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Beyond chemoreception: diverse tasks of soluble olfactory proteins in insects

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ABSTRACT

Odorant-binding proteins (OBPs) and chemosensory proteins (CSPs) are regarded as carriers of pheromones and odorants in insect chemoreception. These proteins are typically located in antennae, mouth organs and other chemosensory structures; however, members of both classes of proteins have been detected recently in other parts of the body and various functions have been proposed. The best studied of these non-sensory tasks is performed in pheromone glands, where OBPs and CSPs solubilise hydrophobic semiochemicals and assist their controlled release into the environment. In some cases the same proteins are expressed in antennae and pheromone glands, thus performing a dual role in receiving and broadcasting the same chemical message. Several reports have described OBPs and CSPs in reproductive organs. Some of these proteins are male specific and are transferred to females during mating. They likely carry semiochemicals with different proposed roles, from inhibiting other males from approaching mated females, to marking fertilized eggs, but further experimental evidence is still needed. Before being discovered in insects, the presence of binding proteins in pheromone glands and reproductive organs was widely reported in mammals, where vertebrate OBPs, structurally different from OBPs of insects and belonging to the lipocalin superfamily, are abundant in rodent urine, pig saliva and vaginal discharge of the hamster, as well as in the seminal fluid of rabbits. In at least four cases CSPs have been reported to promote development and regeneration: in embryo maturation in the honeybee, limb regeneration in the cockroach, ecdysis in larvae of fire ants and in promoting phase shift in locusts. Both OBPs and CSPs are also important in nutrition as solubilisers of lipids and other essential components of the diet. Particularly interesting is the affinity for carotenoids of CSPs abundantly secreted in the proboscis of moths and butterflies and the occurrence of the same (or very similar CSPs) in the eyes of the same insects. A role as a carrier of visual pigments for these proteins in insects parallels that of retinol-binding protein in vertebrates, a lipocalin structurally related to OBPs of vertebrates. Other functions of OBPs and CSPs include anti-inflammatory action in haematophagous insects, resistance to insecticides and eggshell formation. Such multiplicity of roles and the high success of both classes of proteins in being adapted to different situations is likely related to their stable scaffolding determining excellent stability to temperature, proteolysis and denaturing agents. The wide versatility of both OBPs and CSPs in nature has suggested several different uses for these proteins in biotechnological applications, from biosensors for odours to scavengers for pollutants and controlled releasers of chemicals in the environment.

Key words: odorant-binding proteins, chemosensory proteins, chemical communication, pheromone glands, proboscis, visual pigments, development, seminal fluid, biosensors, scavengers.

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I. INTRODUCTION

In insects, chemoreception is mediated by transmembrane receptors (olfactory receptors: ORs, gustatory receptors: GRs and ionotropic receptors: IRs) that are responsible for recognising and discriminating a variety of semiochemicals and environmental odours (Clyne *et al.*, 1999; Vosshall *et al.*, 1999; Leal, 2013; see Chapter 2 in Wicher, 2015; Carraher *et al.*, 2015). However, before reaching the dendrites of sensory neurons, volatile molecules, which are generally very hydrophobic, have to be solubilised and ferried from the external environment to the membrane of chemosensing neurons. This task is performed by small soluble proteins, highly concentrated in the lymph of chemosensilla, belonging to two major families, odorant-binding proteins (OBPs) and chemosensory proteins (CSPs) (Vogt & Riddiford, 1981; Vogt, Prestwich & Lerner, 1991; Angeli *et al.*, 1999; Wanner *et al.*, 2004; Pelosi *et al.*, 2006, 2014; Vieira & Rozas, 2011). A sub-group of OBPs, specifically tuned to pheromones and recognisable in Lepidoptera for their conserved sequences, are referred to as PBPs (pheromone-binding proteins) (Vogt *et al.*, 1991).

Thanks to their abundance, stability and easy expression as recombinant proteins, OBPs and CSPs have been widely investigated, particularly since sequencing techniques have provided easy access to an increasing amount of genomic and transcriptomic data. Most studies on both classes of proteins have focused on their activity within the insect chemosensory system, trying to understand their role in detecting and recognising environmental chemical stimuli. However, there is much more to these highly efficient proteins, that in recent years have been reported to be endowed with different functions in non-sensory organs of the insect body, such as pheromone delivery, solubilisation of nutrients, development and insecticide resistance. Thanks to their small size and compact structure, these proteins represent highly efficient tools as carriers for hydrophobic compounds, a fact which may have favoured the expansion and evolution of gene families coding for these proteins and their involvement in different contexts of insect physiology.

After a brief introduction to structural, functional and phylogenetic aspects, we focus herein on members of both OBPs and CSPs involved in non-chemosensory functions and discuss their proposed modes of action in different organs of the insect. A parallel will be drawn with vertebrate lipocalins, a large family of ligand-binding proteins, to which OBPs of vertebrates (structurally distinct from those of insects) belong, but also including several other members endowed with different tasks and functions. Finally, we consider prospective uses of these proteins in odour-detection devices, as suggested by their versatility and exceptional structural stability.

II. STRUCTURE AND FUNCTIONS OF OBPS AND CSPS

The first insect OBP was discovered at the protein level, following a traditional approach where a radioactive pheromone was used to reveal binding proteins from an antennal extract of the giant moth *Antheraea polyphemus* (Vogt & Riddiford, 1981). By coincidence, this discovery occurred at about the same time but independently from that of the first mammalian OBP, a functionally similar but structurally different protein found using a similar ligand-binding approach (Pelosi *et al.*, 1981; Pelosi, Baldaccini & Pisanelli, 1982).

Genes encoding CSPs were reported much later in *Drosophila melanogaster* and named OS-D (McKenna *et al.*, 1994) and A10 (Pikielny *et al.*, 1994), but their expression products were not recognised as semiochemical-binding proteins until later. Subsequently, studies at the protein level on the antennae of several phasmids isolated abundant polypeptides similar to *Drosophila* OS-D (Mameli *et al.*, 1996; Tuccini *et al.*, 1996; Marchese *et al.*, 2000). A protein of the same class was identified in the maxillary palps of the lepidopteran *Cactoblastis cactorum* and was suggested to be involved in carbon dioxide sensing (Maleszka & Stange, 1997). However, it was only when CSPs were shown to be abundantly expressed in the sensillar lymph of the antennae of the desert locust *Schistocerca gregaria* that a

role in chemodetection appeared reasonable and the name 'chemosensory proteins' was proposed (Angeli *et al.*, 1999).

Particularly interesting for the aims of this review, which focuses on non-chemosensory functions of OBPs and CSPs, is the fact that before CSPs were identified in the antennae of *Drosophila*, a small soluble protein, later recognised as a CSP, was described in relation to limb regeneration in the cockroach (Nomura *et al.*, 1992; Kitabayashi *et al.*, 1998).

Since these early reports, very large numbers of both OBPs and CSPs have been identified in different insect species, particularly in recent years, due to genome projects and transcriptome sequencing.

(1) Structure of OBPs and CSPs

Both OBPs and CSPs are small compact polypeptides, mainly made of α-helical domains which define a hydrophobic binding cavity (Sandler *et al.*, 2000; Campanacci *et al.*, 2003; Tegoni, Campanacci & Cambillau, 2004). The structure of OBPs is further stabilised by three interlocked disulfide bridges between conserved cysteines (Leal, Nikonova & Peng, 1999; Scaloni *et al.*, 1999), while in CSPs two disulphide bonds connect adjacent cysteines (Angeli *et al.*, 1999). The family of OBPs includes members with a smaller (C-minus OBPs) or higher number (C-plus OBPs) of cysteines, as well as atypical OBPs containing additional domains (Xu, Zwiebel & Smith, 2003; Zhou *et al.*, 2004; Lagarde *et al.*, 2011; Spinelli *et al.*, 2012). CSPs, instead, seem to form a more homogeneous group of proteins, some with only five instead of six α-helical domains, as predicted by primary sequence modelling (Kulmuni & Havukainen, 2013). Figure 1 shows the structures of the first OBP [*Bombyx mori* pheromone-binding protein 1 (PBP1); Sandler *et al.*, 2000] and the first CSP (*Mamestra brassicae* CSP1; Campanacci *et al.*, 2003) to be solved.

