Chem. Senses 36: 335–344, 2011

doi:10.1093/chemse/bjq137
Advance Access publication January 10, 2011

Odorant-Binding Proteins and Chemosensory Proteins in Pheromone Detection and Release in the Silkmoth *Bombyx mori*

Francesca R. Dani[1], Elena Michelucci[1], Simona Francese[2], Guido Mastrobuoni[3], Silvia Cappellozza[4], Giancarlo La Marca[5], Alberto Niccolini[6], Antonio Felicioli[6], Gloriano Moneti[1] and Paolo Pelosi[7]

[1]CISM, Centro Interdipartimentale di Spettrometria di Massa, University of Firenze, Viale G. Pieraccini n.6, 50139 Firenze, Italy, [2]Biomedical Research Centre, Sheffield Hallam University, Howard Street S1 1WB, Sheffield, UK, [3]Max Delbrück Center for Molecular Medicine, Berlin, Germany, [4]CRA, Consiglio per la Ricerca e la Sperimentazione in Agricoltura, Unità Ricerca Apicoltura & Bachicoltura, Via Dei Colli, n. 28, I-35143 Padova, Italy, [5]Department of Pharmacology, University of Florence, Viale G. Pieraccini, n.6 50139 Florence, Italy, [6]Department of Physiological Sciences, University of Pisa, Viale delle Piagge, n.2 56124 Pisa, Italy and [7]Department of Biology of Agricultural Plants, University of Pisa, Viale delle Piagge, n. 23 56124 Pisa, Italy

Correspondence to be sent to: Francesca R. Dani, Centro Interdipartimentale di Spettrometria di Massa CISM, University of Firenze, Viale G. Pieraccini, n.6, 50139, Firenze, Italy. e-mail: francescaromana.dani@unifi.it

Accepted November 23, 2010

Abstract

The genome of the silkmoth *Bombyx mori* contains 44 genes encoding odorant-binding proteins (OBPs) and 20 encoding chemosensory proteins (CSPs). In this work, we used a proteomic approach to investigate the expression of proteins of both classes in the antennae of adults and in the female pheromone glands. The most abundant proteins found in the antennae were the 4 OBPs (PBP, GOBP1, GOBP2, and ABP) and the 2 CSPs (CSP1 and CSP2) previously identified and characterized. In addition, we could detect only 3 additional OBPs and 2 CSPs, with clearly different patterns of expression between the sexes. Particularly interesting, on the other hand, is the relatively large number of binding proteins (1 OBP and 7 CSPs) expressed in the female pheromone glands, some of them not present in the antennae. In the glands, these proteins could be likely involved in the solubilization of pheromonal components and their delivery in the environment.

Key words: *Bombyx mori*, chemosensory protein, MALDI profiling, odorant-binding protein, pheromone glands, proteomics

Introduction

The silkmoth *Bombyx mori* is one of the most studied model species for chemical communication in insects. Bombykol, the sex attractant for males of this species, was the first pheromone to be identified (Butenandt et al. 1959) and the pheromone-binding protein (PBP) was among the very first proteins of this class to be purified and characterized (Maida et al. 1993). This was also the first odorant-binding protein (OBP) of an insect, whose structure has been resolved (Sandler et al. 2000) and the first to exhibit a conformational change triggered by pH and ligand binding (Horst et al. 2001). However, the complexity of protein repertoire possibly involved in chemical communication in this species was not evident, until the sequencing of its genome (Xia et al. 2004, 2007) revealed the presence of 41 genes encoding olfactory receptors (Wanner et al. 2007), 44 encoding OBPs (Gong et al. 2009), and 20 encoding chemosensory proteins (CSPs) (Gong et al. 2007).

OBPs and CSPs are small soluble polypeptides present in the lymph of chemosensilla (Picimbon 2003, Wanner et al. 2004; Vogt 2005; Pelosi et al. 2006). OBPs are characterized by 6 conserved cysteines paired in 3 interlocked disulfide bridges (Scaloni et al. 1999; Leal et al. 1999; Tegoni et al.

2004). In some insect species other proteins, assigned to the OBP family contain a greater or lower number of cysteines and have been classified as C-plus and C-minus, respectively (Zhou et al. 2004; Forêt and Maleszka 2006). Others have been termed as "atypical" (Xu et al. 2003). CSPs present only 4 conserved cysteines with a different topology, where adjacent residues are paired (Angeli et al. 1999).

OBPs were regarded at the beginning as passive carriers of hydrophobic semiochemicals across the aqueous sensillar lymph. Recent data, however, have indicated more important and specific functions for these proteins in chemoreception. First, the discovery of conformational changes associated with ligand binding in OBPs of different species (Horst et al. 2001; Wogulis et al. 2006; Pesenti et al. 2008) suggested the possibility of interactions with membrane-bound olfactory receptors. Then evidence was provided that an OBP mediates pheromone response in the silkmoth *B. mori* (Popof 2004; Grosse-Wilde et al. 2006) and that silencing the gene for LUSH, one of the *Drosophila* OBPs, suppresses both the electrophysiological and behavioral response to the male sex pheromone (Xu et al. 2005). Moreover, using mutants of this protein, the same authors demonstrated that LUSH can activate the specific olfactory receptor even in the absence of pheromone but provided it assumes the correct conformation (Laughlin et al. 2008). The role of OBPs not only in olfactory detection but in the fine discrimination of chemical stimuli was also confirmed at the behavior level by switching 2 genes that are involved in attraction or repulsion by 2 fatty acids between 2 species of *Drosophila* (Matsuo et al. 2007).

In *B. mori*, a single PBP and 2 general odorant-binding proteins (GOBPs) were first described at the protein level (Vogt et al. 1991). A gene encoding a fourth OBP, named antennal binding protein X (ABPX), was also identified (Krieger et al. 1996). The expression of these 4 proteins was studied in various sensillum types through immunocytochemisty (Maida et al. 2005). Later, 2 additional genes encoding proteins of the PBP subfamily have been described (Forstner et al. 2006). In the same report, polymerase chain reaction (PCR) experiments show that PBP2 and PBP3 are almost equally expressed in the antennae of both sexes and that their genes are localized only in 1 or 2 sensilla, different from those expressing PBP1. The presence of microheterogeneity of OBPs was suggested in a study where proteins were separated by preparative isoelectric focusing. Four protein bands cross-reacting with anti-PBP serum and 5 with anti-GOBP2 serum were detected, but no evidence was provided on the number of genes involved (Maida et al. 1997).

