol binding site (Fig. 5b), thus assisting the release of the pheromone molecule from the cavity. Such behaviour is absent in other OBPs, such as the PBP of the cockroach *L. maderae* [82], which lacks the C-terminus common to Lepidopteran OBPs, being 19 residues shorter than *B. mori* PBP. Therefore, the formation of a seventh α-helix for pushing its ligand out of the binding cavity cannot occur in this protein. Interestingly, components of the pheromonal blend of *L. maderae*, such as 3-hydroxy-butan-2-one, which bind the PBP with good affinity, are highly hydrophilic, unlike moth pheromones [83]. Therefore, as the authors suggest, an active mechanism for releasing the ligand would not be needed in this case.

Folding of the PBP of the giant moth *A. polyphemus* was resolved as a complex with the specific pheromone at pH 6.3 using NMR spectroscopy [84]. At pH 4 and 5 the authors recorded an overall change of the spectrum, accompanied by loss of binding. A conformational change, similar to that observed for the PBP of *B. mori*, also seems to occur in this protein, as reported by more recent studies [85, 86]. At 5.2, in fact, the PBP of *A. polyphemus* exhibits a pH-induced structural change, where the protonation of His69, His70 and His95 in the binding pocket causes a reorientation of α-helices 1, 3 and 4, thus providing the driving force for the release of the pheromone molecule from the cavity. At this pH, binding of the pheromone is drastically reduced.

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Pdom-CSP1                EEEELYSDKYDYIDPMEIVNNDRLRDQYYNCFMNTG--PCVTPDAIYFKEHFPE
Amel-ASP3                  DESYTSKFDNINVDEILHSDRLLNNYFKCLMDEG-R-C-TAEGNELKRVLPD
Ecal-CSP1                 EGGKYTTKYDNVNLEEVFGNERLLESYRKCLMDEGL--C-APDAEELKKAIPD
Ecal-CSP3                 EGGKYTTKYDNVNLKEIFENERLFASYKECLL--GNRPC-PPDGQELKDAIPD
Sgre-CSP4                  EEKYTTKYDNVNLDEILANDRLLNKYVQCLLEDDESNC-TADGKELKSAVPD
Mbra-CSPA6                 EDKYTDKYDNINLDEILANKRLLVAYVNCVMERG-K-C-SPEGKELKEHLQD
Bmor-CSP1                  DDKYTDKYDKINLQEILENKRLLESYMDCVLGKG-K-C-TPEGKELKDHLQE
Bmor-CSP2                  QDKYEPIDDSFDASEVLSNERLLKSYTKCLLNQG--PC-TAELKKIKDKIPE
Ccac-CLP1                ARPSDTYTDKYDNINIQEILENKRLLEAYVNCVLDKG-K-C-TPEGKELKEHLQE
Dmel-OS-D PHPPATSPSPMMERMVEQAYDDKFDNVDLDEILNQERLLINYIKCLEGTG--PC-TPDAKMLKEILPD
Dmel-EjB                   EDKYTTKYDNIDVDEILKSDRLFGNYFKCLVDNG-K C-TPEGRELKKSLPD
Agam-CSP1                   QKYTDKFDNIDVDRVLSNDRILNNYLKCLLDKG--PC-TQEGRELK-TLPD
Pame-p10                   DDKYTTKYDNIDLDEILASDRLLANYHKCLIEEG-K-C-TPDGEELKSHVSD
Afra-CSP1                HNRVRRQKPGQLENIDVDSLLKNKKYVQTQIKCILNEG-K-C-DKTGRDMKDLLPE
                                                          *       *

Pdom-CSP1  AVVTKCKKCTEIQKTNFEKLAIWYNENRPDEWTALIKKFMEDAKKQNS              100
Amel-ASP3  ALATDCKKCTDKQREVIKKVIKFLVENKPELWDSLANKYDPDKKYRVKFEEEAKKLGINV   109
Ecal-CSP1  ALENECAKCSEKQKAGVETTIVFLIKNKPEIWESFKKKYDPTHKYEKIYERYIKQAEEKARKS 113
Ecal-CSP3  ALENECAKCSEKQKAGVETTIVFLIKNKPEVWESFKKKYDPTHKYQTFYDNLLKQAEEKAKSS 113
Sgre-CSP4  ALSNECAKCNEKQKEGTKKVLKHLINHKPDVWAQLKAKYDPDGTYSKKYEDREKELHQ      109
Mbra-CSPA6 AIENGCKKCTENQEKGAYRVIEHLIKNEIEIWRELTAKYDPTGNWRKKYEDRAKAAGIVIPEE 112
Bmor-CSP1  ALETGCEKCTEAQEKGAETSIDYLIKNELEIWKELTAHFDPDGKWRKKYEDRAKAKGIVIPE  111
Bmor-CSP2  ALETHCAKCTDKQKQMAKQLAQGIKKTHPELWDEFITFYDPQGKYQTSFKDFLES         104
Ccac-CLP1  AIENGCEKCTEAQEKGAYTVIEHLIKNEIEIWRQLADKFDPERKYRKKYEDRARAKGIEIPE 114
Dmel-OS-D  AIQTDCTKCTEKQRYGAEKVTRHLIDNRPTDWERLEKIYDPEGTYRIKYQEMKSKANEEP   125
Dmel-EjB   ALKTECSKCSEKQRQNTDKVIRYIIENKPEEWKQLQAKYDPDEIYIKRYRATAE         109
Agam-CSP1  ALKTNCEKCSEKQRTSSRKVIAHLEERKPQEWKKLLDKYDPEGIYKSKFEKINKRS      104
Pame-p10   ALQ--CAKCSDKQRAGAEKVINFLYNKKKPMWESLQKKYDPENTYVTKYADRLKELHD     105
Afra-CSP1  VLQRNCRKCSEVQKVNADKIINYMKQNHPSGVDQNFQHLQQNLSY                   98
                    *  *
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Figure 3. Sequences of representative CSPs from different insect species. Conserved residues are highlighed. Lepidoptera: *B. mori* (Bmor), *Cactoblastis cactorum* (Ccac); Diptera: *D. melanogaster* (Dmel); Hymenoptera: *A. mellifera* (Amel), *P. dominulus* (Pdom); Orthoptera: *Schistocerca gregaria* (Sgre); Dictyoptera*: Periplaneta americana* (Pame); Phasmatodea*: Eurycantha calcarata* (Ecal). A CSP-like protein has been described in a non-insect arthropod, the brine shrimp *Artemia franciscana* (Afra), suggesting that CSPs might be present all across arthropods.

The structure of LUSH, another OBP identified in *Drosophila*, shows its C-terminus folded back into the core of the protein [87]. Such a conformation is similar to that assumed by moth PBPs in acidic conditions, but occurs in this protein at neutral pH (Fig. 5c). The authors have crystallised LUSH in the presence of small alcohols that have been found to occupy the binding cavity. However, binding assays in solution have measured good affinity only to large aromatic molecules [88]. It has been proposed that in order to let such ligands enter the binding cavity, the C-terminus of the protein should move out, with a mechanism similar to that described for the *B. mori* PBP [87]. Like LUSH, the C-terminus of the honeybee's OBP, ASP1, which is shorter than in *B. mori* PBP but longer

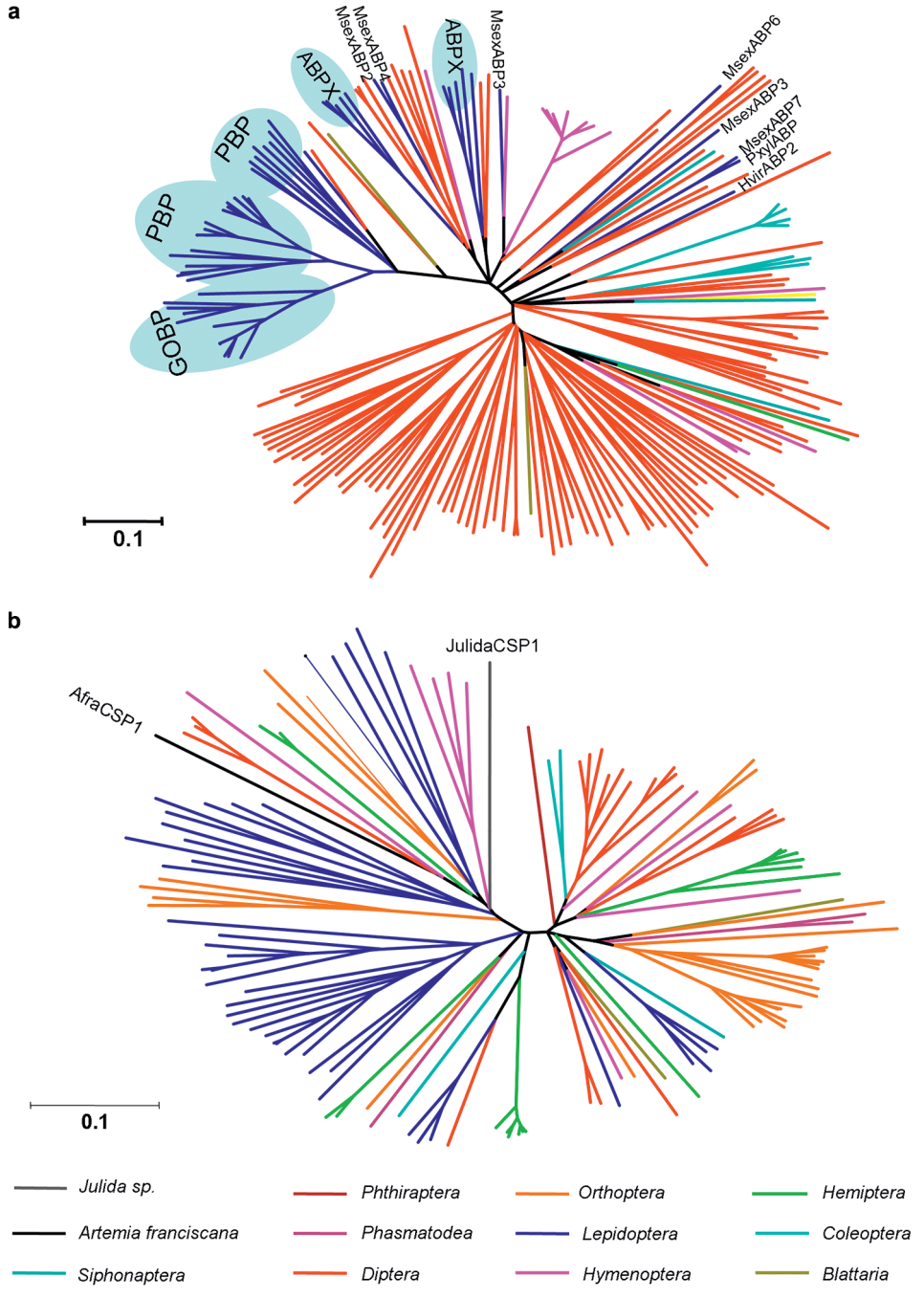

Figure 4. Cladograms of all OBPs (*a*) and CSPs (*b*) of insects, whose sequences have been annotated. For some OBPs and all the CSPs, a three-letter code at the end of each line indicates the order, followed by the sequence number. The trees were constructed using the MEGA3 program with neighbour-joining phylogeny and the p-distances model. The mature amino acid sequences were aligned with ClustalW 8 [73] with default gap-penalty parameters of gap opening 10 and extension 0.2. The cladograms were then constructed from these multiple alignments using MEGA3 software [74]. The final unrooted consensus tree was generated with 1050 bootstrap trials using the neighbour-joining method [75].