The folding of both classes of proteins, forming a hydrophobic pocket, is well conserved across species and orders, although amino acid sequences, particularly for OBPs, can be highly divergent. It has been observed, however, that the length of the C-terminus can differ, with consequences on the mechanism of ligand-binding (Tegoni *et al.*, 2004). In particular, the C-terminus can be long enough to enter the binding pocket, as in *B. mori* PBP1 (Sandler *et al.*, 2000). Medium-length C-terminus segments act as a lid covering the entrance to the binding pocket, as in the case of honeybee *Apis mellifera* PBP1 (Lartigue *et al.*, 2004). Finally, OBPs with a short C-terminus, as in the case of a PBP of the cockroach *Leucophaea maderae*, have their binding pocket open to the external environment (Riviere *et al.*, 2003). To date, the structures of more than 20 OBPs have been solved by X-ray crystallography and/or nuclear magnetic resonance (NMR) spectroscopy, some also complexed with ligands (reviewed in Brito, Moreira & Melo, 2016). By contrast, the structures of only three CSPs are currently available (Lartigue *et al.*, 2002; Tomaselli *et al.*, 2006; Jansen *et al.*, 2007).

The compact structure of both OBPs and CSPs makes these proteins highly resistant to temperature, withstanding boiling for several minutes, as well as to the action of organic solvents and proteolytic agents (Ban *et al.*, 2002; Calvello *et al.*, 2003).

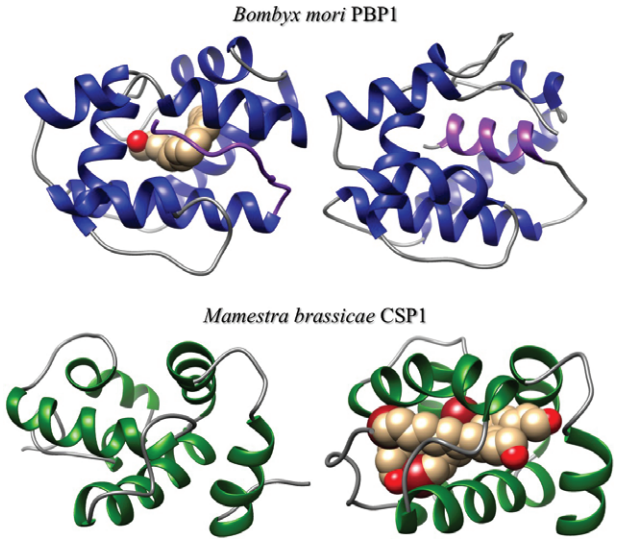

Fig. 1. Three-dimensional structures of *Bombyx mori* pheromone-binding protein (PBP1) and *Mamestra brassicae* chemosensory protein 1 (CSP1), representative examples of the two classes of ligand-binding proteins involved in insect chemical communication. Both proteins have been shown to undergo major conformational changes related to ligand-binding. In PBP1 of *B. mori* the C-terminus is unstructured at neutral pH and does not interfere with the binding of the pheromone. At low pH values, it folds into an α-helix that fits the binding pocket, thus ejecting the pheromone molecule. CSP1 of *M. brassicae* has been shown to swell significantly to incorporate three molecules of the large ligand 12-bromo-dodecanol.

During evolution, CSPs appear to be more conserved than OBPs, with often 40–50% identical residues between orthologues from phylogenetically distant species. By contrast, OBPs only share on average 10–15% of their residues between species (Pelosi *et al.*, 2006). One reason for this could be related to the different arrangement of disulfide bridges in the two classes of proteins. In OBPs the three interlocked S—S bonds contribute to a stable and conserved structure of the protein, wherein residue substitutions would have limited effects, as compared to CSPs which instead have to rely on sequence conservation to maintain folding, to provide a binding pocket and ensure overall stability.

The lower variability of CSPs compared to OBPs could also be linked to other features, such as their lower binding specificity or their affinity for common environmental volatiles rather than semiochemicals involved in communication. In fact, being more flexible than OBPs, CSPs may adapt to bind different ligands, with a larger range of sizes and shapes than in the case of most OBPs.

(2) Physiological functions of OBPs

Although olfactory receptors are responsible for detecting the chemical signals, there is evidence that OBPs are also required for physiological sensitivity of the olfactory system

(Xu *et al.,* 2005; Biessmann *et al.,* 2010; Pelletier *et al.,* 2010) and play a role in the discrimination of chemical signals (Matsuo *et al.,* 2007; Qiao *et al.,* 2009; Swarup, Williams & Anholt, 2011; Y.F. Sun *et al.,* 2012*a*). Moreover, several reports have shown that OBPs can modulate the response of ORs to odorants, although the detailed mechanism of such complex interplay is not yet clear (Grosse-Wilde, Svatos & Krieger, 2006; Forstner, Breer & Krieger, 2009; Sun *et al.,* 2013; Chang *et al.,* 2015).

Conformational changes of OBPs have been observed as a result of ligand binding and/or pH changes (Fig. 1). This phenomenon was observed first in PBP1 of *Bombyx mori* (Sandler *et al.,* 2000), where the C-terminus, rich in acidic residues, loses its negative charge at low pH and folds into a seventh α-helix, which enters the binding pocket and expels the ligand present inside (Damberger *et al.,* 2000). This mechanism has been suggested as an active mechanism for presenting the pheromone to the transmembrane receptor. Analogous conformational changes have been observed with other insect OBPs (Zubkov *et al.,* 2005; Wogulis *et al.,* 2006; Leite *et al.,* 2009; Xu *et al.,* 2010; Han *et al.,* 2014), while a different mechanism triggered by pH and involving dimerisation has been proposed for the release of the bound pheromone in a honeybee OBP (Pesenti *et al.,* 2008). In all cases, however, the details of the interplay between OBPs and receptors remain elusive and controversial (Gong *et al.,* 2009). For *D. melanogaster* LUSH, an OBP with binding affinity to the male pheromone vaccenyl acetate (Xu *et al.,* 2005), interaction with the specific olfactory receptor has been proposed (Laughlin *et al.,* 2008). Accordingly, the pheromone would induce a conformational change in the protein, triggering specific binding of the LUSH–pheromone complex with the receptor. This mechanism was supported by the observation that a specific mutant of LUSH could activate the receptor even in the absence of the pheromone (Laughlin *et al.,* 2008). However, later work failed to confirm this mechanism, showing that the LUSH mutant did not affect pheromone-triggered activity or basal firing of the specific neurons (Gomez-Diaz *et al.,* 2013). Moreover, these authors compared the structures of LUSH alone and in complex with the pheromone or other ligands and did not find structural differences that could justify the previously proposed interaction with the receptor.

Major conformational changes in the structure of CSPs upon binding have also been reported, but with a different mechanism. Using X-ray diffraction on crystals of the CSP1 from *Mamestra brassicae* (MbraCSP1; Fig. 1) it was observed that this protein can swell significantly while encapsulating in its binding pocket three molecules of the ligand 12-bromo-dodecanol (Campanacci *et al.,* 2003). A similar change could occur in the CSP4 of the lepidopteran *Helicoverpa armigera* (HarmCSP4), given that this protein binds the very large molecule of ß-carotene with good affinity. Although structural evidence is lacking, docking simulations have shown that only by modelling HarmCSP4 on MbraCSP1 in its swollen form could it present a cavity large enough to accommodate a molecule of ß-carotene (Zhu *et al.,* 2016*a*).

CSPs are able to enlarge their binding cavities because the two disulfide bridges do not place constraints on the scaffolding of the protein, unlike the three interlocked disulfide bridges of OBPs. From a functional point of view, this might suggest a role of CSPs as reservoirs for physiologically relevant chemicals, without any necessary link to signal transduction.

(3) OBPs and CSPs across evolution

During evolution, both OBPs and CSPs seem to have undergone much duplication and differentiation in Hexapoda (Pelosi *et al.,* 2014) similarly to ORs that evolved in insects after the emergence of Archaeognatha and Zygentoma (Missbach *et al.,* 2014).

In Chelicerata, Crustacea and Myriapoda, the presence of only one or two CSP sequences (sometimes together with a couple of isoforms) (Pelosi *et al.,* 2006, 2014; Vieira & Rozas, 2011; Chipman *et al.,* 2014; Qu *et al.,* 2015; Gulia-Nuss *et al.,* 2016) does not seem to support a chemosensory role (Fig. 2). Genes coding for OBP-like proteins, with a cysteine pattern similar to C-minus OBPs, have been reported recently in chemosensory organs of two chelicerates, the tick *Amblyomma americanum* (Renthal *et al.,* 2016) and the hunter spider *Dysdera silvatica* (Vizueta *et al.,* 2016). Proteins named as OBPs are known for vertebrates and are particularly well studied in rodents; however, these proteins belong to the lipocalin superfamily and therefore represent a structurally different class to the OBPs of Hexapoda (Pelosi, 1994; Bianchet *et al.,* 1996; Tegoni *et al.,* 1996, 2000).