Concerning CSPs, 2 abundant proteins of this class have been purified from the antennae and characterized by N-terminal sequencing. Cloning of the relative genes allowed unambiguous assignment of these proteins to the CSP family (Picimbon et al. 2000).

Recently, based on genome information, the expression pattern of all the genes encoding OBPs (Gong et al. 2009) and CSPs (Gong et al. 2007) in different organs of the silkmoth has been studied. Of the 44 genes present in the genome and encoding OBP-like proteins, 6 belong to the GOBP/PBP group, including the 3 previously described proteins, 9 are classified as plus-C, containing more than the 6 conserved cysteines and 5 as minus-C, presenting only 4 of such residues. The others include 2 groups of ABPs and another classified as chemoreceptor lymph–binding protein. It is remarkable that of the 44 genes present in the genome, 11 were not detected, using microarray techniques, at any developmental stage and most of the others were not specific to sensory organs. The expression of 4 genes, assigned on the basis of sequence similarity to the PBP group, has been monitored in the cited paper (Gong et al. 2009) by quantitative PCR in the antennae of both sexes. OBP3 (formerly named PBP1) is expressed significantly more in males than in females, in agreement with previous reports, while OBP4 and OBP6 are expressed in similar amounts in both sexes. OBP5, on the other hand, is the least abundant of these proteins and strongly female biased. The expression of genes encoding CSPs in *B. mori* has been investigated using microarray techniques and revealed interesting expression patterns during developmental stages, with members expressed also in non-sensory organs (Gong et al. 2007). A total of 20 sequences encoding proteins similar to CSPs is present in the genome, whereas the transcription products of 14 of these have been identified in different organs at various stages of the larval and adult life (Gong et al. 2007).

The great number of genes encoding OBPs and CSPs in the genome of several insects and their expression in non-sensory organs suggests alternative functions for these soluble proteins. In some species, it has been reported that OBPs or CSPs, depending on the species, are also expressed in pheromone producing glands and could likely be involved in the delivery of these semiochemicals. Known examples include CSPs expressed in the female pheromone glands of the moth *Mamestra brassicae* (Jacquin-Joly et al. 2001) and EjB, the ejaculatory bulb protein of *Drosophila melanogaster* produced in the male pheromone glands (Dyanov and Dzitoeva 1995) as well as OBP22 secreted in the sperm of *Aedes aegypti* (Li et al. 2008). In mammals, the occurrence of the same OBPs in the olfactory organs and in the pheromone delivery glands has been well documented. The mouse urinary proteins (Cavaggioni et al. 1990; Cavaggioni and Mucignat-Caretta 2000) and the pig salivary proteins (Marchese et al. 1998) represent the best examples.

In this work, we applied a proteomic approach to detect the expression of OBPs and CSPs in the antennae of both sexes of *B. mori* in order to verify which and to what extent the genes transcripted in these organs were also translated, identifying at the same time major differences, if present, between sexes. Moreover, using the same strategy, we have searched for OBPs and CSPs expressed in the female's pheromone glands.

Materials and methods

Insects

Cocoons of *B. mori* were reared at the Unità Ricerca Apicoltura & Bachicoltura, Padova, Italy. Pupae were separated according to their sex and stored at room. Adults were frozen at –20 °C within few hours after emerging and transferred to a –80 °C few days later. Antennae and female pheromonal glands were dissected under a stereoscopic microscope and stored at –80 °C until extraction or were immediately used for profiling experiments.

2D electrophoresis

For 2D gel electrophoresis, 60 antennae or 30 female pheromonal glands were homogenized in 0.5 mL of 0.1% aqueous trifluoroacetic acid (TFA). The suspension was sonicated and then centrifuged at 12 000 rpm for 40 min at 4 °C. Supernatants were concentrated to 50 μL and then diluted to 400 μL with a buffer containing 7 M urea, 2 M thiourea, 2% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 1% (v/v) Immobilized pH gradient (IPG) buffer (GE-Healthcare), and 60 mM dithiothreitol (DTT). The samples were loaded by rehydration for 13.5 h in IPG strips (pH 3–11, 11 cm). Isoelectrofocusing was performed with an Ettan IPG Phor III system (GE-Healthcare) using the following conditions. For the antennal samples: 50 V (2 h), 100 V (2 h), 500 V (1 h), 1000 V (2 h), 3000 V (1.5 h), and 4000 V (1 h); for the gland extract: 100 V (2 h), 500 V (2 h), 1000 V (3.5 h), 3000 V (2.5 h), and 5000 V (0.5 h). Strips were then equilibrated for 15 min in a Tris–HCl 1.5 M pH 8.8 solution containing glycerol 29.3%, urea 6 M, sodium dodecyl sulfate (SDS) 2% (w/v), DTT 1% and then for further 15 minutes in a Tris–HCl 1.5 M pH 8.8 solution, containing glycerol 29.3%, urea 6 M, SDS 2%, and iodacetamide 2.5%.

The 2D electrophoresis was performed in 14% acrylamide gels using a SE 600 Ruby equipment (GE-Healthcare). Gels were stained with Brilliant Blue G-Colloidal Concentrate (Sigma).