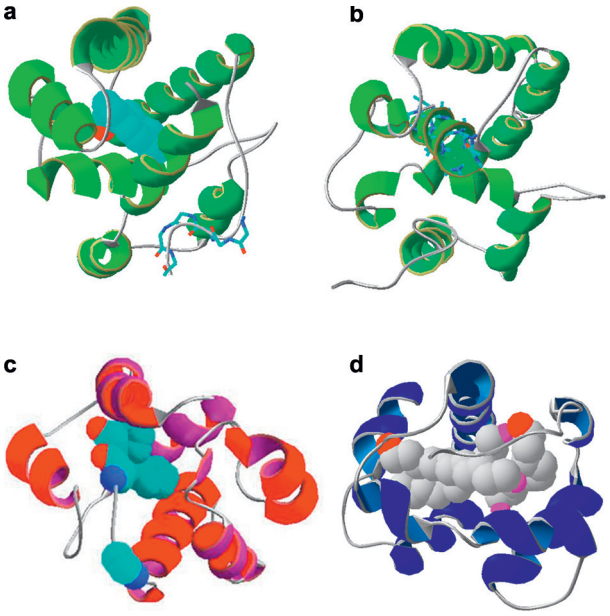

Figure 5. Three-dimensional structures of OBPs and CSP. (*a*): PBP of the silkmoth *B. mori* at pH 6.5, complexed with a molecule of its ligand, the specific pheromone bombykol. The C-terminus, whose residues are shown with their side chains, is not structured at this pH [77]. (*b*) the same protein at pH 4.5; the C-terminus is folded into an α-helical segment that fits into the bombykol binding pocket, chasing out the ligand [78, 79]. (*c*) structure of *D. melanogaster* LUSH, one of the classical OBPs [87]. In neutral conditions this proteins presents its bulky C-terminus (a triptophan and a proline residue shown in space-filling mode) folded back into the core of the protein. The binding of large aromatic molecules likely involves a conformational change, where the C-terminus is folded out, rotating around the flexible glycine (shown in space-filling mode). (*d*) CSP of *M. brassicae*: the protein is mainly constituted by α-helical domains, like OBPs, but folded in a motif different from that of OBPs and unlike any other protein [91]. Three molecules of bromododecanol are shown inside the protein. A major conformational change has been observed consequent to the process of ligand binding [92].

than in *L. maderae* PBP, folds back into the protein core without forming an α-helix, and partially occupies the binding cavity [89].

Finally, one of the OBPs of *A. gambiae*, the last whose structure has been resolved, presents the common motif of the six α-helices, but with an additional interesting feature. The ligand-binding pocket is a sort of tunnel running from one end of the protein to the other, thus potentially allowing a ligand to pass through the protein. Moreover, in the crystal structure this OBP is present as a dimer, with the binding pockets of the two units connected to make a continuous long tunnel [90].

The structure of a single CSP has been published so far, that of *M. brassicae* A6, resolved both in its crystallized form and in solution [91–93]. This protein is folded into a very compact and stable structure (Fig. 5d), with more than half of the residues involved in six α-helical domains. This folding is different from that of OBPs and any other class of proteins. One of the major differences with insect OBPs is that the two disulphide bridges present in CSPs do not contribute to their stability, connecting two adjacent conserved cysteine residues in the sequence. Another difference is the shape of the binding site, a sort of 14-Å-long channel, which can be further extended by rotation of a tyrosine ring. Major conformational changes, triggered by ligand binding, have also been observed with this CSP. In fact, the protein can swallow three molecules of 12-bromododecanol (Fig. 5d), thus increasing its size in the process [92]. Such a mechanism is also likely to occur with other ligands, and it is unclear whether the binding site is made to take more molecules of the same ligand with a positive cooperative kinetic, as observed, or a single large molecule of about the size of a triglyceride.

Tissue expression and cellular localisation

Another important piece of information towards understanding the physiological function of a protein is its expression both at the tissue and subcellular levels. Generally, OBPs and several CSPs are present in the lymph of chemosensilla, bathing the dendrites of chemosensory neurons. Chemosensilla tuned to different classes of semiochemicals can be morphologically different, regardless of their anatomical localisation. Therefore, although antennae are generally regarded as olfactory organs and tarsi and mouth as taste organs, it is the type of sensillum where an OBP or a CSP is expressed, rather than the organ, that might indicate whether the protein is involved in olfaction or taste.

In earlier studies, one of the criteria adopted for the identification of OBPs in Lepidoptera was their specific expression in the antennae. Moreover, those OBPs specifically or preferentially produced by one sex were initially classified as PBPs, a suggestion later supported by ligand-binding experiments and by their selective expression in sensilla that are electrophysiologically active to the specific pheromones [31]. Such an approach was also applied to insects of other orders, as in the case of the cockroach *L. maderae*, whose PBP is uniquely expressed in the antennae of the females. In this species the sex pheromone is in fact produced by the males [83]. In other cases, proteins classified as OBPs on the basis of their sequences could in fact be involved in taste. Thus in the fly *Phormia regina* an OBP, CRLBP, is expressed in taste sensilla of palpi and tarsi, but also in wings and antennae [94, 95]. In *D. melanogaster* and *A. gambiae*, the expression of OBPs in different organs has been monitored using OBP promoter-driven expression of the LacZ reporter gene [40], *in situ* hybridisation [41] and reverse transcriptase-polymerase chain reaction (RT-PCR) [44, 96, 97]. Few members are specifically present in single organs, while a relatively large number of OBPs have

been detected in different sensory parts, such as antennae, mouth and tarsi, as well as in other parts of the body. We cannot exclude that some of these genes, classified as OBPs on the basis of their sequences, could encode proteins that are not involved in chemoreception. This might be the case for the so-called Gp-9, an OBP expressed in the thorax of queens of the red imported fire ant, *Solenopsis invicta*. Its absence produces colonies with more than one queen [98]. Rather than being involved in the perception of the pheromone, as suggested by the authors, this protein could be active in the release of the queen pheromone, thus inhibiting the fertility of other females.

For CSPs, the relationship between tissue expression and function is even less conclusive, as these proteins appear to be more widely distributed in the insect's body, although most of them have been isolated from sensory organs. Thus, while in the lepidopteran *C. cactorum* [55] CSP is abundant in sensilla of the maxillary palps, in another lepidopteran species, *M. brassicae*, proteins of the same family have been detected in antennae, proboscis, labial palps and pheromone glands [59]. In locusts, CSPs have been purified from antennae, tarsi and mouth organs, but are also expressed in large amounts in the wings [54, 69, 70]. Another CSP is secreted by the ejaculatory bulb of *D. melanogaster* [99], where again a role in pheromone release could be hypothesised. The idea that OBPs and CSPs are two sets of functionally different proteins is also supported by the observation that in moths the synthesis of CSPs starts before the synthesis of OBPs, suggesting that the expression of these two classes of proteins is controlled by independent mechanisms [60].

The prevailing idea that OBPs rather than CSPs are the proteins of chemoreception was suggested by the observation that in many species OBPs are antennal specific, while CSPs are also expressed in other parts of the body. However, a few cases have been reported where the situation is reversed. In some Hymenoptera, such as the argentine ant *Linepithema humile* [63], the paper wasp *P. dominulus* [37] and the hornet *V. crabro* [64], CSPs are only expressed in the antennae. OBPs, in contrast, at least in the last two species, were also isolated from tarsi and wings. Such 'reversed' expression, however, is not a general rule in hymenopterans. In fact, the five OBPs and three CSPs of the honeybee *A. mellifera* revealed a more complex expression pattern, as monitored by RT-PCR [64]. A complex pattern of expression of some OBPs has also been observed in *Anopheles* [97] and in *Drosophila* [40], where several members are expressed in taste organs, such as the labellum, the pharyngeal labral sense organ, dorsal and ventral cibarial organs, as well as taste bristles located on the wings and tarsi.

More specific information has been obtained at subcellular level by immunocytochemical localisation of OBPs and CSPs within chemosensilla. In several studies, performed by using polyclonal antibodies, OBPs were always shown to be highly concentrated in the extracellular sensillum lymph, but never on the neuron dendrites. Moreover, of the three cells – tormogen, trichogen and techogen – that are located at the base of the sensillum, only the first two were labelled, indicating that synthesis of OBPs is restricted to those cells. In Lepidoptera, a simple classification of OBPs into four subclasses (PBP, GOBP1, GOBP2 and ABPX) parallels a relatively simple classification of types of chemosensilla. All pheromone-sensitive sensilla trichodea express PBPs, while most sensilla basicona, which respond to general odorants, mainly express GOBPs [100–103]. This correlation, although being an oversimplification of more complex and diversified situations, represents a general guideline that can be broadly applied to Lepidoptera. Studies performed mainly with *A. polyphemus* and *B. mori* have also indicated that the expression of a particular type of OBP is not related to a specific morphogical type of sensillum, but rather to the classes of semiochemicals (pheromones or general odorants) to which the sensillum responds. Thus, long sensilla trichodea that are present on the male and female antennae of *B. mori* respond to different stimuli and express different proteins in the two sexes. In the males they are pheromone specific and express PBP, whereas in the females they respond to benzoic acid and linalool and express GOBPs [101]. Expression of GOBP1 in both basiconic and trichoid sensilla was also observed by *in situ* hybridisation in *M. sexta* [43]. As a general rule, in Lepidoptera each sensillum expresses a single type of OBP, with sensilla trichodea mainly expressing PBPs and sensilla basiconica GOBPs. However, the presence of a great number of non-labelled sensilla, the heavy expression of PBP in female sensilla and a number of morphological subtypes of sensilla in lepidopteran species make the picture less defined, and general conclusions are difficult to draw [101, 103].

The situation is even more complex in other orders of insects, where OBPs cannot be segregated into well-defined subclasses as in Lepidoptera. This is partly due to the limited information available, and also to the great variety of sensilla, which do not always have their counterparts in Lepidoptera. Moreover, two OBPs of *Drosophila*, OS-E and OS-F, were always found to be co-localised in the same sensillum [104]. These two proteins represent a unique case of high similarity (60% of identical residues) among *Drosophila*'s OBPs. Some of these sensilla also express LUSH, a third protein of the same family [105]. Expression of OBPs has also been reported in gustatory sensilla of *Drosophila* [40]. Finally, two other *Drosophila* OBPs, PBPRP2 and PBPRP5, present in the adult quite distinct expression patterns, the first being present in the outer lymph of sensilla coeloconica, the second in the lymph of olfactory basiconica sensilla. Interestingly, both proteins are also expressed in the larvae [106].