Soluble proteins of a different class (Niemann-Pick type 2) have been suggested to function as semiochemical carriers in Chelicerata and Crustacea (Pelosi *et al.,* 2014). However, while carrier proteins for hydrophobic odorants are expected to enhance olfactory sensitivity in terrestrial arthropods, such proteins do not seem to be necessary in aquatic animals which mainly rely on water-soluble semiochemicals (Derby *et al.,* 2016).

Finally, it is interesting to note that although plant transcriptomes are often reported to contain a large number of sequences belonging to both OBPs and CSPs, the presence of these genes is obviously due to contamination by insect samples (Zhu, Wang & Pelosi, 2016*b*).

Within the Hexapoda, the number of genes encoding for OBPs and CSPs is highly variable among species, ranging from 12 to about 100 for OBPs and from 4 to 70 for CSPs (Pelosi *et al.,* 2014). Some representative examples are reported in Fig. 2. Such information is only reliable in species for which genome sequencing has been carried out; for other species the data remain preliminary. For example, within the Entognatha, the larger number of genes, encoding for OBPs and CSPs reported for Collembola compared to Protura and Diplura (Fig. 2) presumably only reflects the different attention paid to these three orders. Moreover, the number of expressed proteins within each class cannot be predicted in the absence of proteomic data. In addition, two

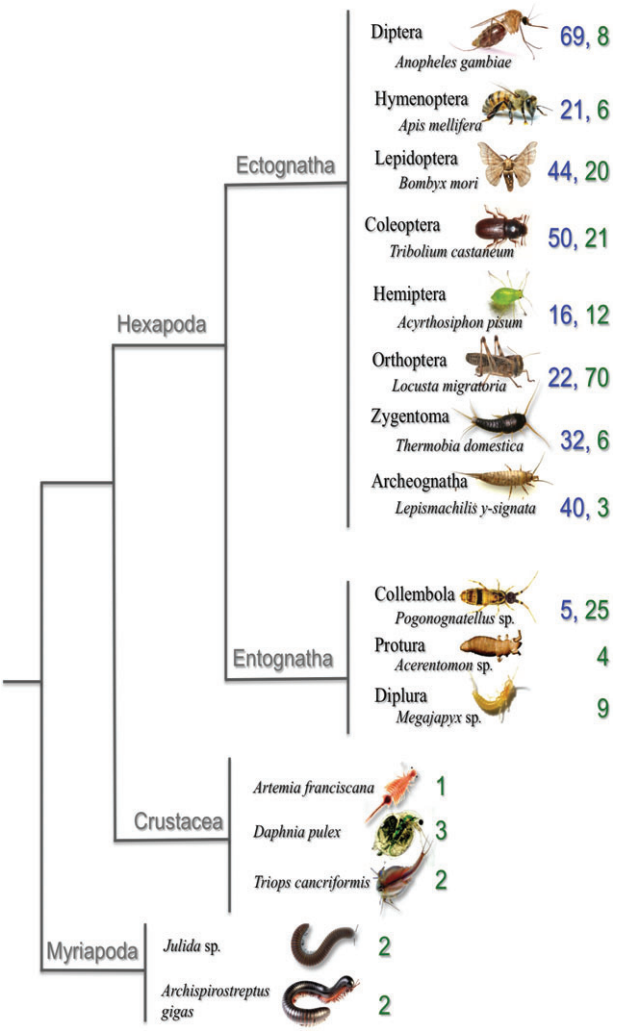

Fig. 2. Odorant binding proteins (OBPs) and chemosensory proteins (CSPs) across arthropods. Gene numbers are shown on the right for selected species in each taxon. OBPs (in blue) are only present in Hexapoda; the number of genes coding for these proteins in insect species supports a role in chemical communication. By contrast, CSPs (in green) are also expressed in Crustacea and Myriapoda, although the small number of genes involved might exclude a function in chemosensing. In insects, the number of genes expressing OBPs and CSPs does not show any trend with phylogenetic position nor with other major species characteristics.

or more OBPs could cooperatively bind ligands, as has been demonstrated for *Anopheles gambiae* OBP1 and OBP4 (Qiao *et al.*, 2011), thus increasing the variety of carrier proteins with different affinities. On the other hand, some OBPs and CSPs could have further functions beside chemodetection or not be involved in chemodetection at all.

The wide variability in the number of OBP and CSP genes found to date (Fig. 2) appears to show little correlation with the phylogeny of Hexapoda or with particular lifestyles. For example, within the Diptera, the number of OBP genes varies from 41 to 62 in different species of the genus *Drosophila*, while the number of CSP genes is limited to three or four (Hekmat-Scafe *et al.*, 2002; Vieira & Rozas, 2011; Almeida *et al.*, 2014). A recent study on three members of Culicidae (Manoharan *et al.*, 2013) reported a higher number and non-orthologous OBP genes in *Culex quinquefasciatus* and *Aedes aegypti* (109 and 111, respectively) with respect to *An. gambiae* (69), which implies an important expansion of this gene family. Morevover, C-minus OBPs, which are present in several Holometabola orders besides Diptera, have not been found in *Anopheles*. However, despite such large numbers of genes, proteomic analysis showed that less than half of them were expressed in the antennae of *An. gambiae* (Mastrobuoni *et al.*, 2013).

Within Muscidae, 87 OBP genes have been reported for *Musca domestica*, while the number is only 20 in *Glossina morsitans* (International *Glossina* Genome Initiative, 2014; Liu *et al.*, 2010). This reduced number of OBP genes does not appear to be balanced by expansion of the CSP family, which as in the other dipterans only contains a few members (Liu *et al.*, 2012).

Remarkable differences in the numbers of OBP and CSP genes have also been found within Hymenoptera. The honeybee genome is endowed with 21 OBPs, of which nine are C-minus OBPs, and six CSPs, small numbers when compared to the 170 olfactory receptors. Only 12 OBPs and 2 CSPs were detected at the protein level in the antennae of forager honeybees (Dani *et al.*, 2010). Slightly fewer (16) OBP genes were found in two species of bumblebee, none of which encoded for C-minus OBPs (Sadd *et al.*, 2015). Among ant species with sequenced genomes, 105–400 OR, 8–27 OBP (Bonasio *et al.*, 2010; Wurm, Wang & Keller, 2010; Smith *et al.*, 2011*a*,*b*; McKenzie, Oxley & Kronauer, 2014) and 11–21 CSP (Kulmuni, Wurm & Pamilo, 2013) genes have been reported, these latter figures being more than twice the number of genes codifying CSP proteins in the honeybee. A particularly high number of CSP (21; Kulmuni *et al.*, 2013) with respect to OBP genes (12; Wurm *et al.*, 2010) has been identified in the fire ant *Solenopsis invicta*, although only two OBPs and one CSP have been reported at the protein level in the antennae (González *et al.*, 2009). A CSP specifically expressed in *Camponotus japonicus* antennae has been reported to bind cuticular hydrocarbons, which in social insects constitute the pheromones underlying nest-mate recognition (Ozaki *et al.*, 2005; Hojo *et al.*, 2014). A different CSP expressed in the antennae of fire ants, not an orthologue of *C. japonicus* CSP, was found to have affinity for fatty acids and fatty esters rather than for hydrocarbons (González *et al.*, 2009). Since nest-mate recognition is considered pivotal to the onset and evolution of sociality in insects, several studies have recently focused on the evolution and expression of CSPs (Kulmuni & Havukainen, 2013; Kulmuni *et al.*, 2013; Hojo *et al.*, 2014; McKenzie *et al.*, 2014), finding that some lineages of genes encoding for CSPs specifically transcripted in antennae have expanded in ants (Hojo *et al.*, 2014; McKenzie *et al.*, 2014). Surprisingly, the genome of the parasitoid wasp *Nasonia vitripennis* has been reported to contain a much larger number

of OBPs (90), together with 10 CSPs (Werren *et al.*, 2010; Vieira *et al.*, 2012).

In Lepidoptera olfaction is mainly used for sexual-partner localization and to find host plants for oviposition. Unexpectedly, the silk moth *B. mori* has 44 genes encoding OBPs and 20 encoding CSPs (Gong *et al.*, 2007, 2009), although only seven OBPs and four CSPs could be detected at the protein level in the antennae of this species (Dani *et al.*, 2011).