Identification of proteins from 2-D gel spots

Spots of interest (in the range of 10–20 kDa) were excised from the gel and individually transferred to a 1.5-mL microcentrifuge tube. Spots were washed 3 times for about 10 min in 40 μL of acetonitrile and then in 40 μL of a 0.1 M ammonium bicarbonate water solution. The solution was removed and 30 μL of a 1 ng/μL of modified trypsin (Promega) in 10 mM ammonium bicarbonate was added to each spot. Tube were kept at 4 °C for 30 min, then the solution was removed and replaced with 25 μL of 10 mM ammonium bicarbonate. After overnight digestion at 37 °C, the supernatant was recovered and the reaction blocked by addition of 1 μL of 10% TFA. Peptides were analyzed through nano High Performance Liquid Chromatography (HPLC)-Electrospray Ionization (ESI) Fourier Transform Mass Spectrometry (FTMS) analysis on an Ultimate 3000 (LC Packings Dionex) coupled with an LTQ Orbitrap mass spectrometer (Thermo Fisher). They were concentrated on a precolumn cartridge PepMap100 C18 (300 μm inner diameter [i.d.] × 5 mm, 5 μm, 100 Å, LC Packings Dionex) and then eluted on a C18 PepMap100 column (75 μm i.d. × 15 cm, 5 μm, 100 Å, LC Packings Dionex) at 300 nL/min. The mobile phases composition was H_2O 0.1% formic acid/CH_3CN 97/3 (phase A) and CH_3CN 0.1% formic acid/H_2O 97/3 (phase B). The gradient program was 0 min, 4% B; 10 min, 40% B; 30 min, 65% B; 35 min, 65% B; 36 min, 90% B; 40 min, 90% B; 41 min, 4% B; 60 min, 4% B. Mass spectra were acquired in positive ion mode, setting the spray voltage at 1.9 kV, the capillary voltage and temperature, respectively, at 40 V and 200 °C, and the tube lens at 130 V. Data were acquired in data-dependent mode with dynamic exclusion enabled (repeat count 2); survey mass spectroscopy (MS) scans were recorded in the Orbitrap analyzer in the mass range 300–2000 Th at a 15 000 nominal resolution, then up to 3 most intense ions in each full MS scan were fragmented and analyzed in the Orbitrap analyzer at a 7500 nominal resolution. Monocharged ions did not trigger MS/MS experiments. The acquired data were searched using Bioworks 3.2 (Thermo Fisher) using Sequest as search algorithm against a database created by merging the sequences contained in the database silkpept.fasta (downloaded from http://silkworm.swu.edu.cn/silkdb/doc/download.html) with the mature OBP and CSP sequences reported in Gong et al. (2007, 2009). Searches and acceptance of the proteins identified followed the same criteria as described in Dani et al. (2010). Each identified protein present in the gel at a position compatible with its calculated molecular weight was submitted to a basic alignment search tool in NCBI.

MALDI profiling

Experiments were performed on a Matrix Assisted Laser Desorption Ionization Time of Flight/Time of Flight mass spectrometer Ultraflex III (Bruker Daltonics) by using Flex Control 3.0 as data acquisition Software, with a method similar to that previously reported for *Anopheles gambiae* (Dani et al. 2008). Single antennae of *Bombyx* females and males and freshly dissected pheromonal glands were laid on an MALDI target; 0.3 μL of matrix solution (sinapinic acid 20 mg/mL in 50:25:25 acetonitrile:acetone:water, 0.1% TFA) were added all along the antennae and glands and allowed to dry. This made the sample to stick to the target. Spectra were acquired in linear mode over the m/z range 5000–22 000 (for a total of 800 shots) all along the antennal surface and the gland area. The most intense signals were obtained where the antennal cuticle was fractured, probably because the extraction of internal soluble proteins was more efficient. The instrumental parameters were chosen by

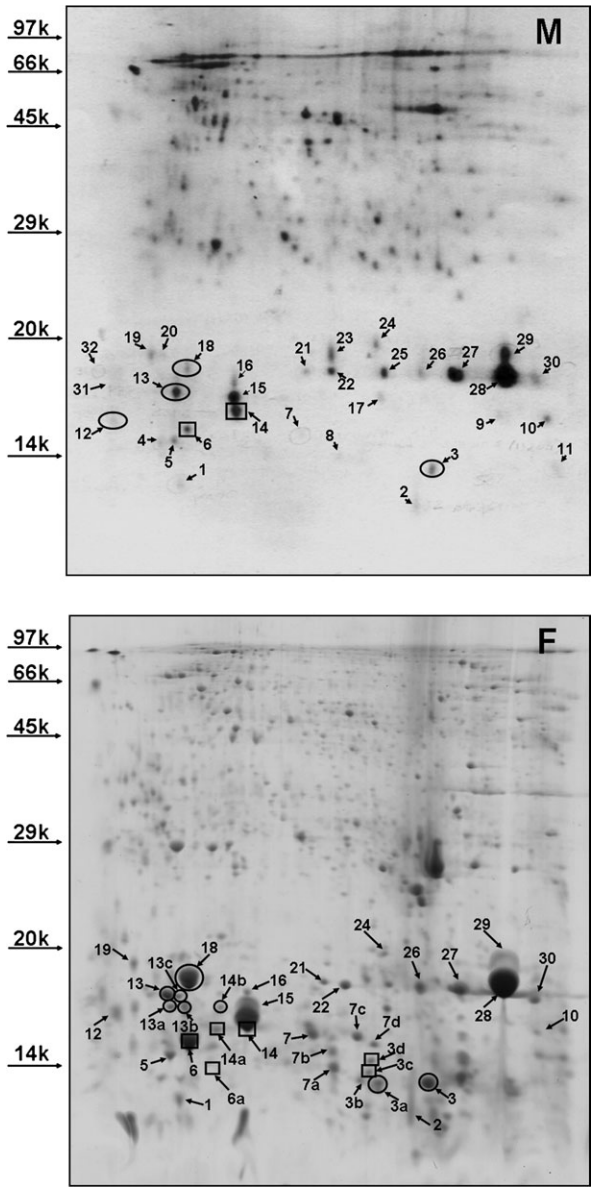

Figure 1 2D gel electrophoretic separation of proteins extracted from male (M) and female (F) antennae of *Bombyx mori*. Samples obtained from 60 antennae were loaded onto the gels. Circles indicate spots that have been identified as OBPs, squares indicate CSPs. The full set of data on the identified proteins is reported in Table 1 for OBPs and CSPs and in Supplementary Table S1 for other proteins.

setting ion source 1 at 25 kV, ion source 2 at 23.45 kV, lens at 6.0 kV, and pulsed ion extraction at 100 ns. The instrument was externally calibrated prior to analysis using the Bruker Protein I calibrant standard kit (5000–17 000 Da).

Protein nomenclature

For OBPs and CSPs, we have adopted the nomenclature of Gong et al. (2007, 2009). For proteins previously described, we also cited the traditional name.