Apart from moths and *Drosophila*, in only two other species has the immunocytochemical localization of OBPs

been investigated. The OBP of the hemipteran *Lygus lineolaris*, LAP, is specifically expressed in olfactory multipore sensilla of the antennae [107]. In the oriental locust *Locusta migratoria,* OBP is only expressed in olfactory hairs, trichodea and basiconica, of the antennae, but not in the gustatory sensilla coeloconica and chaetica [70]. Moreover, only two – trichogen and tormogen – of the three cells at the base of the sensillum were stained, indicating that the synthesis of OBP takes place in these two cells, as previously observed in Lepidoptera. A very recent study, performed on the labial palps of the same species, reports that OBP and CSPs are differentially expressed in this organ. In fact, OBP was detected only in sensilla basiconica and CSP-I only in some subtypes of sensilla chaetica, while CSP-II was not found in any of the observed hairs [108].

The first immunocytochemical study with CSPs revealed that in the desert locust *Schistocerca gregaria* these proteins are associated with contact chemosensory hairs [54]. Such sensilla present two cavities, both filled with lymph, but not in contact with each other. CSPs have been always found in the lymph of the outer cavity that is not in contact with neuronal dendrites, but communicates with the external environment through the apical pore. In subsequent experiments, using antisera against three different CSPs, only sensilla chaetica of the oriental locust *L. migratoria* were stained, irrespective of the organs, antennae, tarsi or palpi [70].

In other cases, the expression of CSPs in the regions that are dense with sensilla coeloconica has suggested that such proteins could be involved in the perception of carbon dioxide. Examples of these CSPs include the CLP-1 of *C. cactorum* [55] and the *Drosophila* OS-D [38]. In the moth *Heliothis virescens* CSP messenger RNAs (mRNAs) were detected in various parts of the body, particularly in legs. The expression of HvirCSP1 in legs started early during adult development, in parallel with the appearance of the cuticle. HvirCSP1 mRNA was detectable five days before eclosion (day E-5), increased dramatically on day E-3 and remained at a high level into adult life. The authors suggest that in this species CSPs may be involved in contact chemosensation [60]. Such variety in the tissue and cellular distribution of CSPs could indicate that these proteins may have adapted to different roles according to the species.

Ligand binding

The binding activity of OBPs and CSPs to small organic compounds, such as pheromones and other semiochemicals, is the key feature of these proteins and provides essential information for understanding their physiological function. Most of the binding assays have been performed with proteins expressed in heterologous systems. This, however, seems not to be a problem because both OBPs and CSPs do not carry post-translational modifications apart from the establishment of disulphide bridges [37, 53, 54, 57, 69]. Moreover, it has been experimentally demonstrated that such bridges form spontaneously in the same pattern observed in the natural proteins [37]. Different approaches were adopted to demonstrate the formation of a complex between protein and ligand, and to measure the relative dissociation constants. Each method presents advantages and drawbacks.

To identify the first OBP of insects, Vogt and Riddiford [1] analysed an antennal extract, previously incubated with the tritium-labelled pheromone (E,Z)-6,11-hexadecadienyl acetate by native gel electrophoresis, followed by autoradiography. Later, this method was widely used either as described in the original paper or with modifications. In some studies, radioactive photoaffinity labels with structural similarity to the pheromone were used [109]. Such ligands establish a covalent bond with the protein, thus preventing dissociation of the complex that otherwise would extensively occur during the electrophoretic run. On the other hand, the preparation of radioactive photoaffinity labels is often complicated and expensive. An alternative to autoradiography is slicing the gel into thin horizontal sections after the electrophoretic run, and counting them for radioactivity. All the methods that include an electrophoretic separation step do not allow estimation of the dissociation constants although they present the advantage of associating the radioactivity of the ligand to a specific electrophoretic band and do not require a pure sample of the protein. Unfortunately, often the great majority of the radioactivity is lost during the electrophoresis, and this raises some questions about the significance of the final measurements.

More recently, fluorescent binding assays have been applied to study the binding of both OBPs [110] and CSPs [111] to putative ligands. This technique uses a fluorescent probe that upon binding modifies its emission spectrum. Generally, a blue shift is observed, accompanied by a marked increase in intensity. The method requires a fluorescent compound that exhibits some affinity for the protein being studied. But, when applicable, it presents the unique advantage of allowing binding measurements at the equilibrium. Moreover, it is simple, rapid and safe, not requiring the use of radioactive ligands. Fluorescence measurements have been currently the method of choice for OBPs and CSPs. In some cases where a suitable probe is not available, the intrinsic fluorescence of tryptophan can be measured to monitor the presence of a ligand, provided that a tryptophan residue is positioned in the binding pocket and that its interaction with the ligand appreciably affects its fluorescence properties. Typically, aromatic ligands are good quenchers, but bromo derivatives of potential ligands have also been used successfully. The quenching of intrinsic fluorescence of

Table 1. Binding of pheromones and odorants to OBPs and CSPs of insects from different species.

Species	Protein	Ligands	Method	K_D (μM)	Ref.
Antheraea polyphemus	ApolPBP1	^{3}[H]-(E,Z)-6,11-C16-Ac	native PAGE	–	[1, 121–124]
Antheraeapolyphemus	ApolPBP1	^{3}[H]-(E,Z)-6,11-C16-DzAc	photoaffinity	–	[125]
Antheraea polyphemus	ApolPBP1	1-AMA + ligands	fluorescence	0.5–1.4	[110]
Antheraea polyphemus	ApolPBPs	pheromones	W fluorescence	0.6- 0.8	[114]
Antheraea polyphemus	ApolPBPs	tritiated pheromones	native PAGE	–	[124]
Antheraea pernyis	AperPBPs	tritiated pheromones	native PAGE	–	[124]
Bombyx mori	PBP	tritiated bombykol	chromatography	–	[126]
Bombyx mori	PBP	bombykol	W fluorescence	1.1	[110]
Bombyx mori	PBP	bombykol	extraction + GC-MS	–	[119]
Lymantria dispar	PBP1, PBP2	tritiated pheromones	gel filtration	1.8–7.1	[115]
Lymantria dispar	PBP1, PBP2	pheromones and analogues	DNS-PBP and W fluorescence	0.1–0.3	[116]
Mamestra brassicae	MbraPBPs	tritiated pheromones	native PAGE	–	[127]
Mamestra brassicae	MbraPBP1	1-AMA + ligands	fluorescence	0.1–0.6	[110]
Mamestra brassicae	CSPMbraA6	12-bromo-dodecanol	W fluorescence		[91]
Mamestra brassicae	CSPMbraA6 CSPMbraB3	tritiated pheromones	native PAGE		[56, 59]
Thaumatopoea pityocampa	PBP	^{3}[H]-(Z)-13-hexadecen-11-ynyl DzAc	photoaffinity	–	[128]
Manduca sexta	GOBP	general odorants	photoaffinity	–	[109]
Apis melliphera	ASP1 (OBP)	pheromones	extraction	–	[129]
Apis melliphera	ASP2 (OBP)	2-heptanone, other ligands	calorimetry, VOBA	0.14–0.45	[35]
Apis melliphera	ASP3 (CSP)	ASA + ligands	fluorescence	0.57 (ASA)	[62]
Drosophila melanogaster	LUSH (OBP)	1-NPN, ligands	fluorescence	1.5 (1-NPN)	[88]
Polistes dominulus	OBP-1	1-NPN, ligands	fluorescence	2.1 (1-NPN)	[37]
Polistes dominulus	CSP-1	1-NPN + amides	fluorescence	2.2 (1-NPN)	[37]
Camponotus japonicus	CSP	long-chain hydrocarbons	extraction + GLC	–	[65]
Schistocerca gregaria	CSP-I	1-NPN + ligands	fluorescence	4.0 (1-NPN)	[111]
Locusta migratoria	OBP-1	1-NPN, ligands	fluorescence	1.7 (1-NPN)	[36]
Locusta migratoria	CSP-II	1-NPN + ligands	fluorescence	6.2 (1-NPN)	[69]
Leucophaea maderae	PBP	ANS, ligands	fluorescence	2.1 (ANS)	[83]

For explanation of the methods, see text. Dissociation constants refer, where applicable, to the radioactive ligand or the fluorescent probe. 1-AMA, 1-aminoanthracene; ANS, 1-anilinonaphthalene-8-sulphonic acid; 1-NPN, N-phenyl-1-naphthylamine; DzAc, diazoacetate; DNS-PBP, dansylated PBP.

tryptophan residues in the binding pocket was adopted to measure the binding of aromatic ligands to the CSP of *S. gregaria* [111] or bromo derivatives to the CSP of *M. brassicae* [92]. The results of binding experiments so far published for insect OBPs and CSPs are summarised in Table 1, while Figure 6 reports the structures of the fluorescent probes and their spectral characteristics. The most widely used probes are 1-aminoanthracene (1-AMA) and N-phenyl-1-naphthylamine (1-NPN); both were previously employed in binding experiments with vertebrate OBPs [112, 113]. 1-AMA was first adopted with the PBPs of the moths *M. brassicae* and *A. polyphemus* [110]. This compound, when excited in water at the wavelength of 256 nm [112] or 298 nm [110], produces a weak emission spectrum with a maximum at 563 nm. When 1-AMA is bound to a protein, such as *M. brassicae*

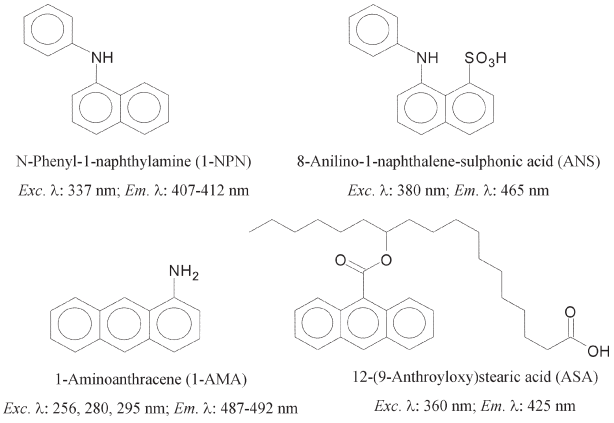

Figure 6. Structures and spectral characteristics of the fluorescent probes used in binding experiments with insect OBPs and CSPs.