Unlike the species discussed above, whose genomes have been sequenced, only limited information is available for Entognatha, Archaeognatha and Zygentoma, mostly based on transcriptome projects. However, 12 OBP transcripts have been deposited for the collembolan *Folsomia candida* and five for *Orchesella cincta* and *Onychiurus articus*, while 11 and 25 CSP sequences are available for the collembolans *F. candida* and *Pogonognatellus* sp., nine for the dipluran *Megajapyx* sp. and four for the Proturan *Acerentomon* sp. The greater numbers found in Collembola, with respect to the other two classes simply reflects the greater attention given to this group to date. Similarly, transcriptome studies of *Lepysmachilis* (order Archaeognatha) and *Thermobia* (order Zygentoma) identified 40 and 32 OBP genes, and three and six CSP genes, respectively (Missbach *et al.*, 2015). Such relatively large numbers of soluble olfactory proteins indicate that extensive duplication and differentiation must have taken place in basal Hexapoda, especially for OBPs, while CSPs expansion may be limited to Collembola. The reverse is found in the oriental locust *Locusta migratoria*, with 22 OBP and 70 CSP sequences reported in EST databases (Zhou *et al.*, 2013). A large number of these 70 genes are expressed in the antennae of the locust and include several isoforms (Picimbon *et al.*, 2000*b*; Ban *et al.*, 2003).

The high divergence of genes encoding both OBPs and CSPs of Entognatha, within and between species, can be appreciated by the phylogenetic trees in Fig. 3 that report representative examples for the two classes of proteins. The CSP tree also includes members of Crustacea and Myriapoda suggesting that these genes have undergone extensive duplication after the separation of these two Pancrustacea clades.

Information regarding OBPs and CSPs in Arthropoda is certainly going to increase thanks to current and future transcriptome projects; however, the numbers of CSP genes so far reported for non-Hexapoda species appear too limited to support an important function in chemical detection.

In conclusion, from an evolutionary perspective, we can postulate that CSPs first appeared within the Mandibulata as a few genes. These genes, possibly not endowed with sensory functions, underwent duplication and differentiation within Hexapoda leading sometimes to remarkable differences in gene numbers even within the same order, and acquiring a role as a carrier of volatile odorants. The appearance and expansion of OBP genes in Hexapoda, suggested by the high number of sequences recently reported as transcripts for some species of Collembola, indicates that OBP genes withstood duplication and differentiation during the approximately 100 million years that separate the split between Entognatha and Ectognatha from the Hexapoda–Crustacea division. Genome sequencing of additional species of Entognatha are necessary to confirm this. The origin of these successful and versatile proteins remains an open question. OBP genes have been suggested to originate from the CSP family (Vieira & Rozas, 2011) although these two families do not show significant sequence homology within Entognatha. More recently Vizueta *et al.* (2016) suggested that members of an OBP superfamily, similar to the OBP-like proteins found in chelicerates and myriapods were already present in arthropod ancestors.

III. MULTIPLE FUNCTIONS OF OBPS AND CSPS

It is now well recognised that OBPs and CSPs represent complex families of proteins, including members with diverse and unrelated functions, of which chemodetection is only one. Their stable nature and simple structure made these proteins adaptable to various tasks. In vertebrates, a similar position is occupied by the superfamily of lipocalins, also small and robust polypeptides, which include the OBPs of vertebrates, and are utilised in a variety of different roles.

While CSPs were immediately understood to be a complex family of proteins with members involved in different tasks, insect OBPs were regarded until recently as proteins exclusively related to chemodetection. In the last few years, however, several studies have reported the occurrence of OBPs in non-sensory organs with diverse physiological roles. Most of the functions documented or proposed for both OBPs and CSPs, however, are related to the ability of these proteins to bind small molecules, from semiochemicals to nutrients, hormones or toxic compounds. Below we examine evidence for different organs expressing OBPs and/or CSPs and discuss the physiological roles demonstrated or suggested for these proteins.

(1) Chemosensory organs: detecting chemosignals

A role for OBPs in detecting chemical stimuli was clear from the discovery of the first member of this group (Vogt & Riddiford, 1981) and evidence for their involvement in the detection and identification of odorants and pheromones is described above (see Section II.2). For CSPs, a role as semiochemical carriers, similar to that of OBPs, has been long debated. However, several pieces of evidence now indicate strongly that at least some members of the CSP family are involved in chemodetection and should be regarded as a second class of binding proteins. In particular: (*i*) CSPs are abundant in the lymph of chemosensory hairs, both in olfactory and contact sensilla, in locusts (Angeli *et al.*, 1999; Jin *et al.*, 2005), phasmids (Monteforti *et al.*, 2002), Lepidoptera (Jacquin-Joly *et al.*, 2001) and Coleoptera (Sun *et al.*, 2014); (*ii*) they bind semiochemicals with micromolar dissociation constants, similarly to OBPs (Iovinella *et al.*, 2013). In particular, CSP3 of the honeybee [reported in

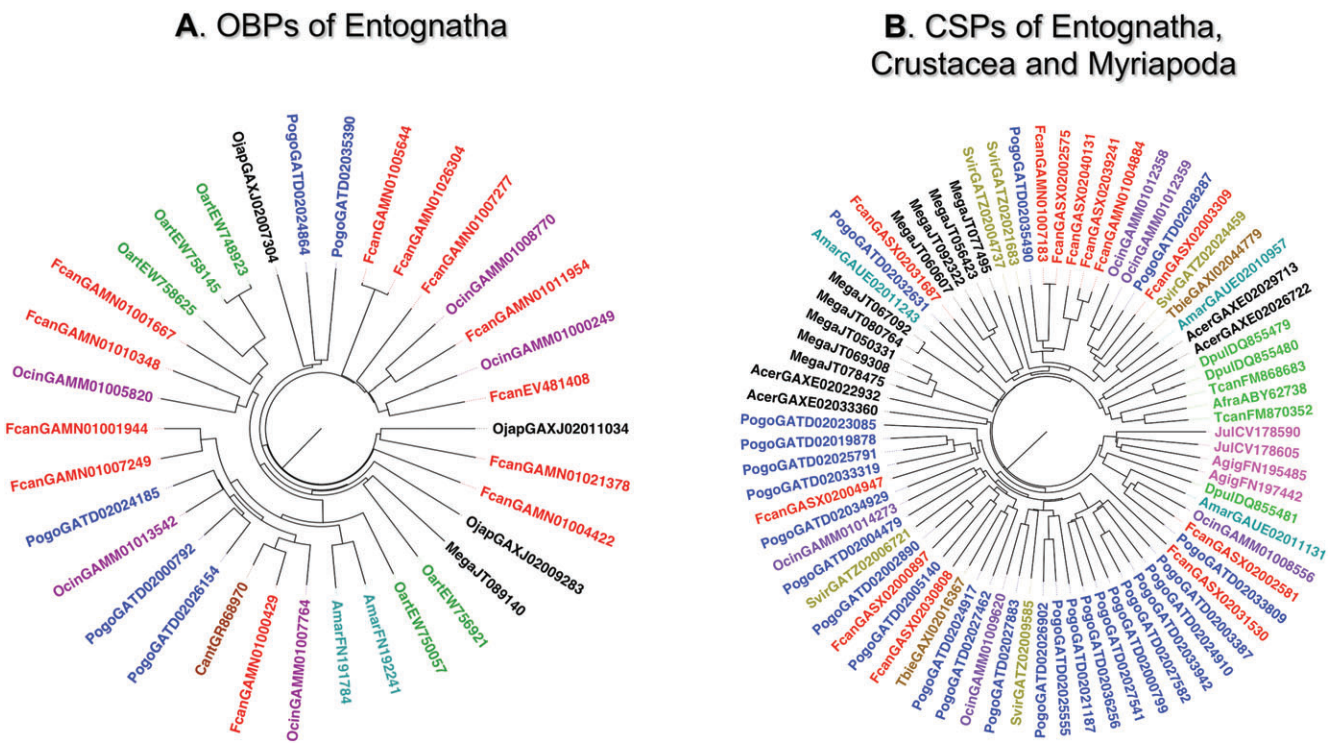

Fig. 3. Phylogenetic trees of odorant-binding proteins (OBPs) and chemosensory proteins (CSPs) in Entognatha and non-insect Mandibulata. (A) OBPs are only expressed in Hexapoda. The presence of up to a dozen of these proteins in Entognatha, together with high divergence both within and between species supports their importance in olfaction in basal Hexapoda. (B) CSPs are also widely represented in Entognatha, where they might be involved in chemical communication. CSPs are also present in Crustacea and Myriapoda; however, since only 1–3 sequences are expressed in each species, their role in chemical sensing may be limited. Different colours indicate different species. Black is used for Protura and Diplura. Sequences are indicated by the first letter of the genus name followed by the first three letters of the species name and the NCBI accession number. Entognatha: Collembola – Fcan, *Folsomia candida*; Ocin, *Orchesella cincta*; Oart, *Onychiurus arcticus*; Amar, *Anurida maritima*; Cant, *Cryptopygus antarcticus*; Pogo, *Pogonognathellus* sp.; Svir, *Sminthurus viridis*; Tbie, *Tetrodontophora bielanensis*; Protura – Acer, *Acerentomon* sp.; Diplurans – Ojap, *Occasjapyx japonicus*; Mega, *Megajapyx* sp.; Crustacea – Dpul: *Daphnia pulex*, Afra: *Artemia franciscana*, Tcan: *Triops cancriformis*; Myriapoda: Jul: *Julida sp.*; Agig: *Archispirostreptus gigas*.

the original work as antennal specific protein 3 (ASP3)], specifically binds some components of brood pheromone (Briand *et al.*, 2002); (*iii*) in some species, such as the paper wasp *Polistes dominulus* (Calvello *et al.*, 2003) and the Argentine ant *Linepithema humile* (Ishida, Chiang & Leal, 2002), some CSPs seem to be exclusive to or most abundant in the antennae. A more recent analysis, performed on several species of ants at the RNA level, shows that genes encoding both OBPs and CSPs are specifically expressed in antennae, suggesting that proteins of both families can be involved in olfaction in social insects (McKenzie *et al.*, 2014).