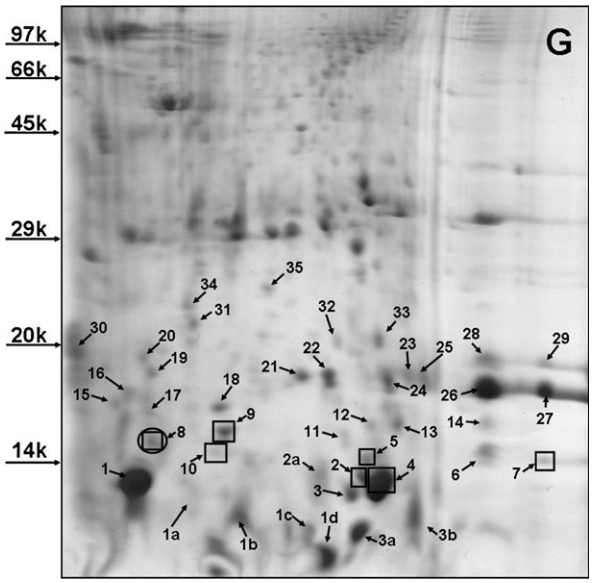

Figure 2 2D gel electrophoretic separation of an extract from 30 female pheromone glands of *Bombyx mori*. Circles indicate spots that have been identified as OBPs, squares indicate CSPs. The full set of data on the identified proteins is reported in Table 1 for OBPs and CSPs and in Supplementary Table S2 for other proteins.

Results and discussion

The high number of genes encoding OBPs (44) and CSPs (20) in the genome of *B. mori*, compared to the very few proteins of both classes identified so far through classic biochemistry (3 OBPs and 2 CSPs), prompted us to investigate the expression of these genes in the antennae of both sexes as well as in the female pheromone glands of the adult moth.

In a first series of experiments, we separated the proteins of crude extracts from the antennae of both sexes, as well as from female pheromone glands, by 2D gel electrophoresis (Figures 1 and 2). Spots were then selected in the low molecular weight region and subjected to digestion with trypsin, followed by nano-HPLC mass spectrometry analysis, as described in the Material and Methods. Peptides eluted from the column, were characterized by their molecular mass and partial sequence information, that enabled us to uniquely identify the corresponding peptide among those predicted in the genome.

In the male antenna, we were able to identify 4 OBPs, the most abundant being the OBP3 (formerly named PBP1). The other 3, present in much lower concentrations, were OBP2 (previously known as GOBP2), OBP20 (previously named ABPX), and OBP27. Among the CSPs, only CSP1 and CSP2 were clearly recognized, the same that had been previously identified at the protein level (Picimbon et al. 2000). The gel of female antennae is richer in both OBPs and CSPs. The most abundant spot is due to OBP2. Other very intense spots are those of OBP27, CSP1, and CSP2. In addition, there are 4 more OBPs (1 and 3 and 27 previously described, respectively, as GOBP1, PBP1 and ABP, and OBP25) and

2 more CSPs, 8 and 9, that have been unambiguously recognized but are present at much lower levels (see Figure 1 and Table 1).

MALDI profiling experiments, performed on single antennae, confirmed the results obtained by 2D gels (Figure 3). In fact, the most abundant peak in the male antenna is due to CSP2, which also gave the most intense spot in the gel, whereas other peaks can be assigned to CSP1, OBP3, and OBP27, all present with spots of medium intensity in the gel.

In the female antenna, again CSP2 corresponds to the most intense peak, while CSP1 and OBP27 can also be detected. Although there is fairly good agreement between the calculated masses and those measured for CSP1 (calc.12 888, accounting for 2 disulfide bridges and one proton added by the matrix, measured: 12 893 and 12 892) and OBP3 (calc.15 879, accounting for 3 disulfide bridges and one proton added by the matrix, measured: 15 879), the calculated mass of CSP2 (12 048) is higher than the measured values of 12 031 and 12 029, suggesting the occurrence of an aminoacidic substitution. Unexpectedly, OBP2, that is present in the gel with the largest spot, could not be identified in the MALDI profiling. Instead a large unidentified peak of m/z 11168.8 could indicate a degradation product of OBP2. The same peak, but of lower intensity, is also present in the MALDI spectrum of male antennae, whose 2D gel also contains a spot identified as OBP2, but weaker than in the female gel. However, no conclusions can be drawn on the basis of the available data.

Of the 4 proteins belonging to the PBP group (OBPs 3, 4, 5, and 6), whose expression had been monitored by PCR in the antennae of both sexes, we could only detect OBP3 (the only PBP previously described, Maida et al. 1993; Krieger et al. 1996), whereas we found no evidence of the other proteins in our experimental conditions. In fact, PCR experiments indicated levels of expression for OBP6, OBP4, and OBP5 about 1, 2, and 3 orders of magnitude lower than OBP3, respectively (Gong et al. 2009). It is possible, therefore, that these proteins are present in the extract, but at levels lower than our detection limit. Moreover, we could not detect PBP2 and PBP3 at the protein level neither in the MALDI spectra nor in the 2D gels. This also could be due to the small amount of these proteins expected to be present in the antennae as the relative genes have been reported to be expressed only in 1 or 2 sensilla (Forstner et al. 2006).

Several CSPs had been reported to be expressed in the antennae of adults, on the basis of PCR experiments (Gong et al. 2009). High amplification was obtained for CSPs 1, 2, 4, 9, 11, 14, 15, 16, whereas CSPs 6, 7, 8, 12, 13, and 17 gave much weaker bands. Some of these were also found at the protein level in our investigation, but there is no complete agreement between our protein data and the gene analysis. In fact, among all the genes reported in the antennae, we could detect the abundant expression products only for CSP1 and CSP2, whereas CSP8 and CSP9 were present at much lower levels.

Most interestingly, the analysis of the pheromone glands extract (Figure 2) revealed the presence of 7 members of the CSP family, no. 1, 2, 6, 8, 9, 11, 15, and one OBP (OBP11).

In particular, CSP6 produced the most intense spot while was not detected in the antennae of either sex. Interestingly, some of the other CSPs 8 and 9 were also found in female antennae (but not in males), whereas others (11, and 15) were not detected in the antennae of neither sexes.