MbraPBP1, a severalfold increase in the intensity is observed with a shift of the maximum to 492 nm. By using this probe together with different organic compounds, it was possible to measure the affinities of several ligands in competitive binding assays. Thus, MbraPBP1 binds all three components of the pheromone (Z)11-hexadecenol, (Z)11-hexadecenal and (*Z*)11-hexadecenyl acetate with dissociation constants between 0.17 and 0.29 μM [110]. Fatty acids also bind well to MbraPBP1, especially palmitic acid, with a dissociation constant of 0.12 μM. The same study reported that the PBP of the moth *A. polyphemus*, ApolPBP1, binds the specific pheromone and a series of structurally related compounds. In this case, the components of the pheromonal blend (E)6,(Z)11-hexadecadienal, (E)6,(Z)11-hexadecadienyl acetate and (E)4,(Z)9-tetradecadienyl acetate display identical dissociation constants (0.50, 0.48 and 0.51 μM, respectively). Moreover, pheromones of other insect species, such as bombykol of *B. mori* and several fatty acids, also strongly bind to ApolPBP1 with similar affinities (K_D between 0.56 and 1.36 μM). The quenching of tryptophan intrinsic fluorescence has been used to measure the affinity of pheromone components to the PBP of *A. polyphemus* [114]. All these results and other data obtained with different OBPs indicate a rather broad specificity of binding, in contrast with the extremely high selectivity exhibited by insects in recognising their specific pheromones. Dissociation constants in the micromolar range have also been measured with the two PBPs of *Lymantria dispar* using tritium-labelled ligands and a binding assay in heterogeneous phase [115]. In the same work, the authors also observed some selectivity of binding between the two enantiomers of the pheromone. More recently, the same group reported competitive binding data with the same proteins and some pheromone analogues in fluorescent assays [116]. The OBP of the paper wasp *P. dominulus* also exhibits a marked selectivity of binding to oleoamide: both the *trans* isomer, elaidic amide, and the corresponding saturated compound, stearic amide, bind to the OBP with dissociation constants more than one order of magnitude higher than that of oleoamide [37]. The OBP of *L. migratoria* that also binds oleamide with a constant of micromolar order, in contrast, shows a much broader spectrum of ligands, including also large aromatic molecules [36]. The PBP of the cockroach *L. maderae* is particularly interesting for the fact that it is the only insect OBP so far reported to bind a hydrophilic ligand, 3-hydroxy-butan-2-one, a component of the pheromonal blend for this species [83]. The dissociation constant for this compound has been estimated as 3.8 μM. It is noteworthy that only a closely related compound, butane-2,3-diol, binds to the protein with comparable strength, while other components of the pheromonal blend (3-methyl-2-butenoic acid and E-2-octenoic acid), as well as other organic compounds failed to show any measurable affinity [83]. Finally, the *Drosophila* OBP LUSH, whose absence had been previously related to an anomalous behaviour of the fly towards high concentrations of alcohols [117], failed to bind alcohols as well as a great number of volatiles known to elicit electrophysiological responses in the fly's antenna, but showed good affinity to a series of large aromatic compounds, including some phthalates [88]. Although phthalates cannot obviously be considered its natural ligands, this protein seems nevertheless to be tailored to large-size organic compounds. This is in agreement with a later report showing that LUSH is expressed in sensilla responding to the male-produced aggregation pheromone vaccenyl acetate [118]. However, ligand-binding experiments of this compound to LUSH have not been reported.

A recently described binding protocol for OBPs involves separation of the complex from free ligand by rapid ultrafiltration and evaluation of bound ligand following extraction using gas chromatography-mass spectrometry (GC-MS) analysis [119]. This method presents the advantage that it can be applied to mixtures of organic compounds, all incubated at the same time with the protein, allowing the identification of the best ligands in a single experiment. Other protocols used to measure dissociation constants of ligands to insect OBPs also involve calorimetry and VOBA (volatile odorant binding assay) [35].

To address the question whether OBPs could discriminate between similar ligands and perhaps act as signalling proteins, an interesting study [120] looked for conformational changes of the protein during ligand binding. Using circular dichroism measurements, the authors monitored the secondary structure of two PBPs of *A. polyphemus* upon binding the pheromone components. The interesting result was that although both proteins bind all three components of the pheromonal blend equally well, only one of them produced a conformational change in any given protein. The conclusion of this study was that OBPs can exhibit a higher selectivity when studying the phenomenon of binding at the microscopic level.

Fewer binding data have been published for CSPs with respect to OBPs, using both radioactive and fluorescent probes. Certainly, CSPs can be classified as binding proteins based on the fact that they possess a hydrophobic binding cavity in their three-dimensional structure and have been shown to bind a variety of different compounds, depending on the insect species. Using the fluorescent probe 1-NPN, binding affinities to a wide range of compounds were measured for CSPs of *S. gregaria* [111] and *L. migratoria* [69]. In some cases, however, CSPs have been found to be more narrowly tuned to specific compounds. For instance, the CSP of the paper wasp *P. dominulus* – as was the case for the OBP of the same species mentioned above – has shown a significant specificity towards amides of 16–18 carbon fatty acids [37]. Similarly, the honeybee CSP, ASP3c, which is expressed

in drones, selectively binds fatty-acid ester components of the brood pheromone [62]. This work used a novel fluorescent probe, 12-(9-anthroyloxy)stearic acid (ASA), a large molecule containing the fluorescent moiety of anthracene attached to a long fatty-acid chain, mimicking the protein's ligands. In another study, the CSPs of *M. brassicae* are reported to bind the pheromone components (*Z*)-11-hexadecenyl acetate and (*Z*)-11-octadecenyl acetate, but the method used did not allow evaluation of the dissociation constants [59].

A very recent paper suggests cuticular hydrocarbons as the ligands for one of the CSPs of the carpenter ant *Camponotus japonicus* [65]. This protein is expressed in a single type of sensilla that responds to blends of cuticular hydrocarbons and mediates recognition of nest mates. Due to the complete insolubility in water of long-chain hydrocarbons, these compounds were deposited on the wall of glass test tubes by evaporating their solutions in organic solvents. The protein solution was then added to the tubes, and the amount of ligand bound to the protein was evaluated by gas-liquid chromatography (GLC) after extraction from the aqueous solution with an organic solvent. The CSP described in this paper showed no specificity for any single component of the hydrocarbon blend, but reproduced the pattern of the original mixture in the bound fraction.

Endogenous ligands

An interesting phenomenon, well documented in some mammalian species, is that proteins structurally very similar, or even identical, to OBPs are involved in the delivery of pheromonal signals. Typical examples are the urinary proteins of mouse and rat [130–136] and the salivary proteins of pig [137, 138]. These proteins are male specific and secreted at high concentrations in the respective biological fluids complexed with the specific volatile pheromones. Another protein with analogous function is aphrodisin, a component of hamster vaginal discharge [139, 140]. In insects some data seem to suggest that a parallel phenomenon may be present, where OBPs as well as CSPs might help in some circumstances delivering hydrophobic pheromones in the environment. It has already been mentioned – when discussing tissue expression – that in some cases OBPs and CSPs are expressed in non-sensory organs. When purified from these sources, proteins of both classes have been found complexed with endogenous ligands. This phenomenon has been observed with CSPs in the locust *L. migratoria* [69] and with OBPs of the wasp *P. dominulus* [37]. Both proteins, when isolated from the wings, are associated with long-chain amides. In other cases, the co-occurrence of pheromones and binding proteins in the same organ could suggest that the two components may be associated, although direct experimental evidence has not yet been provided. One example is the EjB protein, a CSP of *D. melanogaster* which is expressed in the ejaculatory bulb, where the putative male pheromone vaccenyl acetate is also synthesised [99]. Another case is the CSP of *M. brassicae,* which is expressed in the glands where the moth's pheromones are secreted [59]. An OBP of *Solenopsis invicta* could also be included in such a group of carrier proteins. This protein, as already mentioned, is expressed in the thorax and reported to be required for the formation of monogyne colonies [98]. Although a potential ligand has not been identified, a role of pheromone carrier for this protein is plausible, in analogy with the cases described above.

In all these instances, a role of OBPs or CSPs in chemoreception cannot be proposed in organs lacking sensory structures. Instead, a function in releasing the chemical messages in the environment seems more likely and is supported by analogy with mammalian systems. Moreover, it has also been shown that by regulating the synthesis of the different OBPs in urine, a mouse can modulate the release of a specific bouquet of pheromones, giving the chemical message its own fingerprint [135]. In insects, a similar mechanism for adjusting the chemical message seems plausible, although direct evidence has not been provided.

We can conclude this section on binding properties of OBPs and CSPs with a cautionary note. However informative the results of binding experiments are and however unique for probing the function of these proteins, it is worth remembering that all such data have been obtained in media and conditions very different from those present '*in vivo*'. As an example, the concentration of OBPs (and very likely also CSPs) in the sensillum lymph is of the order of 10 mM [141], four orders of magnitude higher than the micromolar levels normally used in ligand-binding experiments in the laboratory. In this respect, probably X-ray diffractometry and NMR, requiring very high concentrations of proteins, are likely to give more realistic pictures of the proteins and their interactions with ligands.

Physiological and behavioural data

Since their discovery, different hypotheses and models of the role of OBPs in olfaction have been formulated [12]. However, few experiments have been designed and performed to address the question whether and how the presence of OBPs and CSPs could affect the physiology and the behaviour of insects. In particular, the central question of whether such soluble proteins are involved in the recognition and coding of olfactory stimuli has not yet received a convincing answer. Indeed, such research is quite complicated due to several factors, such as the great number of these proteins often expressed in the

same species and their extremely high concentration. In fact, the number of OBPs and CSPs – unknown in most cases, unless genome information is available – makes silencing of all their genes very complicated. Moreover, due to their exceptionally high concentration in the sensillar lymph, any method used to inactivate the protein or silence the gene – very rarely reaching 100% efficiency – might leave enough protein in the sensillar lymph for a normal functioning.

A reversed approach to this problem, however, has provided interesting results, showing that OBPs are not required for olfactory perception. When olfactory receptors have been expressed in heterologous systems such as *Xenopus* oocytes, thus in the complete absence of insect OBPs or CSPs, they were able to respond to semiochemicals with good selectivity [10]. In a very elegant experiment, olfactory receptors of *A. gambiae* have been expressed in the antennae of *D. melanogaster* and shown to respond to the same odours and with the same specificity as in *A. gambiae* [8]. It is worth emphasising, in this respect, that OBPs of the two species are structurally very different.

Assuming, therefore, that odorants and pheromones directly activate olfactory receptors, different theories have been formulated where OBPs act as more or less passive carriers, helping hydrophobic semiochemicals to cross the aqueous barrier of the sensillar lymph and reach the membrane-bound receptors and/or facilitating their desorption from the membrane after the signal has been delivered. Models have also been proposed where the same OBP might perform both functions [140–144]. This mechanism requires that the OBP continuously switch between two conformational states, active one in the binding, the other in the releasing phase. That an OBP could exist in two conformations with different affinities for the ligand was proposed a decade ago, before any structural information on these proteins was available. Ziegelberger [122] reported two electrophoretic bands in native conditions for the PBP of *A. polyphemus* and assigned them to a fully oxidized (with all six cysteines forming disulphide bridges) and a partially reduced form. Pheromone binding seemed to induce a slow partial oxidation of the reduced form, as monitored by the relative amounts of pheromone bound to the two bands. On this basis, a model was proposed where the PBP could bind the pheromone in its reduced form and deliver it to the membrane receptor, before being oxidized and act in this form as a scavenger.

More recently, structural evidence has suggested a different mechanism, still involving two forms, for the PBP of *B. mori*. This protein undergoes a major conformational change, triggered by pH and pheromone, as reported above. These observations, together with the low pH calculated in the close proximity of the membrane, has suggested that the PBP could pick up the pheromone at neutral pH in the periplasm, carry it to the membrane and release it there in the proximity of the receptor [79]. The acidity of the membrane would stabilise the C-terminal α-helix, which would push the molecule of bombykol out of the core of the protein. This mechanism has been questioned on the basis that the corresponding increase in potassium ions near the membrane would counteract the effect of pH on binding. Also, the calculated pH in the proximity of the membrane only reaches a value of 6 near the membrane and is still higher than 6.8 at a distance of 15 Å , corresponding to the radius of a PBP molecule [145]. However, later experiments have shown that the conformational changes also occur at high values of ionic strength, being not influenced by saline concentration [146].