These pieces of evidence indicate that CSPs might have a similar role in chemodetection to OBPs; however, unlike the situation for OBPs, there is no direct experimental evidence that CSPs are required for insect olfaction, nor that their absence can affect the detection of pheromones and odorants. Most functional studies with OBPs were focused on the detection of pheromones, and in such systems (*i*) a specific pheromone (sexual for *D. melanogaster* and Lepidoptera, alarm for aphids) was targeted, and (*ii*) the proteins (both receptors and OBPs) responsible for detecting that specific pheromone were known. For CSPs, specific information on pheromone binding is not generally available; therefore, the presence or absence of a single CSP is not expected to produce major effects on the response to odours. While several OBPs have been linked to detection of pheromones, for CSPs there is only scant experimental evidence suggesting a function in semiochemical sensing. In addition to the cases reported above, in the ant *Camponotus japonicus* CSPs have been reported to bind cuticular hydrocarbons and mediate recognition of nest mates (Ozaki *et al.*, 2005; Hojo *et al.*, 2014).

(2) Pheromone glands: releasing semiochemicals

The best documented role of OBPs and CSPs in non-sensory organs of insects is in storing pheromones in specific glands and delivering them gradually to the environment. Thus, structurally similar or even identical proteins can perform the dual role of participating in the detection of semiochemicals in sensory organs and acting as releasers of semiochemicals in secretory glands. This is analogous to having a transmitter and a receiver of radio signals tuned

to the same wavelength. That CSPs could be involved in broadcasting chemical signals was documented immediately following their discovery, although the protein involved was not recognised as a CSP at the time. This protein was identified in the ejaculatory bulb of *D. melanogaster*, the organ that produces the male pheromone vaccenyl acetate (Mane, Tompkins & Richmond, 1983; Benton, 2007) and was named ejaculatory bulb protein III (EjB-III) (Dyanov & Dzitoeva, 1995), but no function was suggested. Much later, two CSPs (named CSPMbraA6 and CSPMbraB1) were identified in the pheromone glands of the cabbage moth *M. brassicae* (Jacquin-Joly *et al.*, 2001). The first is identical to a CSP in the antennae, the second is identical to a CSP previously identified in the proboscis (Nagnan-Le Meillour *et al.*, 2000). Binding experiments with the radioactively labelled pheromone on antennal and pheromone gland extracts showed good affinity of both tissues for the ligand, likely to be due to the CSPs present (Jacquin-Joly *et al.*, 2001). The latter authors proposed that CSPs in the pheromone glands might act by solubilising hydrophobic pheromones produced by the glands and releasing them into the environment. Transcriptome projects have identified genes encoding CSPs in the pheromone glands of the lepidopteran *Spodoptera litura* (Zhang *et al.*, 2015), *Chilo suppressalis* (Xia *et al.*, 2015), *Agrotis ipsilon* (Gu *et al.*, 2013), *Agrotis segetum* (Strandh, Johansson & Löfstedt, 2009) and *Sesamia inferens* (Zhang *et al.*, 2013). The number of such reports is increasing rapidly with examples from different orders of insects.

By contrast, proteomic and transcriptomic projects performed on the antennae and other sensory organs of several species of insects have failed to detect the expression of all the OBP- and CSP-encoding genes predicted by the genomes, implying that some members of both families of proteins may only be expressed in non-sensory organs.

In the honeybee, only 12 of the 21 OBPs and two of the six CSPs predicted by the genome have been identified in the antennae through proteomics (Dani *et al.*, 2010), while nine OBPs, most of them also expressed in antennae, were identified in the mandibular glands, together with the two antennal CSPs (Iovinella *et al.*, 2011). These proteins are likely carriers for the pheromone components produced by the same glands and are expressed with different patterns according to age and caste (Iovinella *et al.*, 2011).

Honeybees are known to possess a complex chemical language based on several pheromones acting as primers or releasers (Le Conte & Hefetz, 2008). These semiochemicals mediate queen and worker reproduction and brood rearing into different castes, as well as regulating various activities within the colony. Some pheromones impact on several different aspects of colony organisation. For example, queen mandibular pheromone (QMP) prevents workers from laying eggs, regulates the diet supplied to larvae by nurse bees and acts as a sexual pheromone during mating flights. Other pheromones with several different functions have been described, such as alarm pheromones, pheromones used to mark foraging sites, pheromones of low volatility involved in nestmate recognition, and compounds released by larvae (brood pheromones) that stimulate nurse care. Although specific studies on pheromone release have not been performed, it seems likely that compounds broadcasting these messages are encapsulated in binding proteins, probably to extend their lifetime and protect them from chemical degradation. It has also been suggested that OBPs and CSPs in glands secreting complex blends of different pheromones might allow adjustment of the relative concentrations of the components according to specific and temporal requirements (Iovinella *et al.*, 2011). It may be easier and more economical for the insect to regulate the expression of a protein, which needs activation of a single gene, rather than regulating the synthesis of a pheromone, often requiring the expression and action of several enzymes.

A proteomic analysis on the antennae of the silkmoth *B. mori* detected just seven of the 44 predicted OBPs and only four of the 20 predicted CSPs (Dani *et al.*, 2011), two of which had been previously identified at the protein level (Picimbon *et al.*, 2000*a*). However, seven CSPs were reported in the pheromone glands of females (Dani *et al.*, 2011). Variants in the predicted amino acid sequence, due to RNA editing, have been reported by Xuan *et al.* (2014) for several CSPs and for some OBPs expressed in different tissues of this same species. They hypothesised that the extremely high number of CSP variants observed in pheromone glands (27 for CSP1) could be due to the involvement of these proteins in the biosynthesis of pheromones.

OBPs and CSPs are likely to be involved in binding and releasing pheromones in seminal fluid. The Ejb-III of *D. melanogaster*, found in seminal fluid containing the male pheromone cis-vaccenyl acetate, was the first member of this family described in connection with such a role (Dyanov & Dzitoeva, 1995). Later work, based on a proteomic approach, added five further OBPs to the composition of the seminal fluid of *D. melanogaster* (Takemori & Yamamoto, 2009). In *L. migratoria*, male reproductive organs contain large quantities of a specific CSP (LmigCSP91) with good affinity to a putative pheromone (a mixture of α- and ß-naphthylpropionitrile) produced in the same organ (Ban *et al.*, 2013; Zhou *et al.*, 2013). LmigCSP91, which is the only CSP identified in male reproductive organs, could not be found in the reproductive organs of virgin females, but was detected there after mating, suggesting that males use this protein to transfer putative pheromones during copulation. The ovaries and accessory glands of female locusts contained at least 16 other CSPs, accounting for most of the low-molecular weight proteins of these organs (Zhou *et al.*, 2013). Although there is no experimental evidence to suggest functions for these proteins in the female reproductive organs, we could speculate that they might play roles in egg formation and embryo development, based on reports in honeybees (Maleszka *et al.*, 2007) and mosquitoes (Costa-da-Silva *et al.*, 2013; Marinotti *et al.*, 2014), as explained in Section III.3.

Other examples of OBPs produced in the sperm and transferred to females during mating include OBP22 of the mosquito *A. aegypti* (Li *et al.*, 2008; Sirot *et al.*, 2008), CSP3 and OBP9 of *A. mellifera* in the seminal fluid (Baer *et al.*, 2012), two

OBPs of *Tribolium castaneum* (Xu, Baulding & Palli, 2013) and OBP9 of the moth *H. armigera* (Y.L. Sun *et al.*, 2012*b*). While the function of binding proteins in pheromone glands is easy to understand, the presence of such proteins in reproductive organs may require further investigation.