A comparison of our results with the gene expression of CSPs in the pheromone glands, evaluated by PCR (Gong et al. 2009) does not show good agreement. In fact, such analysis indicated CSPs 9 and 11 as the most expressed, whereas CSPs 6, 14, and 17 gave weaker amplification signals. Such disagreement, when it only concerns different levels of expression of RNA and protein, as in the cases of CSPs 6, 9, and 11, could be still acceptable. More difficult to justify are the strong spots produced in the gel by CSPs 2 and 8, as well as the weaker but reliable spots of CSP1 and CSP15, although their RNA had not been detected by PCR. We could speculate that perhaps the samples used in the 2 types of experiments were from insects in different physiological states, but such aspect needs to be further investigated before any hypothesis could be suggested.

The abundant expression of CSP6 is also evident from the MALDI profiling spectrum (Figure 4), where it gives by far the most intense signal. Also the presence of CSP2 is clearly confirmed in the same spectrum of pheromone gland with a peak of moderate intensity.

Our results show that of the 44 OBPs and 20 CSPs predicted in the genome of *B. mori*, only a small number of these genes are expressed at the protein level, at least in concentrations above our detection limits. In particular, we have identified OBPs 1, 2, 3, 20, 27, and 25 in the antennae of adults and OBP11 in the female pheromone glands. We can incidentally observe that only the last 2 proteins had not been previously described.

As for CSPs, besides the known CSP1 and CSP2, we detected CSP8 and CSP9 in the antennae and 3 more (CSPs 6, 11, and 15) in the pheromone glands that are not expressed in the antennae.

The fact that only few of the proteins predicted by the genome can be detected, at least in reasonable concentrations, deserves some comment. At the beginning, OBPs of insects were regarded as a class of proteins specifically involved in chemoreception with the defined role of binding odorants and pheromones. For CSPs, instead, the idea that only a subset of these proteins could be directly involved in the perception of chemical stimuli was more acceptable, on the basis that CSPs were detected also in nonsensory organs and sometimes could be almost ubiquitous. Apart from those expressed in pheromone glands, such as the ones described and those cited in this work (for which a function in chemical communication is still likely), CSPs could be performing

Table 1 OBPs and CSPs identified in male and female antennae and in female pheromone gland

Spot #	Code (Gong et al. 2007, 2009)	Name[a]	No. of peptides	Coverage[b]	MW[c]	P(pro) consensus score
Male and female antennae						
3 (M, F)	BGIBMGA002626	OBP27[d] (ABP)	16	1–119	13180[f]	2.3×10^{-13}
				100%		170.3
3a (F)	BGIBMGA002626	OBP27[d] (ABP)	16	1–119	13180[f]	2.3×10^{-13}
				100%		170.3
3a (F)	BGIBMGA002627	OBP25	2	81–91; 115–119	13077	2.8×10^{-7}
				13.45%		20.2
3c (F)	Bmb018112	CSP9[d]	9	23–61; 98–108	12533	2.1×10^{-8}
				46.30%		110.2
3d (F)	Bmb018111	CSP8[d]	9	44–59; 69–81; 90–109	12769	1.5×10^{-6}
				44.04%		90.1
6 (M, F)	Bmb008613	CSP1[d,e]	5	22–35; 42–57; 83–93	12883[f]	2.6×10^{-7}
				36.13%		50.2
6a (F)	Bmb008613	CSP1[d,e]	4	42–57; 83–93	12883[f]	2.2×10^{-7}
				30.63%		40.2
12 (M)	BGIBMGA002308	OBP20[d] (ABPX)	5	15–44	12791	3.5×10^{-9}
				25.42%		20.3
13 (M, F)	BGIBMGA012615	OBP3[d,e] (PBP1)	11	1–21;39–119	15868[f]	9.1×10^{-12}
				71.83%		190. 3
13 (F)	BGIBMGA012611	OBP1[d,e] (GOBP1)	6	47–78; 90–110; 114–124	17179	6.5×10^{-8}
				43.84%		60.2
13a (F)	BGIBMGA012611	OBP1[d,e] (GOBP1)	19	1–29; 47–124	17179	1.1×10^{-16}
				73.29%		230.4
13b (F)	BGIBMGA012611	OBP1[d,e] (GOBP1)	5	58–78; 100–110; 114–124	17179	1.4×10^{-5}
				29.45%		50.2
13b (F)	BGIBMGA012614	OBP2[d,e] (GOBP2)	12	1–14; 59–78; 90–119	16130	1.1×10^{-11}
				45.39%		150.3
13b (F)	BGIBMGA012615	OBP3[d,e] (PBP1)	3	1–14; 79–99	15868[f]	2.8×10^{-8}
				24.65%		50.1
13c (F)	BGIBMGA012611	OBP1[d,e] (GOBP1)	14	1–20; 47–110; 114–124	17179	3.9×10^{-11}
				80.14%		190.2
14 (M, F)	Bmb030868	CSP2[d,e]	5	43–57; 94–104	12046[f]	1.1×10^{-09}
				25.00%		30.3
14a (F)	Bmb030868	CSP2[d,e]	3	43–57; 68–74; 94–104	12046[f]	1.48×10^{-5}
				31.73%		30.1
14b (F)	BGIBMGA012611-PA	OBP1[d,e] (GOBP1)	16	1–20; 47–124	17179	1.1×10^{-9}
				67.12%		160.2
18 (M, F)	BGIBMGA012614	OBP2[d,e] (GOBP2)	4	1–34; 47–78; 90–141	16130	1.1×10^{-14}
				83.69%		350.4

Table 1 Continued

Spot #	Code (Gong et al. 2007, 2009)	Name[a]	No. of peptides	Coverage[b]	MW[c]	P(pro) consensus score
Female pheromone glands						
2	Bmb018112	CSP9[d]	19	22–61; 65–108	12533	1.2×10^{-9}
				77.78%		190.3
2	Bmb018109	CSP6[d]	5	1–27; 87–105	12273[f]	5.4×10^{-9}
				43.81%		40.2
2a	Bmb018109	CSP6[d]	4	1–27; 44–56	12273[f]	5.2×10^{-6}
				38.10%		40.1
4	Bmb018109	CSP6[d]	14	1–27; 41–56; 71–105	12273[f]	6.3×10^{-11}
				74.29%		190.3
5	Bmb018111	CSP8	8	44–59; 70–105	12769	7.2×10^{-7}
				47.71%		80.1
7	Bmb028718	CSP1[d]	6	47–57; 69–78; 97–108	12646	1.7×10^{-76}
				27.78%		60.1
8	BGIBMGA008354	OBP11	8	1–53; 55–72; 76–88; 111–122	13981	9.2×10^{-9}
				73.85%		100.2
8	Bmb008613	CSP1	4	22–33; 42–64	12883	4.2×10^{-9}
				33.33%		50.2
9	Bmb030868	CSP2	13	3–57; 68–104	12047[f]	2.7×10^{-010}
				85.0%		130.3
10	Bmb037504	CSP15	3	7–20; 45–56; 92–105	12370	3.0×10^{-5}
				38.1%		40.1