Despite the evidence that olfactory receptors can be directly activated by odorants and pheromones, some data indicate that OBPs might be required for the correct perception of chemical stimuli. A recent publication provides experimental evidence that the presence of LUSH in the sensillum lymph is necessary for an electrophysiological and behavioural response in *D. melanogaster* [118]. Based on the fact that trichoid sensilla of type T1 respond to the aggregation pheromone vaccenyl acetate (VA), the authors tested the behaviour and electrophysiological response of flies lacking the gene for LUSH. In both types of experiments, the flies were reported to be insensitive to VA, but normal response was restored after reintroduction of the *lush* gene. Quite interestingly, the absence of the binding protein also reduces spontaneous firing rate by a factor of more than 400, suggesting that in the wild-type and in the absence of ligands, such activity could be due to direct interaction of the protein with the receptor. Finally, introduction of LUSH protein through the recording pipette restores the sensitivity of the neuron to VA in the mutant flies. The lag time for such recovery is of the order of 5–20 min, in accordance with a diffusion-limited event.

The idea that OBPs might be required for olfactory perception in insects had been proposed in the past, and data supporting this view had also been produced. The first indication came from the work of Van der Berg and Ziegelberger [147]. They managed to push the lymph out of the relatively large sensilla of the moth *A. polyphemus* and found that the electrophysiological response to the moth pheromone was abolished. Addition of the moth OBP in the sensillum restored the response, but addition of BSA also showed the same effect.

Later, the electrophysiological experiments of Pophof [148] suggested that both pheromones and binding proteins could be required and involved in the response. Her work focused on two of the three components of the sex pheromone – hexadecadienyl acetate (AC) and haxadecadienal (AL) – of the moth *A. polyphemus*, and their two binding proteins, PBP1 and PBP2, respectively. Sensilla

trichodea of this species contain two or three receptor cells. Each pheromone component elicited a response from either one or the other cell when it was presented in air, but no response was observed when the pheromone component was supplied in a buffer. In combination with its own binding protein (AC/PBP1; AL/PBP2), each pheromone component specifically and independently excited one of the two receptor cells, as expected. However, when they were applied with the 'wrong' combination of protein (AC/PBP2 and AL/PBP1), both cells responded. This suggests that each olfactory receptor could be activated not only by its specific ligand, but also by the other ligand, provided the PBP of the 'correct' ligand is present. Similar results were obtained with the moth *B. mori* [149]. It is tempting to conclude that the specificity resides in the PBPs rather than in the structures of the pheromones, in contrast to the results obtained in the heterologous expression of olfactory receptors cited above. However, the data of Pophof, although clear and complete, are not easy to explain and cannot suggest a realistic model, without further support being provided from complementary approaches.

Behavioural observation supporting the view that OBPs are required for olfaction is even scarcer. Only two reports have been published; neither of them provides convincing evidence. The first indicated that LUSH was essential for a correct perception of ethanol in *D. melanogaster*, on the observation that flies lacking the *lush* gene were not repelled by high concentrations of ethanol, unlike normal flies [117]. Later work has indicated that LUSH does not bind ethanol, but large aromatic molecules, such as phthalates, and that normal flies are not repelled by ethanol at any concentration [88]. Moreover, LUSH could be involved in the perception of the aggregation pheromone vaccenyl acetate, as suggested in a later work [118].

The second observation relating a specific behaviour to the presence of an OBP comes from the fire ant *Solenopsis invicta* [98]. Failure in expressing the OBP Gp-9 results in the production of abnormal multigene colonies. As described in previous section, however, this OBP was isolated not from sensory organs, but from the thorax of the queens. Therefore, an impaired perception related to the lack of this protein is difficult to explain. Alternatively, the OBP could be involved in the release of pheromones – not yet identified – from the queen, that could inhibit the maturation of other females. Thus, the absence of this OBP would reduce or modify the pheromonal signal, performing a role similar to that of urinary proteins in mice [130–136].

Conclusions

More than 2 decades of research have accumulated a very large amount of structural data on insect binding proteins, but have failed to produce a convincing model for the role of OBPs and CSPs in chemoreception. Although structurally different, both OBPs and CSPs (i) reversibly bind organic molecules, including semiochemicals, with micromolar affinities and (ii) are present at high concentration in the lumen of chemosensilla. These are the two basic requirements for binding proteins active in chemoreception. However, while for OBPs binding of pheromones and specific expression in olfactory sensilla responding to pheromones or odours are well-documented phenomena, for CSPs such data are fewer and weaker. Moreover, several members, identified as CSPs based only on sequence similarity, are certainly not involved in chemoreception. It would be more appropriate to classify as CSPs only those proteins that are expressed in sensory organs in addition to structural requirements. A similar situation is observed with vertebrate OBPs, which are a small subset of lipocalins, a very large superfamily of proteins that includes members involved in many different functions [23].

In vertebrates, it is well documented and described that proteins similar or identical to nasal OBPs are also present in secretions, such as urine or saliva, where they are involved in the delivery of pheromones [130–138, 150]. Few data seem to indicate that the same phenomenon occurs in insects and that both OBPs (in the paper wasp) and CSPs (in locusts and in *M. brassicae*) can act as carriers of hydrophobic endogenous ligands for external delivery.

Regarding the physiological function of OBPs, the current view assigns a role to these proteins as carriers for odorants and pheromones across the sensillar lymph. This concept was first suggested about 20 years ago for OBPs of both vertebrates [151] and insects [152] and remains the most widely accepted hypothesis. The different models proposed under such common assumption [153], however, present some difficulties. The exceptionally high concentration of 10 mM reported for OBPs in the sensillar lymph means that these proteins completely fill the space between the cuticle and the neuronal dendrites, with individual molecules of proteins nearly touching one another. Travelling across such a crowded environment would be extremely difficult for a protein molecule, and even more for a hydrophobic compound such as a pheromone or an odorant. Therefore, a passive diffusion of semiochemicals or of their complexes with binding proteins would be too slow compared with the fast responses of insects to environmental odours. The field is still open to alternative hypotheses. For instance, the hydrophobic ligand could be handed over from one OBP to the next in a process faster than diffusion in the aqueous medium or on the back of a protein. Alternatively, the molecules of OBPs could in principle form a sort of hydrophobic channel where the ligand would freely travel to the receptor. This seems a model suggested by the structure of *A. gambiae* OBP [90], but this feature is not shared by other OBPs.

Another unsolved problem is the fate of ligands – pheromones and general odorants – once they have fulfilled their role of activating the transmembrane receptors. A fast reactivation of the olfactory system, as needed by an insect flying in an odour plume, requires that the stimulus be eliminated within fractions of a second. It is true that very active enzymes have been discovered in some species that can rapidly degrade the specific pheromones [1, 20, 152, 154], but the problem is still unsolved for general odorants and other semiochemicals. Even if we hypothesise that the same OBPs could clear off the ligands in the membrane area, the protein-ligand complex should be disposed of before it accumulates in the sensillum lymph. Although no experimental data have been produced on this aspect, it is not unlikely that the OBP-ligand complex is destroyed and the protein recycled in the cells at the base of the sensillum. Such a mechanism, however unlikely and wasteful it might appear – one molecule of protein for each molecule of pheromone – might prove more efficient that other alternatives. On the other hand, this is the high price paid when chemical messages are delivered in the environment. Mice discard in their urine vast amounts of protein to send their pheromones, and insects might well use a similar approach.

1 Vogt, R. G. and Riddiford, L. M. (1981) Pheromone binding and inactivation by moth antennae. *Nature* 293, 161–163.
2 Pelosi, P., Pisanelli, A. M., Baldaccini, N. E and Gagliardo, A. (1981) Binding of [^{3}H]-2-isobutyl-3-methoxypyrazine to cow olfactory mucosa. Chem. Senses 6, 77–75.
3 Pelosi, P., Baldaccini, N. E. and Pisanelli, A. M. (1982) Identification of a specific olfactory receptor for 2-isobutyl-3-methoxypyrazine. Biochem. J. 201, 245–248.
4 Buck, L. and Axel, R. (1991) A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. Cell 65, 175–187.
5 Troemel, E. R., Chou, J. H., Dwyer, N. D., Colbert, H. A. and Bargmann, C. I. (1995) Divergent seven transmembrane receptors are candidate chemosensory receptors in *C. elegans*. Cell 83, 207–218.
6 Clyne, P. J., Warr, C. G., Freeman, M. R., Lessing, D., Kim, J. and Carlson, J. R. (1999) A novel family of divergent seven-transmembrane proteins: candidate odorant receptors in *Drosophila*. Neuron 22, 327–338.
7 Vosshall, L. B., Amrein, H., Morozov, P. S., Rzhetsky, A. and Axel, R. (1999) A spatial map of olfactory receptor expression in the *Drosophila* antenna. Cell 96, 725–736.
8 Hallem, E. A., Fox, A. N., Zwiebel, L. J. and Carlson, J. R. (2004) Olfaction: mosquito receptor for human-sweat odorant. Nature 427, 212–213.
9 Krautwurst, D., Yau, K. W. and Reed, R. R. (1998) Identification of ligands for olfactory receptors by functional expression of a receptor library. Cell 95, 917–926.
10 Wetzel, C. H., Behrendt, H. J., Gisselmann, G., Stortkuhl, K. F., Hovemann, B. and Hatt, H. (2001) Functional expression and characterization of a *Drosophila* odorant receptor in a heterologous cell system. Proc. Natl. Acad. Sci. USA 98, 9377–9380.
11 Zhao, H., Ivic, L., Otaki, J. M., Hashimoto, M., Mikoshiba, K. and Firestein, S. (1998) Functional expression of a mammalian odorant receptor. Science 279, 237–242.
12 Pelosi, P. (1994) Odorant binding proteins. Crit. Rev. Biochem. Mol. Biol. 29, 199–228.
13 Pelosi, P. (1996) Perireceptor events in olfaction. J. Neurobiol. 30, 3–19.
14 Pelosi, P. (1998) Odorant-binding proteins: structural aspects. Ann. N. Y. Acad. Sci. 855, 281–293.
15 Pelosi, P. (2001) The role of perireceptor events in vertebrate olfaction. Cell. Mol. Life Sci. 58, 503–509.
16 Pelosi, P. and Maida, R. (1995) Odorant-binding proteins in insects. Comp. Biochem. Physiol. 111B, 503–514.
17 Picimbon (2003) Biochemistry and evolution of OBP and CSP proteins. In: Insect Pheromone Biochemistry and Molecular Biology, (Blomquist, G. J. and Vogt, R. G., Eds.), pp. 539–566. Elsevier Academic Press, London.
18 Steinbrecht, R. (1998) Odorant-binding proteins: expression and function. Ann. N. Y. Acad. Sci. 855: 323–332.
19 Tegoni, M., Pelosi, P., Vincent, F., Spinelli, S., Campanacci, V., Grolli, S., Ramoni, R. and Cambillau, C. (2000) Mammalian odorant binding proteins. Biochim. Biophys. Acta 1482, 229–240.
20 Vogt, R. G. (2003) Biochemical diversity of odor detection: OBPs, ODEs and SNMPs. In: Insect Pheromone Biochemistry and Molecular Biology. (Blomquist, G. J. and Vogt, R. G., Eds.), Elsevier Academic Press, London. pp. 391–446.
21 Vogt, R. G. (2005) Molecular basis of pheromone detection in insects. In: Comprehensive Insect Physiology, Biochemistry, Pharmacology and Molecular Biology, vol. 3, Endocrinology, (Gilbert, L. I., Iatro, K. and Gill S., Eds.). pp. 753–804. Elsevier, London.
22 Wanner, K. W., Willis, L. G., Theilmann, D. A., Isman, M. B., Feng, Q. and Plettner, E. (2004) Analysis of the insect OS-D-like gene family. J. Chem. Ecol. 30, 889–911.
23 Flower, D. R. (1996) The lipocalin protein family: structure and function. Biochem. J. 318, 1–14.
24 Tegoni, M., Campanacci, V. and Cambillau, C. (2004) Structural aspects of sexual attraction and chemical communication in insects. Trends Biochem. Sci. 29, 257–264.
25 Raming, K., Krieger, J. and Breer, H. (1989) Molecular cloning of an insect pheromone-binding protein. FEBS Lett. 256, 215–218.
26 Vogt, R. G., Köehne, A. C., Dubnau, J. T. and Prestwich, G. D. (1989) Expression of pheromone binding proteins during antennal development in the gypsy moth *Lymantria dispar. J. Neurosci.* 9, 3332–3346.
27 Gyorgyi, T. K., Roby-Shemkovitz, A. J. and Lerner, M. R. (1988) Characterization and cDNA cloning of the pheromone-binding protein from the tobacco hornworm, *Manduca sexta*: a tissue-specific developmentally regulated protein. Proc. Natl. Acad. Sci. USA 85, 9851–9855.
28 Maida, R., Steinbrecht, R. A., Ziegelberger, G. and Pelosi, P. (1993) The pheromone-binding protein of *Bombyx mori*: purification, characterisation and immunocytochemical localisation. Insect Biochem. Mol. Biol. 23, 243–253.
29 Krieger, J., Von Nickisch, E., Mameli, M., Pelosi, P. and Breer, H. (1996) Binding proteins from the antennae of *Bombyx mori*. Insect Biochem. Mol. Biol. 26, 297–307.
30 Vogt, R. G. and Lerner, M. R. (1989) Two groups of odorant binding proteins in insects suggest specific and general olfactory pathways. *Neurosci. Abstr.* 15, 1290.
31 Vogt, R. G., Prestwich, G. D. and Lerner, M. (1991) Odorant-binding-protein subfamilies associate with distinct classes of olfactory receptor neurons in insects. J. Neurobiol. 22, 74–84.
32 Vogt, R. G., Prestwich, G. D. and Lerner, M. R. (1991). Molecular cloning and sequencing of general-odorant binding proteins GOBP1 and GOBP2 from tobacco hawk moth *Manduca sexta*: comparisons with other insect OBPs and their signal peptides. *J. Neurosci.* 11, 2972–2984.
33 Scaloni, A., Monti, M., Angeli, S. and Pelosi, P. (1999) Structural analyses and disulfide-bridge pairing of two odorant-binding proteins from *Bombyx mori*. Biochem. Biophys. Res. Commun. 266, 386–391.