In *H. armigera* OBP9 was detected on the surface of eggs, thus marking only fertilized eggs. Although specific behavioural experiments have not been carried out, we can speculate that volatile compounds, found to be associated with the protein, might act as oviposition deterrents for the female during oviposition, prompting her to move away, with the effect of avoiding cannibalism among larvae and increasing their survival rate (Y.L. Sun *et al.*, 2012*b*).

In *D. melanogaster* six members of the OBP family were found among seminal fluid proteins transferred during mating (Findlay *et al.*, 2008; Takemori & Yamamoto, 2009); three of these are expressed in the seminal receptacle, together with an odorant receptor, probably playing a role in sperm–egg communication (Prokupek *et al.*, 2010).

In addition to pheromone glands and reproductive organs, OBPs and CSPs have also been detected in the venom of some stinging Hymenoptera. The parasitic wasp *Leptopilina heterotoma* expresses at least one OBP and one CSP in its venom gland (Heavner *et al.*, 2013). In another parasitic wasp, *Pteromalus puparum*, an OBP was identified and located in all parts of the venom apparatus through immunofluorescence (Wang *et al.*, 2015). In the venom sac of the woodwasp *Sirex noctilio* four genes encoding OBPs and five encoding CSPs have been detected; three of these CSPs have been identified also at the protein level (Wang *et al.*, 2016). Interestingly, a proteomic analysis of *A. mellifera* revealed that OBP21 is present in venom extracted manually from the venom gland, but is absent when extracted with electrical stimulation (Li *et al.*, 2013).

In the absence of more-specific information, we can speculate that OBPs and CSPs in the venom glands of hymenopterans might act as carriers of alarm pheromones. In fact, such semiochemicals have been reported as venom components of some species of wasps (Ono *et al.*, 2003; Bruschini *et al.*, 2006, 2008).

Figure 4 summarises the OBPs and CSPs known to be present in pheromone glands, reproductive organs and the venom apparatus of insects.

In some cases, the same OBP or CSP is present in the antenna to detect a specific pheromone and in the glands to release the same molecule. This is suggested by the presence of OBPs and CSPs in pheromone glands or reproductive organs that are identical to those expressed in antennae and other chemosensory structures. For example, in the honeybee, several OBPs and CSPs have been detected both in antennae of foragers and in mandibular glands of different castes and ages (Iovinella *et al.*, 2011); in the silkmoth *B. mori*, the two most abundant CSPs are expressed in antennae and pheromone glands (Dani *et al.*, 2011). The male reproductive organs of the mosquito *Ae. aegypti* (Li *et al.*, 2008; Sirot *et al.*, 2008) and the lepidopteran *H. armigera* (Y.L. Sun *et al.*, 2012*b*) produce OBPs which are also expressed in the antennae,

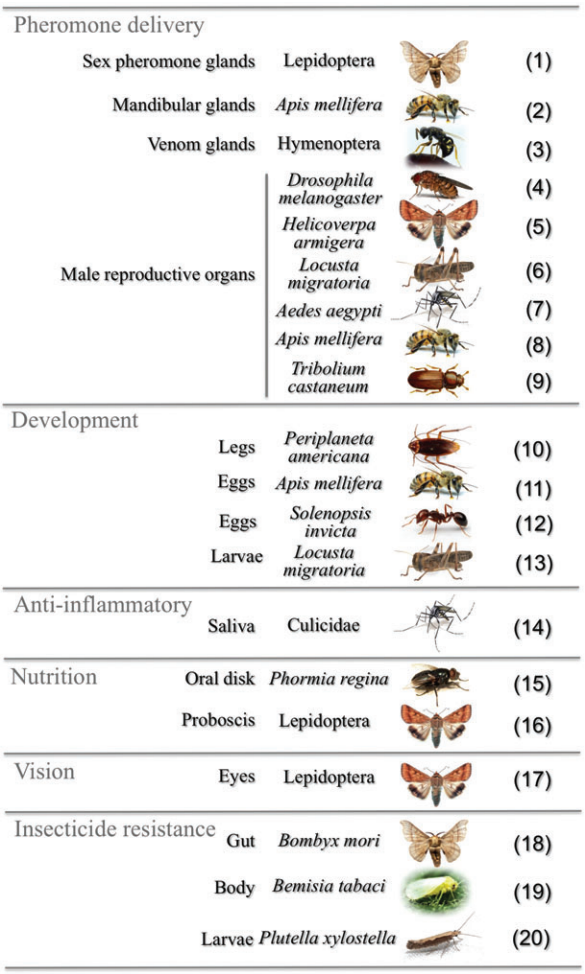

Fig. 4. Functions other than chemoreception reported for insect odorant-binding proteins (OBPs) and chemosensory proteins (CSPs). In most cases, the role of the binding proteins has been demonstrated or suggested to be that of a carrier for semiochemicals, hormones or other biologically active chemicals. Numbers refer to species studied and references, as follows: (1) *Spodoptera litura* (Zhang *et al.*, 2015); *Chilo suppressalis* (Xia *et al.*, 2015); *Agrotis ipsilon* (Gu *et al.*, 2013); *Agrotis segetum* (Strandh *et al.*, 2009); *Sesamia inferens* (Zhang *et al.*, 2013); *Bombyx mori* (Dani *et al.*, 2011); (2) *Apis mellifera* (Iovinella *et al.*, 2011); (3) *Leptopilina heterotoma* (Heavner *et al.*, 2013); *Pteromalus puparum* (Wang *et al.*, 2015); *Sirex noctilio* (Wang *et al.*, 2016); *Apis mellifera* (Li *et al.*, 2013) (4) *Drosophila melanogaster* (Dyanov & Dzitoeva, 1995; Takemori & Yamamoto, 2009); (5) *Helicoverpa armigera* (Y.L. Sun *et al.*, 2012*b*); (6) *Locusta migratoria* (Ban *et al.*, 2013; Zhou *et al.*, 2013); (7) *Aedes aegypti* (Li *et al.*, 2008; Sirot *et al.*, 2008); (8) *Apis mellifera* (Baer *et al.*, 2012); (9) *Tribolium castaneum* (Xu *et al.*, 2013); (10) *Periplaneta americana* (Nomura *et al.*, 1992; Kitabayashi *et al.*, 1998); (11) *Apis mellifera* (Maleszka *et al.*, 2007); (12) *Solenopsis invicta* (Cheng *et al.*, 2015); (13) *Locusta migratoria* (Guo *et al.*, 2011); (14) *Aedes aegypti* (Calvo *et al.*, 2006); *Anopheles stephensi* (Isawa *et al.*, 2002); (15) *Phormia regina* (Ishida *et al.*, 2013); (16) *Mamestra brassicae* (Nagnan-Le Meillour *et al.*, 2000); *Helicoverpa armigera* (Y.L. Liu *et al.*, 2014*b*; Zhu *et al.*, 2016*a*); (17) *Helicoverpa armigera* and other species (Zhu *et al.*, 2016*a*); (18) *Bombyx mori* (Xuan *et al.*, 2015); (19) *Bemisia tabaci* (G.X. Liu *et al.*, 2014*a*, 2016); *Plutella xylostella* (Bautista *et al.*, 2015).

while the only CSP found in reproductive organs of male locusts is also expressed in chemosensory structures, such as antennae, mouthparts and tarsi (Zhou *et al.*, 2013). This strategy, using the same tools to carry ligands in and out makes sense as a simple and economical management of the insect's resources. What appears more puzzling is the use of an OBP in detecting the chemosignals and a CSP in releasing the same molecules. This is the case in *D. melanogaster*, where a CSP is produced in the ejaculatory apparatus secreting the male pheromone vaccenyl acetate (Dyanov & Dzitoeva, 1995), while an OBP (LUSH) binds the same molecule in the antennae (Laughlin *et al.*, 2008; Gomez-Diaz *et al.*, 2013). We could explain this from an evolutionary perspective if CSPs were first utilised for both purposes, but subsequently the more-efficient and more narrowly tuned OBPs assumed the role of discriminating pheromones, a task requiring accuracy and specificity, while CSPs continued in the less-demanding general role of maintaining a reservoir.