[a]Names as reported in Gong et al. (2007, 2009). In brackets traditional names.
[b]Aminoacidic positions in the mature protein (Gong et al. 2007, 2009).
[c]Average molecular weights of the mature proteins.
[d]Previously reported as transcript (see text) in the same organ.
[e]Previously reported as protein (see text) in the same organ.
[f]Signals corresponding to the expected mass for the mature protein were observed in profiling experiments.

completely different physiological functions. The best described example is perhaps the CSP5 of the honeybee, that is only found in the eggs (Forêt et al. 2006) and was clearly shown to be essential for embryo development (Maleszka et al. 2007). Although we do not have similar examples for insect OBPs, the expression of some of them in nonsensory organs, particularly in Diptera (Xu et al. 2003; Zhou et al. 2004; Calvo et al. 2009), could suggest roles different from that of binding semiochemicals. Particularly interesting is a group of proteins found also in the saliva of Diptera and containing 2 or more OBP sequences linked in tandem that are shown to bind biogenic amines (Calvo et al. 2009).

On the other hand, vertebrates' OBPs are only a small subset of proteins in the large family of lipocalins (Flower 1996; Breustedt et al. 2006). They include not only binding proteins for different types of molecules, such as retinol-binding protein and fatty acid-binding proteins, but also enzymes and even proteins attached to the membrane. The OBPs of insects, therefore, as we know them now, could be only a part of a larger group of proteins performing different functions in insects.

Particularly interesting is the relatively large number of CSPs in the pheromone glands, 6 in total, and their high abundance in the secretion. The occurrence of CSPs in nonsensory organs has been previously reported and in some cases related to a role of pheromone delivery in the environment. Such cases include the moth *M. brassicae* (Jacquin-Joly et al. 2001), where CSPs with binding activity to the sex pheromone have been identified in the female pheromone glands and the fruit fly *D. melanogaster*, where the so-called EjB protein, also a member of the CSP family is produced in the same site as the male sex pheromone vaccenyl acetate

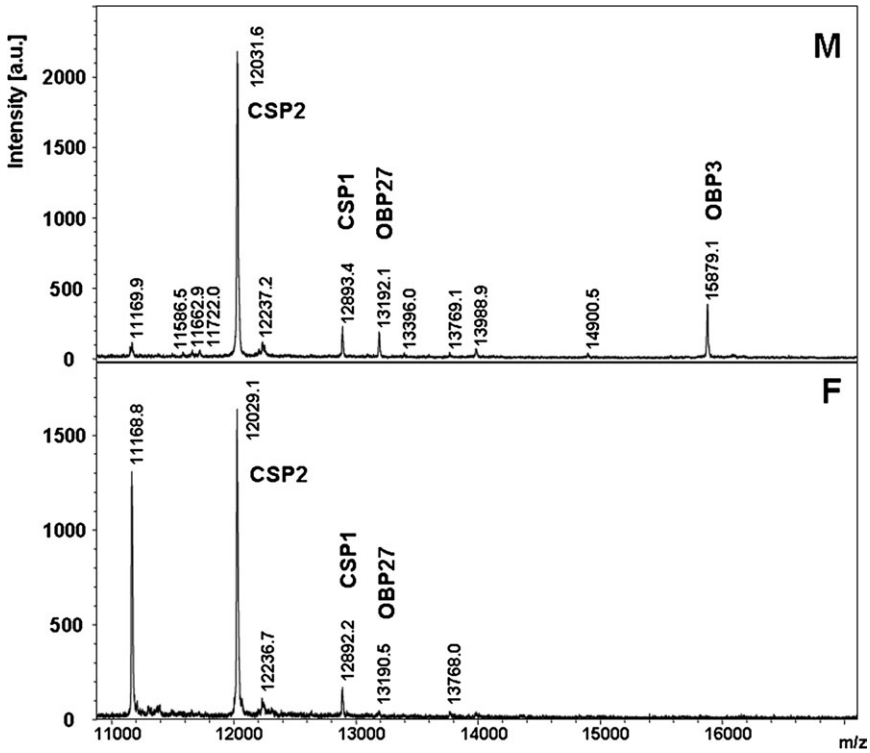

Figure 3 MALDI profiling on antennae of male (M) and female (F) of *Bombyx mori*. The antenna was laid on the MALDI target and the matrix (sinapinic acid) applied across its length. CSPs and OBPs have been identified on the basis of their molecular weight.