34 Leal, W. S., Nikonova, L. and Peng, G. (1999) Disulfide structure of the pheromone binding protein from the silkworm moth, *Bombyx mori*. FEBS Lett. 464, 85–90.
35 Briand, L., Nespoulous, C., Huet, J. C., Takahashi, M. and Pernollet, J. C. (2001) Ligand binding and physico-chemical properties of ASP2, a recombinant odorant-binding protein from honeybee (*Apis mellifera* L.). Eur. J. Biochem. 268, 752–760.
36 Ban, L. P., Scaloni, A., D'Ambrosio, C., Zhang, L., Yan, Y. H. and Pelosi, P. (2003) Biochemical characterisation and bacterial expression of an odorant-binding protein from *Locusta migratoria*. Cell. Mol. Life Sci. 60, 390–400.
37 Calvello, M., Guerra, N., Brandazza, A., D'Ambrosio, C., Scaloni, A., Dani, F. R., Turillazzi, S. and Pelosi, P. (2003) Soluble proteins of chemical communication in the social wasp *Polistes dominulus.* Cell. Mol. Life Sci. 60, 1933–1943.
38 McKenna, M. P., Hekmat-Scafe, D. S., Gaines, P. and Carlson, J. R. (1994) Putative *Drosophila* pheromone-binding proteins expressed in a subregion of the olfactory system. J. Biol. Chem. 269, 16340–16347.
39 Pikielny, C. W., Hasan, G., Rouyer, F. and Rosbach, M. (1994) Members of a family of *Drosophila* putative odorant-binding proteins are expressed in different subsets of olfactory hairs. Neuron 12, 35–49.
40 Galindo, K. and Smith, D. P. (2001) A large family of divergent *Drosophila* odorant-binding proteins expressed in gustatory and olfactory sensilla. Genetics 159, 1059–1072.
41 Hekmat-Scafe, D. S., Scafe, C. R., McKinney, A. J. and Tanouye, M. A. (2002) Genome-wide analysis of the odorant-binding protein gene family in *Drosophila melanogaster*. Genome Res. 12, 1357–1369.
42 Graham, L. A. and Davies, P. L. (2002) The odorant-binding proteins of *Drosophila melanogaster*: annotation and characterization of a divergent gene family. Gene 292, 43–55.
43 Vogt, R. G., Rogers, M. E., Franco, M. D. and Sun, M. (2002) A comparative study of odorant binding protein genes: differential expression of the PBP1-GOBP2 gene cluster in *Manduca sexta* (Lepidoptera) and the organization of OBP genes in *Drosophila melanogaster* (Diptera). *J. Exp. Biol.* 205, 719–744.
44 Zhou, J. J., Huang, W., Zhang, G. A., Pickett, J. A. and Field, L. M. (2004) 'Plus-C' odorant-binding protein genes in two *Drosophila* species and the malaria mosquito *Anopheles gambiae*. Gene 327, 117–129.
45 Biessmann, H., Walter, M. F., Dimitratos, S. and Woods, D. (2002) Isolation of cDNA clones encoding putative odourant binding proteins from the antennae of the malaria-transmitting mosquito, *Anopheles gambiae*. Insect Mol. Biol. 11, 123–132.
46 Vogt, R. G. (2002) Odorant binding protein homologues of the malaria mosquito *Anopheles gambiae*; possible orthologues of the OS-E and OS-F OBPs of *Drosophila melanogaster*. J. Chem. Ecol. 28, 2371–2376.
47 Xu, P. X., Zwiebel, L. J. and Smith, D. P. (2003) Identification of a distinct family of genes encoding atypical odorant-binding proteins in the malaria vector mosquito, *Anopheles gambiae*. Insect Mol. Biol. 12, 549–560.
48 Riviere, S. (2003) PhD Thesis. Acc. no. AAM77026.
49 Nomura, A., Kawasaki, K., Kubo, T. and Natori, S. (1992) Purification and localization of p10, a novel protein that increases in nymphal regenerating legs of *Periplaneta americana* (American cockroach). Int. J. Dev. Biol. 36, 391–398.
50 Kitabayashi, A. N., Arai, T., Kubo, T. and Natori, S. (1998) Molecular cloning of cDNA for p10, a novel protein that increases in the regenerating legs of *Periplaneta americana* (American cockroach) Insect Biochem. Mol. Biol. 28, 785–790.
51 Tuccini, A., Maida. R., Rovero, P., Mazza, M. and Pelosi, P. (1996) Putative odorant-binding proteins in antennae and legs of *Carausius morosus* (Insecta, Phasmatodea). Insect Biochem. Mol. Biol. 26, 19–24.
52 Mameli, M., Tuccini, A., Mazza, M., Petacchi, R. and Pelosi, P. (1996) Soluble proteins in chemosensory organs of phasmids. Insect Biochem. Mol. Biol. 26, 875–882.
53 Marchese, S., Angeli, S., Andolfo, A., Scaloni, A., Brandazza, A., Mazza, M., Picimbon, J.-F., Leal, W. S. and Pelosi, P. (2000) Soluble proteins from chemosensory organs of *Eurycantha calcarata* (Insecta, Phasmatodea). Insect Biochem. Mol. Biol. 30, 1091–1098.
54 Angeli, S., Ceron, F., Scaloni, A., Monti, M., Monteforti, G., Minnocci, A., Petacchi, R. and Pelosi, P. (1999) Purification, structural characterization, cloning and immunocytochemical localization of chemoreception proteins from *Schistocerca gregaria.* Eur. J. Biochem. 262, 745–754.
55 Maleszka, R. and Stange, G. (1997) Molecular cloning, by a novel approach, of a cDNA encoding a putative olfactory protein in the labial palps of the moth *Cactoblastis cactorum*. Gene 202, 39–43.
56 Nagnan-Le Meillour, P., Cain, A. H., Jacquin-Joly, E., François, M. C., Ramachandran, S., Maida, R. and Steinbrecht, R. A. (2000) Chemosensory proteins from the proboscis of *Mamestra brassicae*. Chem. Senses 25, 541–553.
57 Picimbon, J. F., Dietrich, K., Angeli, S., Scaloni, A., Krieger, J., Breer, H. and Pelosi, P. (2000) Purification and molecular cloning of chemosensory proteins from *Bombyx mori*. Arch. Insect Biochem. Physiol. 44, 120–129.
58 Robertson, H. M., Martos, R., Sears, C. R., Todres, E. Z., Walden, K. K. and Nardi, J. B. (1999) Diversity of odourant binding proteins revealed by an expressed sequence tag project on male *Manduca sexta* moth antennae. Insect Mol. Biol. 8, 501–518.
59 Jacquin-Joly, E., Vogt, R. G., Francois, M. C. and Nagnan-Le Meillour, P. (2001) Functional and expression pattern analysis of chemosensory proteins expressed in antennae and pheromonal gland of *Mamestra brassicae.* Chem. Senses 26, 833–844.
60 Picimbon, J. F., Dietrich K., Krieger J. and Breer, H. (2001) Identity and expression pattern of chemosensory proteins in *Heliothis virescens* (Lepidoptera, Noctuidae). Insect Biochem. Mol. Biol. 31, 1173–1181.
61 Danty, E., Arnold, G., Huet, J. C., Huet, D., Masson, C. and Pernollet, J. C. (1998) Separation, characterization and sexual heterogeneity of multiple putative odorant-binding proteins in the honeybee *Apis mellifera,* L. (Hymenoptera: Apidea). Chem. Senses 23, 83–91.
62 Briand, L., Nespoulous, C., Huet, J. C., Takahashi, M. and Pernollet, J. C. (2002) Characterization of a chemosensory protein (ASP3c) from honeybee (*Apis mellifera* L.) as a brood pheromone carrier. Eur. J. Biochem. 269, 4586–4596.
63 Ishida, Y., Chiang, V. and Leal, W. S. (2002) Protein that makes sense in the Argentine ant. Naturwissenschaften 89, 505–507.
64 Calvello, M., Brandazza, A., Navarrini, A., Dani, F. R, Turillazzi, S., Felicioli, A. and Pelosi, P. (2005) Expression of odorant-binding proteins and chemosensory proteins in some Hymenoptera. Insect Biochem. Mol. Biol. 35, 297–307.
65 Ozaki, M., Wada-Katsumata, A., Fujikawa, K., Iwasaki, M., Yokohari, F., Satoji, Y., Nisimura, T. and Yamaoka, R. (2005) Ant nestmate/non-nestmate discrimination by a chemosensory sensillum. Science 309, 311–314.
66 Picimbon, J. F. and Leal, W. S. (1999) Olfactory soluble proteins of cockroaches. Insect Biochem. Mol. Biol. 29, 973–978.
67 Riviere, S. (2003) PhD Thesis. Acc. no. AAM77025.
68 Picimbon, J. F., Dietrich, K., Breer, H. and Krieger, J. (2000) Chemosensory proteins of *Locusta migratoria* (Orthoptera: Acrididae). Insect Biochem. Mol. Biol. 30, 233–241.
69 Ban, L. P., Scaloni, A., Brandazza, A., Angeli, S., Zhang, L., Yan, Y. H. and Pelosi, P. (2003) Chemosensory proteins of *Locusta migratoria*. Insect Mol. Biol. 12, 125–134.