Although the occurrence of similar proteins in organs where pheromones are synthesised and in those dedicated to their detection has been described only recently in insects, this phenomenon has long been recognised in mammals. The 'urinary proteins' of mice and other rodents (reviewed in Cavaggioni & Mucignat-Caretta, 2000) were identified long before the first discovery of OBPs in mammals (Pelosi *et al.*, 1982) and for many years their function was unclear, until they were recognised to be identical or very similar to the OBPs of the nose. Another example of a binding protein synthesised in both the nose and in pheromone glands is a pig OBP, named salivary lipocalin (SAL) because it is produced in male salivary glands which secrete the boar pheromone androstenone (Marchese *et al.*, 1998; Loebel *et al.*, 2000; Spinelli *et al.*, 2002). SAL is abundant in the nose of pigs where it is expressed equally in both sexes and void of ligands, while in the salivary glands SAL is male specific and loaded with the pheromone. OBPs have been reported in the reproductive organs of mammals, where they probably carry pheromones, as has been hypothesised for insects. This is the case in the rabbit where the seminal fluid contains very high levels of an OBP together with its potential ligands (Mastrogiacomo *et al.*, 2014).

(3) Regeneration and development

CSPs have been convincingly shown to be involved in development and regeneration in at least in three cases. The first CSP identified, although not then fully described, was reported to be linked to limb regeneration in the cockroach *Periplaneta americana* (Nomura *et al.*, 1992). This insect, when still in its nymphal stages, can regenerate legs that have been amputated. During this process the expression of a protein of 10 kDa increases dramatically, returning to physiological levels after the process of regeneration is complete. Subsequently, the gene encoding this protein was sequenced, revealing close similarity with other CSPs that had been described (Kitabayashi *et al.*, 1998).

A second member of the same protein family, CSP5 of the honeybee was shown to be essential for the correct development of the embryo. The gene encoding this protein, one of the six predicted by genome sequencing, is specifically expressed in ovaries and eggs, but was not detected in any other part of the body of adults or larvae (Forêt, Wanner & Maleszka, 2007). When the gene encoding CSP5 was silenced by RNA interference (RNAi), the embryos did not develop completely and eggs did not hatch (Maleszka *et al.*, 2007).

The third example of a CSP involved in development is CSP9 of *S. invicta* (Cheng *et al.*, 2015), which belongs to the same clade as *A. mellifera* CSP5 in a neighbour-joining tree of hymenopteran CSPs (González *et al.*, 2009). The expression level of mRNA of *S. invicta*-CSP9 is highest at the end of the third instar; silencing this gene through RNAi affects fatty acid biosynthesis and other metabolic pathways and prevents cuticle development and ecdysis (Cheng *et al.*, 2015).

Although these phenomena have been reported only in these three species, it is likely that they will be present in other insects, with CSPs or OBPs involved in physiological events.

Three studies report the presence of OBPs in the ovaries and in the eggshell of mosquitoes based on proteomic analysis. In *Ae. aegypti*, these proteins were not investigated for their function, but it has been suggested that they might be involved in eggshell formation (Costa-da-Silva *et al.*, 2013; Marinotti *et al.*, 2014). In *An. gambiae* several OBPs were identified at the protein level (Amenya *et al.*, 2010) and the authors suggested that they could carry chemo-attractants for sperm. All the OBPs identified in these studies belong to the atypical OBPs class (Vieira & Rozas, 2011).

Another interesting example, and quite unique in its effects, has been observed in the locust *L. migratoria*. Locusts undergo a physiological transformation from a 'solitary' phase to a 'gregarious' phase, involving morphological and behavioural changes (Nolte, 1963; Nolte, May & Thomas, 1970; Hassanali, Njagi & Bashir, 2005). The same CSP reported to be expressed at high levels in the antennae (Ban *et al.*, 2002, 2003) was recognised, together with the protein 'takeout', as the factor triggering this phase shift (Guo *et al.*, 2011).

At present, we do not have enough information to assume that in the above cases the protein itself is directly responsible for the effects observed. Alternatively, specific chemicals bound to the protein, such as hormones, could be the active agents, while the CSPs or the OBPs act as carriers. In any case, whatever the molecular mechanism producing the physiological effects, the results of such studies could provide health and economic benefits, in view of potential applications interfering with the development of agricultural pests and disease vectors.

Roles of olfactory proteins in development also have been described in vertebrates. Examples of vertebrate lipocalins involved in development further support the functional similarities between OBPs and CSPs in insects and lipocalins in vertebrates. Not long after the first mammalian OBP was discovered (Pelosi *et al.*, 1981, 1982), a new lipocalin was identified that was abundantly produced by chondrocytes of

chick embryos in culture (Descalzi Cancedda *et al.*, 1990). This protein was named extracellular fatty acid binding protein (Ex-FABP) based on its affinity for fatty acids, and was expressed during chicken embryo development not only in hypertrophic cartilage, but also in muscle fibres and in blood granulocytes. At the adult stage, the protein is only detected in cartilage under pathological conditions (Descalzi Cancedda *et al.*, 2000).

In mammals, other lipocalins have been reported to be linked to cell proliferation and cancer. In particular, lipocalin-2, also known as neutrophil gelatinase-associated lipocalin (NGAL), has been associated with several forms of cancer and shown to promote cellular proliferation. High levels of NGAL indicate advanced stages of cancer, making this protein a potential marker in the diagnosis of several types of tumours (Yang *et al.*, 2009; Rodvold, Mahadevan & Zanetti, 2012; Candido *et al.*, 2014, 2016).

(4) Anti-inflammatory action

The saliva of several species of haematophagous insects, including disease-carrying mosquitoes, contains proteins similar to insect OBPs and belonging to the so-called D7-related (D7r) family (Valenzuela *et al.*, 2002). These proteins contain two OBP domains, very different in sequence, connected by a short segment of few amino acids. Both in *An. gambiae* and *Ae. aegypti* these proteins have been functionally characterised; binding studies have shown that the two domains of the proteins act independently, the first binding cysteinyl-leukotrienes, the second exhibiting strong affinity for a number of biogenic amines, such as norepinephrine and serotonin. Both classes of compounds are released immediately after mosquito biting and elicit swelling, erythema, pain, and itching in the host. It is important for the mosquito to reduce such symptoms, because they could cause a reaction from the host leading to interruption of feeding. D7 proteins reduce inflammation through binding of cysteinyl-leukotrienes and biogenic amines (Calvo *et al.*, 2006, 2009; Mans *et al.*, 2007). In *An. stephensi*, a D7-related protein was identified as a blood coagulation inhibitor affecting the activation of the plasma contact system (Isawa *et al.*, 2002). D7-related proteins, being immunogenic, could be used as epidemiological markers of exposure to mosquitoes (Doucoure *et al.*, 2013; Marie *et al.*, 2014; Oktarianti *et al.*, 2015) and sand flies (Martín-Martín, Molina & Jiménez, 2013).

(5) Nutrition

Insect OBPs and CSPs are highly expressed in taste sensilla (Sánchez-Gracia, Vieira & Rozas, 2009) and have also been reported to play roles in feeding, both as solubilisers of hydrophobic nutrients and as surfactants in the proboscis to reduce pressure during sucking.

The blowfly *Phormia regina* feeds on rotting meat and fatty acids are an important component of its diet as they are required for reproduction (Stoffolano *et al.*, 1995). During feeding, the flies secrete in their saliva a lipase that hydrolyses triglycerides, thus producing free fatty acids (Hansen Bay, 1978). These nutrients are not soluble in water and could not be ingested without the cooperation of a carrier. An OBP identified in the oral disk of this species has been suggested to perform this task, on the basis of its affinity for long-chain fatty acids (Ishida, Ishibashi & Leal, 2013). Ishida *et al.* (2013) also suggest a mechanism for releasing these nutrients in the gut, where, due to a lower local pH, the affinity of the protein for fatty acids is drastically reduced. In mammals a lipocalin, FABP, is present in saliva and could perform a similar function in solubilising dietary fatty acids and other lipids (Ghafouri, Tagesson & Lindahl, 2003).

In the cabbage moth *M. brassicae*, several CSPs were identified in the proboscis (Nagnan-Le Meillour *et al.*, 2000) and a role in detecting nutrients was proposed. Other studies have reported unusually high concentrations of CSPs, together with smaller amounts of OBPs, in the proboscis of some moths and butterflies. Such proteins were shown to be secreted in the food canal of this organ, using a new 'drink-blot' approach, where the moth is allowed to feed on a sheet of nitrocellulose membrane that is subsequently developed as in Western blot experiments, thus revealing traces of proteins released through the proboscis (Y.L. Liu *et al.*, 2014*b*; Zhu *et al.*, 2016*a*). A role in chemodetection alone could not explain the exceptionally high levels of these proteins found in the proboscis, although chemosensilla are present on the tip of this organ. A first hypothesis suggested a surfactant role for CSPs to assist sucking of nutritious liquids by reducing the effort required to overcome the hydrostatic pressure (Y.L. Liu *et al.*, 2014*b*). However, the conservation of proboscis CSPs in phylogenetically distant species from moths to butterflies suggests that, in addition to a surfactant effect, a more-specific function may be associated with these proteins. The affinity of such CSPs for ß-carotene suggested that they could act as solubilisers and carriers for important hydrophobic components of the diet (Zhu *et al.*, 2016*a*).