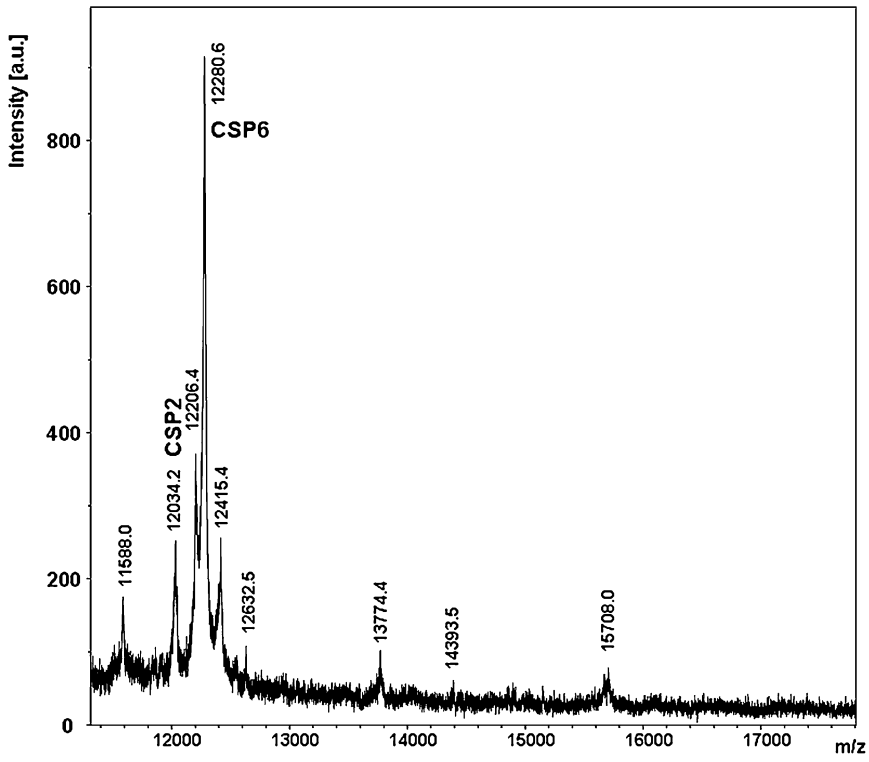

Figure 4 MALDI profiling on female pheromone glands of *Bombyx mori*. Individual glands were laid on the MALDI target and covered with the matrix (sinapinic acid). CSPs and OBPs have been identified on the basis of their molecular weight.

(Dyanov and Dzitoeva 1995). In both cases, a role of pheromone carrier for these proteins has been suggested. In other species, such role could be accomplished by OBPs, as in the mosquito *A. aegypti*, that expresses OBP22 in the male sex organs (Li et al. 2008). In the silkmoth, CSPs seem to be better candidates as pheromone carriers in the pheromone glands, on the basis of their variety and abundance, although we cannot exclude OBP11 from such function that, however, was detected at much lower level. In particular, we can observe that CSP6 (spot #4 in Figure 3) represents one of the most abundant protein in the female pheromone glands and is absent in the antennae of both sexes.

The occurrence of pheromone-binding proteins in both olfactory organs and in pheromone-secreting structures is well documented in mammals. The urinary proteins of mice are very similar or even identical with some OBPs of the nasal tissue (Cavaggioni et al. 1990); the salivary proteins of the boar, carriers of the male pheromones in the saliva, are also expressed in the nose (Marchese et al. 1998) and; the hamster aphrodisin, abundantly secreted in the vaginal fluid, is also a member of the OBP family (Briand et al. 2000). In insects, a similar mechanism seems to be active, where binding proteins function both as receivers and as releasers of the pheromonal messages. However, in insects, the existence of 2 classes of binding proteins, OBPs and CSPs, in the same species, often produces a situation where members of one class are used in detection of semiochemicals and members of the other family in their release. This apparently illogical strategy could be the result of evolution, during which more efficient proteins (OBPs) have replaced CSPs in more critical functions, as in recognizing chemical stimuli, but not in their release, where their role is merely that of a reservoir.

Supplementary material

Supplementary material can be found at http://www.chemse.oxfordjournals.org/.

Funding

Research partly supported by the PRIN Project.

Acknowledgements

Research partly supported by the PRIN Project "Identification and expression analysis of proteins involved in chemical communication in 3 model insects using innovative mass spectrometry technique" (MIUR, Italy). We thank Simona Sagona for assistance in the 2D gel electrophoresis.

References

Angeli S, Ceron F, Scaloni A, Monti M, Monteforti G, Minnocci A, Petacchi R, Pelosi P. 1999. Purification, structural characterization, cloning and immunocytochemical localization of chemoreception proteins from *Schistocerca gregaria*. Eur J Biochem. 262:745–754.

Breustedt DA, Schönfeld DL, Skerra A. 2006. Comparative ligand-binding analysis of ten human lipocalins. Biochim Biophys Acta. 1764:161–173.

Briand L, Huet J, Perez V, Lenoir G, Nespoulous C, Boucher Y, Trotier D, Pernollet JC. 2000. Odorant and pheromone binding by aphrodisin, a hamster aphrodisiac protein. FEBS Lett. 476:179–185.

Butenandt A, Beckmann R, Stamm D, Hecker E. 1959. Über den sexuallockstoff des seidenspinners. *Bombyx mori*: reindarstellung und konstitution. Z Natforsch. 14:283–284.

Calvo E, Mans BJ, Ribeiro JMC, Andersen JF. 2009. Multifunctionality and mechanism of ligand binding in a mosquito antiinflammatory protein. Proc Natl Acad Sci. 106:3728–3733.

Cavaggioni A, Findlay JBC, 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.

Cavaggioni A, Mucignat-Caretta C. 2000. Major urinary proteins, alpha(2U)-globulins and aphrodisin. Biochim Biophys Acta. 1482:218–228.

Dani FR, Francese S, Mastrobuoni G, Felicioli A, Caputo B, Simard F, Pieraccini G, Moneti G, Coluzzi M, Della Torre A, et al. 2008. Exploring proteins in *Anopheles gambiae*male and female antennae through MALDI mass spectrometry profiling. PLoS One. 3:e2822.

Dani FR, Iovinella I, Felicioli A, Niccolini A, Calvello MA, Carucci MG, Qiao H, Pieraccini G, Turillazzi S, Moneti G, et al. 2010. Mapping the expression of soluble olfactory proteins in the honeybee. J Proteome Res. 9: 1822–1833.

Dyanov HM, Dzitoeva SG. 1995. Method for attachment of microscopic preparations on glass for in situ hybridization, PRINS and in situ PCR studies. Biotechniques. 18:822–826.

Flower DR. 1996. The lipocalin protein family: structure and function. Biochem J. 318:1–14.

Forêt S, Maleszka R. 2006. Function and evolution of a gene family encoding odorant binding-like proteins in a social insect, the honey bee (*Apis mellifera*). Genome Res. 16:1404–1413.

Forêt S, Wanner KW, Maleszka R. 2006. Chemosensory proteins in the honey bee: insights from the annotated genome, comparative analyses and expressional profiling. Insect Biochem Mol Biol. 37:19–28.

Forstner M, Thomas G, Breer H, Krieger J. 2006. Candidate pheromone binding proteins of the silkmoth *Bombyx mori*. Invert Neurosci. 6: 177–187.

Gong DP, Zhang HJ, Zhao P, Lin Y, Xia QY, Xiang ZH. 2007. Identification and expression pattern of the chemosensory protein gene family in the silkworm, *Bombyx mori*. Insect Biochem Mol Biol. 37: 266–277.