70 Jin, X., Brandazza, A., Navarrini, A., Ban, L. P., Zhang, S., Steinbrecht, R. A., Zhang, L. and Pelosi, P. (2005) Expression and immunolocalisation of odorant-binding and chemosensory proteins in locusts. Cell. Mol. Life Sci. 62, 1156–1166.

71 Jacobs, S. P., Liggins, A. P., Zhou, J. J., Pickett, J. A., Jin, X. and Field, L. M. 2005. OS-D-like genes and their expression in aphids (Hemiptera: Aphididae). Insect Mol. Biol. 14, 423–432.

72 Kamikouchi, A., Morioka, M. and Kubo, T. (2004) Identification of honeybee antennal proteins/genes expressed in a sex- and/or caste selective manner. Zoolog. Sci. 21, 53–62.

73 Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. and Higgins, D. G. (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.

74 Kumar, S., Tamura, K., Jakobsen, I. B. and Nei, M. (2001) MEGA2: molecular evolutionary genetics analysis software. Bioinformatics 17, 1244–1245.

75 Saitou, N. and Nei, M. (1987) The neighbour-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

76 Krieger, M. J. and Ross, K. G. (2005). Molecular evolutionary analyses of the odorant-binding protein gene Gp-9 in fire ants and other *Solenopsis* species. Mol. Biol. Evol. 22, 2090–2103.

77 Sandler, B. H., Nikonova, L., Leal, W. S. and Clardy, J. (2000) Sexual attraction in the silkworm moth: structure of the pheromone-binding-protein-bombykol complex. Chem. Biol. 7, 143–151.

78 Damberger, F., Nikonova, L., Horst, R., Peng, G., Leal, W. S. and Wuthrich, K. (2000) NMR characterization of a pH-dependent equilibrium between two folded solution conformations of the pheromone-binding protein from *Bombyx mori.* Protein Sci. 9, 1038–1041.

79 Horst, R., Damberger, F., Luginbuhl, P., Guntert, P., Peng, G., Nikonova, L., Leal, W. S. and Wuthrich, K. (2001) NMR structure reveals intramolecular regulation mechanism for pheromone binding and release. Proc. Natl. Acad. Sci. USA 98, 14374–14379.

80 Klusak, V., Havlas, Z., Rulisek, L., Vondrasek J and Svatos, A. (2003) Sexual attraction in the silkworm moth. Nature of binding of bombykol in pheromone binding protein – an ab initio study. Chem. Biol. 10, 331–340.

81 Lee, D., Damberger, F. F., Peng, G., Horst, R., Guntert, P., Nikonova, L., Leal, W. S. and Wuthrich, K. (2002) NMR structure of the unliganded *Bombyx mori* pheromone-binding protein at physiological pH. FEBS Lett. 531, 314–318.

82 Lartigue, A., Gruez, A., Spinelli, S., Riviere, S., Brossut, R., Tegoni, M. and Cambillau, C. (2003) The crystal structure of a cockroach pheromone-binding protein suggests a new ligand binding and release mechanism. J. Biol. Chem. 278, 30213–30218.

83 Riviere, S., Lartigue, A., Quennedey, B., Campanacci, V., Farine, J. P., Tegoni, M., Cambillau, C. and Brossut, R. (2003) A pheromone-binding protein from the cockroach *Leucophaea maderae*: cloning, expression and pheromone binding. Biochem. J. 371, 573–579.

84 Mohanty, S., Zubkov, S. and Gronenborn, A. M. (2004) The solution NMR structure of *Antheraea polyphemus* PBP provides new insight into pheromone recognition by pheromone-binding proteins. J. Mol. Biol. 337, 443–451.

85 Zubkov, S., Gronenborn, A. M., Byeon, I. J. and Mohanty, S. (2005) Structural consequences of the pH-induced conformational switch in *A. polyphemus* pheromone-binding protein: mechanisms of ligand release. J. Mol. Biol. 354, 1081–1090.

86 Leal, W. S., Chen, A. M. and Erickson, M. L. (2005) Selective and pH-dependent binding of a moth pheromone to a pheromone-binding protein. J. Chem. Ecol. 31, 2493–2499.

87 Kruse, S. W., Zhao, R., Smith, D. P. and Jones, D. N. (2003) Structure of a specific alcohol-binding site defined by the odorant binding protein LUSH from *Drosophila melanogaster*. Nat. Struct. Biol. 10, 694–700.

88 Zhou J.-J., Zhang, G.-A., Huang, W., Birkett, M. A., Field, L. M., Pickett, J. A. and Pelosi, P. (2004) Revisiting odorant-binding protein LUSH of *Drosophila melanogaster*: evidence for odour recognition and discrimination. FEBS Lett. 558, 23–26.

89 Lartigue, A., Gruez, A., Briand, L., Blon, F., Bezirard, V., Walsh, M., Pernollet, J. C., Tegoni, M. and Cambillau, C. (2004) Sulfur singlewavelength anomalous diffraction crystal structure of a pheromone-binding protein from the honeybee *Apis mellifera*, L. J. Biol. Chem. 279, 4459–4464.

90 Wogulis, M., Morgan, T., Ishida, Y., Leal, W. S. and Wilson, D. K. (2005) The crystal structure of an odorant binding protein from *Anopheles gambiae*: evidence for a common ligand release mechanism. Biochem. Biophys. Res. Commun. 339, 157–164.

91 Lartigue, A., Campanacci, V., Roussel, A., Larsson, A. M., Jones, T. A., Tegoni M and Cambillau, C. (2002) X-ray structure and ligand binding study of a moth chemosensory protein. J. Biol. Chem. 277, 32094–32098.

92 Campanacci, V., Lartigue, A., Hallberg, B. M., Jones, T. A., Giudici-Orticoni, M. T., Tegoni, M. and Cambillau, C. (2003) Moth chemosensory protein exhibits drastic conformational changes and cooperativity on ligand binding. Proc. Natl. Acad. Sci. USA 29, 5069–5074.

93 Mosbah, A., Campanacci, V., Lartigue, A., Tegoni, M., Cambillau, C. and Darbon, H. (2003) Solution structure of a chemosensory protein from the moth *Mamestra brassicae*. Biochem. J. 369, 39–44.

94 Ozaki, M., Morisaki, K., Idei, W., Ozaki, K. and Tokunaga, F. (1995) A putative lipophilic stimolant carrier protein commonly found in the taste and olfactory systems. Eur. J. Biochem. 230, 298–308.

95 Ozaki, M., Takahara, T., Kawahara, Y., Wada-Katsumata, A., Seno, K., Amakawa, T., Yamaoka, R. and Nakamura, T. (2003) Perception of noxious compounds by contact chemoreceptors of the blowfly, *Phormia regina*: putative role of an odorant-binding protein. Chem. Senses 28, 349–359.

96 Koganezawa, M. and Shimada, I. (2002) Novel odorant-binding proteins expressed in the taste tissue of the fly. Chem. Senses 27, 319–332.

97 Li, Z. X., Pickett, J. A., Field, L. M. and Zhou, J. J. (2005) Identification and expression of odorant-binding proteins of the malaria-carrying mosquitoes *Anopheles gambiae* and *Anopheles arabiensis*. Arch. Insect Biochem. Physiol. 58, 175–189.

98 Krieger, M. J. and Ross, K. G. (2002) Identification of a major gene regulating complex social behavior. Science 295, 328–332.

99 Dyanov, H. M. and Dzitoeva, S. G. (1995) Method for attachment of microscopic preparations on glass for in situ hybridization, PRINS and in situ PCR studies. Biotechniques 18, 822–826.

100 Steinbrecht, R. A., Ozaki, M. and Ziegelberger, G. (1992) Immunocytochemical localization of pheromone-binding protein in moth antennae. Cell Tissue Res. 282, 203–217.

101 Steinbrecht, R. A., Laue, M. and Ziegelberger, G. (1995) Immunolocalization of pheromone-binding protein and general odorant-binding protein in olfactory sensilla of the silk moths *Antheraea* and *Bombyx*. Cell Tissue Res. 282, 203–217.

102 Laue, M., Steinbrecht, R. A. and Ziegelberger, G. (1994) Immunocytochemical localization of general odorant-binding protein in olfactory sensilla of the silkmoth *Antheraea polyphemus*. Naturwissenschaften 81, 178–180.

103 Zhang S.-G., Maida, R. and Steinbrecht, A. (2001) Immunolocalization of odorant-binding proteins in noctuid moths (Insecta, Lepidoptera). Chem. Senses 26, 885–896.