(6) Carriers of visual pigments

The affinity of CSPs in the proboscis for ß-carotene suggested a link with vision. A proteomic analysis applied to the eyes of the lepidopteran *H. armigera*, detected a number of OBPs and CSPs, including members previously identified in the proboscis (Zhu *et al.*, 2016*a*).

CSPs therefore represent likely carriers across aqueous biological fluids for hydrophobic compounds required for vision, from the carotenoids of the diet to their breakdown products, the visual pigments, 3-hydroxyretinol and 3-hydroxyretinal utilised by insects instead of the retinal and retinol of vertebrates. However, other proteins also act in the eyes of insects as carriers for visual pigments. Two larger proteins, a retinoid-binding protein of 273 amino acids, called PINTA [prolonged depolarization afterpotential (PDA) is not apparent], and a retinol-binding protein of 235 amino acids were identified in *D. melanogaster* (Wang & Montell, 2005; Wang, Jiao & Montell, 2007) and in the butterfly *Papilio xuthus* (Wakakuwa, Arikawa & Ozaki, 2003; Wakakuwa, Ozaki & Arikawa, 2004), respectively. These proteins belong

to different families and are completely unrelated to OBPs or CSPs.

It is not surprising that the complex mechanisms of vision, in particular the generation, transport and recycling of visual pigments might require several proteins of different structures. However, in the context of parallels between the functions of OBPs in insects and vertebrates, it is interesting to observe that in vertebrates retinol is carried in the bloodstream by a lipocalin (retinol-binding protein; RBP) from its site of production, the liver, to the retina (Monaco, 2000; Newcomer & Ong, 2000).

(7) Insecticide resistance

A central problem in the use of insecticides for insect population control is the rapid adaptation of insects to the actions of these chemicals. In several cases such adaptation has been related to mutations in the target proteins or in the activation of enzymes degrading the molecules of insecticides.

Another mechanism, still awaiting experimental support, has been suggested by the observation that the genes encoding some CSPs undergo dramatic up-regulation in the gut of insects treated with sub-lethal doses of insecticides. This phenomenon has been observed in the silk moth *B. mori* when treated with avermectins and in the whitefly *Bemisia tabaci* in response to the neonicotinoid thiamethoxam (G.X. Liu *et al.*, 2014*a*, 2016; Xuan *et al.*, 2015). CSPs may act as buffers in the gut by sequestering and masking toxic insecticide molecules, that could then be discarded in the faeces complexed to the proteins. In the diamondback moth, *Plutella xylostella*, three chemosensory genes (CSP4, CSP8 and OBP13) have been reported to be up-regulated in the head after treatment with permethrin (Bautista *et al.*, 2015).

While these findings indicate changes in the expression of chemosensory genes in response to insecticides, the mechanisms by which such changes may contribute to defence against these xenobiotics need to be clarified.

IV. TECHNOLOGICAL APPLICATIONS

The high success of OBPs and CSPs in nature, evidenced by the adaption of these proteins to a large number of diverse tasks, has not escaped the attention of scientists interested in designing biosensors for environmental chemicals. One area of interest is the fabrication of artificial noses: arrays of sensors for the detection and discrimination of environmental odours. OBPs and CSPs of insects (and also the OBPs of vertebrates) are ideal tools to serve as specific biosensing elements for environmental odours. They are: (*i*) easy and cheap to synthesise in heterologous expression systems, due to their small size and the general absence of post-translational modifications; (*ii*) exceptionally stable to denaturing action by temperature and organic solvents, as well as refractory to proteolytic degradation; (*iii*) easily amenable to selective modifications through site-specific mutagenesis in order to tailor their binding affinity and specificity to requirements; (*iv*) suitable for interactions with natural organic compounds, as they have evolved and adapted to detect environmental odours. The large amount of data available on the structure of hundreds of OBPs and CSPs from a variety of insect species represents an invaluable information resource from which to select the proteins best suited for each specific problem.

Biosensors for odours have been successfully produced using OBPs from both vertebrates and insects (Hou *et al.*, 2005, 2007; Sankaran, Panigrahi & Mallik, 2011; Di Pietrantonio *et al.*, 2013). Particularly interesting is the discrimination achieved by a field-effect transistor, based on a pig OBP, that efficiently distinguished between the two enantiomers of carvone (Mulla *et al.*, 2015). In another study, OBP14 from the honeybee was immobilised on graphene and incorporated into a field-effect transistor to produce biosensors able finely to discriminate ligands in a way that parallelled the specificity of the protein when measured in solution (Larisika *et al.*, 2015). These methods, besides representing promising ways to assemble devices for environmental monitoring, offer an alternative method to the current use of a fluorescent reporter for measuring ligand-binding activities in solutions (Ban *et al.*, 2002; Calvello *et al.*, 2003).

There are no reports of similar devices using CSPs as sensing elements. This may reflect the larger volume of information available on OBPs compared to CSPs. However, there is no reason that these proteins could not be used as biodetectors for odours and other organic compounds. Compared to OBPs, CSPs have a more flexible and adaptable structure, as discussed above (see Section II.1), and consequently lower specificity towards ligands. This could be a disadvantage when finely tuned sensors are required, but may be a desirable characteristic if the targets are groups or classes of structurally related chemicals.

The multitask properties of OBPs and CSPs observed in nature could also suggest a variety of biotechnological applications for these proteins, besides their use as biosensing elements. For example, their role in storing semiochemicals for delayed emission could suggest uses as slow releasers of fragrances in the environment or of drugs in the body, or insect pheromones in agriculture. Their proposed insecticide-sequestering action might suggest the use of both OBPs and CSPs to remove dangerous pollutants from the environment. A single report has been published on the use of a mammalian OBP in a filtering trap for the herbicide atrazine (Bianchi *et al.*, 2013). Another interesting application of OBPs for removing unpleasant odours has been proposed by Silva *et al.* (2013), who incorporated the pig OBP on fabrics for clothes. The immobilized protein served a dual purpose, removing or reducing cigarette odour and slowly releasing pleasant fragrances previously bound to the OBP. The high cost involved in such applications, mainly due to the production and purification of large amounts of proteins, as compared to current methods, could be balanced by their selectivity in removing only specific ligands in specific environmental situations. Finally, uses in

analytical chemistry have been demonstrated to be viable. A column for liquid chromatography bearing immobilised *B. mori* PBP was shown to be able to separate structurally related compounds (Margaryan *et al.,* 2006).

V. CONCLUSIONS

(1) Results collected during the last decade have profoundly modified our view of insect OBPs and CSPs, whose functions were previously regarded as confined to chemoreception organs and mechanisms. An increasing number of reports have shown that members of both classes of proteins have been adopted by insects to perform different physiological roles, including in development and insecticide resistance, in most organs of the insect body.

(2) Despite the variety of biological processes in which both OBPs and CSPs are involved, it is reasonable to suspect that the common property linking their very different functions is the ability of these proteins to bind and solubilise small hydrophobic compounds. These can be pheromone components in specialised glands, dietary nutrients such as lipids and carotenoids, visual pigments in the eyes, insecticides in different parts of the body or even hormones promoting development and differentiation.

(3) The versatility of OBPs and CSPs is related to their stable and compact structure that allows a high level of variation within the binding pocket to accommodate different ligands while maintaining conserved overall folding.

(4) The stability and versatility of OBPs and CSPs match similar properties of lipocalins, a superfamily of proteins including vertebrate OBPs. Lipocalins are structurally different from both insect OBPs and CSPs, but are endowed with similar functions, from carriers of pheromones to roles in development, vision, nutrition and regeneration.

(5) The adaptation of OBPs and CSPs to a variety of roles in biological systems has suggested different uses for these binding proteins in technological applications, from the assembly of biosensors for odour monitoring to scavengers for noxious compounds in the environment, as well as in applications where a slow release of chemicals is needed. Moreover, there may be other applications in environmental and food-quality monitoring, as well as in medical diagnostic devices. We can foresee the design of artificial binding proteins, tailored to specific requirements and based on the stable scaffolding of OBPs and CSPs.

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VII. REFERENCES

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