Gong DP, Zhang HJ, Zhao P, Lin Y, Xia QY, Xiang ZH. 2009. The odorant binding protein gene family from the genome of silkworm, *Bombyx mori*. BMC Genomics. 10:332.

Grosse-Wilde E, Svatos A, Krieger J. 2006. A pheromone binding protein mediates the bombykol-induced activation of a pheromone receptor in vitro. Chem Senses. 31:547–555.

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

Jacquin-Joly E, Vogt RG, Francois MC, 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.

Krieger J, Von Nickisch E, Mameli M, Pelosi P, Breer H. 1996. Binding proteins from the antennae of *Bombyx mori*. Insect Biochem Mol Biol. 26:297–307.

Laughlin JD, Soo TH, Jones DNM, Smith DP. 2008. Activation of pheromone-sensitive neurons is mediated by conformational activation of pheromone binding protein. Cell. 133:1255–1265.

Leal WS, Nikonova L, Peng G. 1999. Disulfide structure of the pheromone binding protein from the silkworm moth, *Bombyx mori*. FEBS Lett. 464:85–90.

Li S, Picimbon J-F, Ji SD, Kan YC, Qiao CL, Zhou J-J, Pelosi P. 2008. Multiple functions of an odorant-binding protein in the mosquito *Aedes aegypti*. Biochem Biophys Res Comm. 372:464–468.

Maida R, Mameli M, Müller B, Krieger J, Steinbrecht RA. 2005. The expression pattern of four odorant-binding proteins in male and female silk moths, Bombyx mori. J Neurocytol. 34:149–163.

Maida R, Proebstl T, Laue M. 1997. Heterogeneity of odorant-binding proteins in the antennae of *Bombyx mori*. Chem Senses. 22:503–515.

Maida R, Steinbrecht RA, Ziegelberger G, Pelosi P. 1993. The pheromone-binding protein of *Bombyx mori*: purification, characterisation and immunocytochemical localisation. Insect Biochem Mol Biol. 23:243–253.

Maleszka J, Foret S, Saint R, Maleszka R. 2007. RNAi-induced phenotypes suggest a novel role for a chemosensory protein CSP5 in the development of embryonic integument in the honeybee (*Apis mellifera*). Dev Genes Evol. 217:189–196.

Marchese S, Pes D, Scaloni A, Carbone V, Pelosi P. 1998. Lipocalins of boar salivary glands binding odours and pheromones. Eur J Biochem. 252: 563–568.

Matsuo T, Sugaya S, Yasukawa J, Aigaki T, Fuyama Y. 2007. Odorant-binding proteins OBP57d and OBP57e affect taste perception and host-plant preference in *Drosophila sechellia*. PLoS Biol. 5:e118.

Pelosi P, Zhou J-J, Ban LP, Calvello M. 2006. Soluble proteins in insect chemical communication. Cell Mol Life Sci. 63:1658–1676.

Pesenti ME, Spinelli S, Bezirard V, Briand L, Pernollet JC, Tegoni M, Cambillau C. 2008. Structural basis of the honey bee PBP pheromone and pH-induced conformational change. J Mol Biol. 380: 158–169.

Picimbon JF. 2003. Biochemistry and evolution of OBP and CSP proteins. In: Blomquist GJ, Vogt RG, editors. Insect pheromone biochemistry and molecular biology. London: Elsevier Academic Press. p. 539–566.

Picimbon JF, Dietrich K, Angeli S, Scaloni A, Krieger J, Breer H, Pelosi P. 2000. Purification and molecular cloning of chemosensory proteins from *Bombyx mori*. Arch Insect Biochem Physiol. 44:120–129.

Popof 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.

Sandler BH, Nikonova L, Leal WS, Clardy J. 2000. Sexual attraction in the silkworm moth: structure of the pheromone-binding-protein-bombykol complex. Chem Biol. 7:143–151.

Scaloni A, Monti M, Angeli S, Pelosi P. 1999. Structural analyses and disulfide-bridge pairing of two odorant binding proteins from *Bombyx mori*. Biochem Biophys Res Commun. 266:386–391.

Tegoni MM, Campanacci VM, Cambillau C. 2004. Structural aspects of sexual attraction and chemical communication in insects. Trends Biochem Sci. 29:257–264.

Vogt RG. 2005. Molecular basis of pheromone detection in insects. In: Gilbert LI, Iatro K, Gill S, editors. Comprehensive insect physiology, biochemistry, pharmacology and molecular biology, volume 3, endocrinology. London: Elsevier. p. 753–804.

Vogt RG, Prestwich GD, Lerner MR. 1991. Odorant-binding-protein subfamilies associate with distinct classes of olfactory receptor neurons in insects. J Neurobiol. 22:74–84.

Wanner KW, Anderson AR, Trowell SC, Theilmann DA, Robertson HM, Newcomb RD. 2007. Female-biased expression of odourant receptor genes in the adult antennae of the silkworm, *Bombyx mori*. Insect Mol Biol. 16:107–119.

Wanner KW, Willis LG, Theilmann DA, Isman MB, Feng Q, Plettner E. 2004. Analysis of the insect OS-Dlike gene family. J Chem Ecol. 30:889–911.

Wogulis M, Morgan T, Ishida Y, Leal WS, Wilson DK. 2006. 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.

Xia Q, Zhou Z, Lu C, Cheng D, Dai F, Li B, Zhao P, Zha X, Cheng T, Chai C. 2004. A draft sequence for the genome of the domesticated silkworm (*Bombyx mori*). Science. 306:1937–1940.

Xia QY, Cheng DJ, Duan J, Wang GH, Cheng TC, Zha XF, Liu C, Zhao P, Dai FY, Zhang Z, et al. 2007. Microarray-based gene expression profiles in multiple tissues of the domesticated silkworm, *Bombyx mori*. Genome Biol. 8:R162.

Xu PX, Atkinson R, Jones DN, Smith DP. 2005. *Drosophila* OBP LUSH is required for activity of pheromone-sensitive neurons. Neuron. 45: 193–200.

Xu PX, Zwiebel LJ, Smith DP. 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.

Zhou J-J, Huang W, Zhang GA, Pickett JA, Field LM. 2004. 'Plus-C' odorant-binding protein genes in two *Drosophila* species and the malaria mosquito *Anopheles gambiae*. Gene. 327:117–129.