104 Hekmat-Scafe, D. S., Steinbrecht, R. A. and Carlson, J. R. (1997) Coexpression of two odorant-binding protein homo-

logs in *Drosophila*: implications for olfactory coding. J. Neurosci. 17, 1616–1624.
105 Shanbhag, S. R., Hekmat-Scafe, D., Kim, M. S., Park, S. K., Carlson, J. R., Pikielny, C., Smith, D. P. and Steinbrecht, R. A. (2001) Expression mosaic of odorant-binding proteins in *Drosophila* olfactory organs. Microsc. Res. Tech. 55, 297–306.
106 Park, S. K., Shanbhag, S. R., Wang, Q., Hasan, G., Steinbrecht, R. A. and Pikielny, C. W. (2000) Expression patterns of two putative odorant-binding proteins in the olfactory organs of *Drosophila melanogaster* have different implications for their functions. Cell Tissue Res. 300, 181–192.
107 Dickens, J. C., Callahan, F. E., Wergin, W. P., Murphy, C. A. and Vogt, R. G. (1998) Odorant-binding proteins of true bugs: generic specificity, sexual dimorphism, and association with subsets of chemosensory sensilla. Ann. N. Y. Acad. Sci. 855, 306–310.
108 Jin, X., Zhang, S. and Zhang, L. (2006) Expression of odorant-binding and chemosensory proteins and spatial map of chemosensilla on labial palps of *Locusta migratoria* (Orthoptera: Acrididae). Arthropod. Struct. Dev. 35, 47–56.
109 Feng, L. and Prestwich, G. D. (1997) Expression and characterization of a lepidopteran general odorant binding protein. Insect Biochem. Mol. Biol. 27, 405–412.
110 Campanacci, V., Krieger, J., Bette, S., Sturgis, J. N., Lartigue, A., Cambillau, C., Breer, H. and Tegoni, M. (2001) Revisiting the specificity of *Mamestra brassicae* and *Antheraea polyphemus* pheromone-binding proteins with a fluorescence binding assay. J. Biol. Chem. 276, 20078–20084.
111 Ban, L. P., Zhang, L., Yan, Y. H. and Pelosi, P. (2002) Binding properties of a locust's chemosensory protein. Biochem. Biophys. Res. Commun. 293, 50–54.
112 Paolini, S., Tanfani, F., Fini, C., Bertoli, E. and Pelosi, P. (1999) Porcine odorant-binding protein: structural stability and ligand affinities measured by Fourier-transform infrared spectroscopy and fluorescence spectroscopy. Biochim. Biophys. Acta 1431, 179–188.
113 Marie, A. D., Veggerby, C., Robertson, D. H.L., Gaskell, S. J., Hubbard, S. J., Martinsen, L., Hurst, J. L. and Beynon, R. J. (2001) Effect of polymorphisms on ligand binding by mouse major urinary proteins. Protein Sci. 10, 411–417.
114 Bette, S., Breer, H. and Krieger, J. (2002) Probing a pheromone binding protein of the silkmoth *Antheraea polyphemus* by endogenous tryptophan fluorescence. Insect Biochem. Mol. Biol. 32, 241–246.
115 Plettner E., Lazar J., Prestwich, E. G. and Prestwich G. D. (2000) Discrimination of pheromone enantiomers by two pheromone binding proteins from the gypsy moth *Lymantria dispar*. Biochemistry 39, 8953–8962.
116 Honson, N., Johnson, M. A., Oliver, J. E., Prestwich, G. D. and Plettner, E. (2003) Structure-activity studies with pheromone-binding proteins of the gyspy moth, *Lymantria dispar*. Chem. Senses 28, 479–489.
117 Kim, M. S., Repp, A. and Smith, D. P. (1998) LUSH odorant binding protein mediates chemosensory responses to alcohols in *Drosophila melanogaster*. Genetics, 150, 711–721.
118 Xu, P., Atkinson, R., Jones, D. N. and Smith, D. P. (2005) *Drosophila* OBP LUSH is required for activity of pheromone-sensitive neurons. Neuron 45, 193–200.
119 Leal, W. S., Chen, A. M., Ishida, Y., Chiang, V. P., Erickson, M. L., Morgan, T. I. and Tsuruda, J. M. (2005) Kinetics and molecular properties of pheromone binding and release. Proc. Natl. Acad. Sci. USA 102, 5386–5391.
120 Mohl, C., Breer, H. and Krieger, J. (2002) Species-specific pheromonal compounds induce distinct conformational changes of pheromone binding protein subtypes from *Antheraea polyphemus*. Invert. Neurosci. 4, 165–174.
121 Kaissling, K.-E., Klein, U., De Kramer, J. J., Keil, T. A., Kanaujia, S. and Hemberger, J. (1985) Insect olfactory cells: electrophysiological and biochemical studies. In: Molecular Basis of Nerve Activity. (Changeux, J. P., Hucho, F., Maelicke, A. and Neuman, E., Eds.), pp. 173–183. De Gruyter, Berlin.
122 Ziegelberger, G. (1995) Redox shift of the pheromone-binding protein in the silkmoth *Antheraea polyphemus. Eur. J. Biochem.* 232, 706–711.
123 Maida, R., Krieger, J., Gebauer, T., Lange, U. and Ziegelberger, G. (2000) Three pheromone-binding proteins in olfactory sensilla of the two silkmoth species *Antheraea polyphemus* and *Antheraea pernyi*. Eur. J. Biochem. 267, 2899–2908.
124 Maida, R., Ziegelberger, G. and Kaissling, K. E. (2003) Ligand binding to six recombinant pheromone-binding proteins of *Antheraea polyphemus* and *Antheraea pernyi.* J. Comp. Physiol. [B] 173, 565–573.
125 Prestwich, G. D. (1993) Bacterial expression and photoaffinity labeling of a pheromone binding protein. Protein Sci. 2, 420–428.
126 Maida, R., Steinbrecht, R. A., Ziegelberger, G. and Pelosi, P. (1993) The pheromone-binding protein of *Bombyx mori*: purification, characterisation and immunocytochemical localisation. Insect Biochem. Mol. Biol. 23, 243–253.
127 Maibeche-Coisne, M., Sobrio, F., Delavnay, T., Lettere, M., Dubroca, J., Jacquin-Joly, E. and Nagnam-Le Meillour, P. (1997) Pheromone binding proteins of the moth *Mamestra brassicae*: specificity of ligand binding. Insect Biochem. Mol. Biol. 27, 213–221.
128 Feixas, J., Prestwich, G. D. and Guerrero, A. (1995) Ligand specificity of pheromone-binding proteins of the processionary moth. Eur. J. Biochem. 234, 521–526.
129 Danty, E., Briand, L., Michard-Vanhee, C., Perez, V., Arnold, G., Gaudemer, O., Huet, D., Huet, J. C., Ouali, C., Masson, C. and Pernollet, J. C. (1999) Cloning and expression of a queen pheromone-binding protein in the honeybee: an olfactory-specific, developmentally regulated protein. J. Neurosci. 19, 7468–7475.
130 Cavaggioni, A., Findlay, J. B.C. and Tirindelli, R. (1990) Ligand binding characteristics of homologous rat and mouse urinary proteins and pyrazine binding protein of the calf. Comp. Biochem. Physiol. 96B, 513–520.
131 Bacchini, A., Gaetani, E. and Cavaggioni, A. (1992) Pheromone-binding proteins in the mouse *mus musculus*. Experientia 48, 419–421.
132 Robertson, D. H., Beynon, R. J. and Evershed, R. P. (1993) Extraction characterisation and binding analysis of two pheromonally active ligands associated with major urinary protein of the house mouse (*Mus musculus*). J. Chem. Ecol. 19, 1405–1416.
133 Robertson, D. H., Cox, K. A., Gaskell, S. J., Evershed, R. P. and Beynon, R. J. (1996) Molecular heterogeneity in the Major Urinary Proteins of the house mouse *Mus musculus*. Biochem J. 316, 265–272.
134 Böcskei, Z., Groom, C. R., Flower, D. R., Wright, C. E., Phillips, E. V., Cavaggioni, A., Findlay, J. B.C. and North, A. C. T. (1992) Pheromone binding to two rodent urinary proteins revealed by X-ray crystallography. Nature 360, 186–188.
135 Hurst, J. L. (2002) Polymorphism in major urinary proteins: molecular heterogeneity in a wild mouse population. J. Chem. Ecol. 28, 1429–1446.
136 Beynon, R. J., Veggerby, C., Payne, C. E., Robertson, D. H., Gaskell, S. J., Humphries, R. E. and Hurst, J. L. (2002) Polymorphism in major urinary proteins: molecular heterogeneity in a wild mouse population. J. Chem. Ecol. 28, 1429–1446.
137 Marchese, S., Pes, D., Scaloni, A., Carbone, V. and Pelosi, P. (1998) Lipocalins of boar salivary glands binding odours and pheromones. Eur. J. Biochem. 252, 563–568.
138 Spinelli, S., Vincent, F., Pelosi, P., Tegoni, M. and Cambillau, C. (2002) Boar salivary lipocalin. Three-dimensional X-ray structure and androsterol/androstenone docking simulations. Eur. J. Biochem. 269, 2449–2456.

139 Singer, A. G. and Macrides, F. (1993). Composition of an aphrodisiac pheromone. Chem. Senses 18, 630.
140 Briand, L., Huet, J., Perez, V., Lenoir, G., Nespoulous, C., Boucher, Y., Trotier, D. and Pernollet, J. C. (2000) Odorant and pheromone binding by aphrodisin, a hamster aphrodisiac protein. FEBS Lett. 476, 179–185.
141 Klein, U. (1987) Sensillum-lymph proteins from antennal olfactory hairs of the moth *Antheraea polyphemus* (Saturniidae). *Insect Biochem.* 17, 1193–1204.
142 Kaissling, K. E. (1998) A quantitative model of odor deactivation based on the redox shift of the pheromone-binding protein in moth antennae. Ann. N. Y. Acad. Sci. 855, 320–322.
143 Kaissling, K. E. (2001) Olfactory perireceptor and receptor events in moths: a kinetic model. Chem. Senses 26, 125–150.
144 Kaissling, K. E. (2004) Physiology of pheromone reception in insects. ANIR 6, 73–91.
145 Kowcun, A., Honson, N. and Plettner, E. (2001) Olfaction in the Gypsy Moth, *Lymantria dispar*. J. Biol. Chem. 276, 44770–44776.
146 Leal, W. S., Chen, A. M., Ishida, Y., Chiang, V. P., Erickson, M. L., Morgan, T. I. and Tsuruda, J. M. (2005) Kinetics and molecular properties of pheromone binding and release. Proc. Natl. Acad. Sci. USA 102, 5386–5391.
147 Van den Berg, M. J. and Ziegelberger, G.(1991) On the function of the pheromone-binding protein in the olfactory hairs of *Antheraea polyphemus*. J. Insect Physiol. 37, 79–85.
148 Pophof, B. (2002) Moth pheromone binding proteins contribute to the excitation of olfactory receptor cells. Naturwissenschaften 89, 515–518.
149 Pophof, B. (2004) Pheromone-binding proteins contribute to the activation of olfactory receptor neurons in the silkmoths *Antheraea polyphemus* and *Bombyx mori*. Chem. Senses 29, 117–125.
150 Scaloni, A., Paolini, S., Brandazza, A., Fantacci, M., Bottiglieri, C., Marchese, S., Navarrini, A., Fini, C., Ferrara, L. and Pelosi, P. (2001) Purification, cloning and characterisation of odorant- and pheromone-binding proteins from pig nasal epithelium. Cell. Mol. Life Sci. 58, 823–834.
151 Bignetti, E., Cavaggioni, A. and Pelosi, P. (1987) An odorant binding protein in cow. In: Discussions in Neurosciences, vol. IV, n. 3. Sensory Transduction, (Hudspeth, A. J., MacLeish, P. R., Margolis, F. L. and Wiesel, T. N.), pp. 37–41. FESN, Geneva.
152 Vogt, R. G., Riddiford, L. M. and Prestwich G. D. (1985) Kinetic properties of a sex pheromone-degrading enzyme: the sensillar esterase of *Antheraea polyphemus*. Proc. Natl. Acad. Sci. USA 82, 8827–8831.
153 Leal, W. S. (2005) Pheromone reception. Top. Curr. Chem. 240, 1–36.
154 Ishida, Y. and Leal, W. S. (2005) Rapid inactivation of a moth pheromone. Proc. Natl. Acad. Sci. USA 102, 14075–14079.