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Chemosensory Proteins from the Proboscis of *Mamestra brassicae*

P. Nagnan-Le Meillour, A.H. Cain, E. Jacquin-Joly, M.C. François, S. Ramachandran[1], R. Maida[1] and R.A. Steinbrecht[1]

INRA, Unité de Phytopharmacie et des Médiateurs Chimiques, Bâtiment A, Route de Saint-Cyr, F-78026 Versailles Cedex, France and [1]Max-Planck-Institut für Verhaltensphysiologie, D-82319 Seewiesen, Germany

Correspondence to be sent to: P. Nagnan-Le Meillour, INRA, UPMC, Bâtiment A, Route de Saint-Cyr, F-78026 Versailles Cedex, France. e-mail: nagnan@versailles.inra.fr

Abstract

Soluble, low molecular weight proteins were immunodetected in proboscis extracts of *Mamestra brassicae* males by Western blot, using antibodies raised against the general odorant-binding protein of the moth *Antheraea polyphemus*. The same antibodies weakly labelled the sensillum lymph and subcuticular space of sensilla styloconica on ultrathin sections of the proboscis. The morphology of sensilla styloconica is described. The immunodetected proteins yielded several N-terminal sequences, three of which showed strong affinity for tritiated analogues of pheromonal compounds of *M. brassicae* in binding assays. The cDNAs coding for these sequences were cloned and it was shown that the new proteins are related to the OS-D protein of *Drosophila*. They are named chemosensory proteins (CSP-*Mbra*A1–CSP-*Mbra*A5 and CSP-*Mbra*B1 and CSP-*Mbra*B2) and may have an odorant-binding protein-like function. A common localization in both olfaction and taste organs suggests a physiological role depending on the cellular environment.

Introduction

Insects perceive the chemical cues of their environment by different chemosensory organs. In particular, the proboscis is important in host recognition for oviposition for females and in food uptake for both sexes. The proboscis consists of two elongated galea which originate on basal maxillary structures. This structure, elastic, deformable and capable of extention and recoil, is a sense organ equipped with chemoreceptors and mechanoreceptors.

The sensilla of the lepidopteran proboscis are usually classified into sensilla trichodea, sensilla basiconica and sensilla styloconica, the latter showing an amazing variety of sizes and shapes (Städler *et al.*, 1974; Sellier, 1975; Faucheux, 1991; Büttiker *et al.*, 1996; Paulus and Krenn, 1996). Although the general morphology of the proboscis in Lepidoptera is well known (Eastham and Eassa, 1955), the function of proboscis sensillar organs of adult Lepidoptera have been investigated in only a few species. Most studies used only scanning or transmission electron microscopy (Krenn, 1998; Walters *et al.*, 1998), histology (Städler *et al.*, 1974) or sometimes electrophysiological recordings (Städler and Seabrook, 1975) to make functional considerations. Although the proboscis is first thought of as a taste and food uptake organ, some authors found evidences for both gustatory and olfactory functions (Altner and Altner, 1986). In fact, these authors described sensilla with both a terminal pore and wall pores, which characterize sensilla housing chemoreceptors which react to volatile stimuli, on the proboscis of *Rhodogastria bubo*. Although no electrophysiological experiments were done on this species to support this hypothesis, Städler and Hanson (Städler and Hanson, 1975) were able to demonstrate that receptors on the maxillae of *Manduca sexta* larvea which were classified as contact chemoreceptors due to their structure were responsive to vapours of normal food substances.

The data accumulated on the proboscis establish this organ as a chemosensory organ with an important behavioural role. In this context, we propose exploring the underlying biochemistry by identifying and characterizing proboscis proteins in the cabbage armyworm *Mamestra brassicae*. In another chemosensory organ, the antenna, we have previously characterized odorant-binding proteins (OBPs) specifically expressed in male and female antennae of this species (Nagnan-Le Meillour *et al.*, 1996). The genes coding for three OBPs, MbraPBP1, MbraPBP2 and MbraGOBP2, were cloned (Maïbèche-Coisné *et al.*, 1998a,b). A binding assay with purified proteins has shown that MbraPBP1 specifically binds a tritiated analogue of the major pheromonal component, *cis*-11-hexadecenyl acetate (Z11–16:Ac), in contrast to MbraPBP2, the specific ligand of which remains unknown (Maïbèche-Coisné *et al.*, 1997). Differences in the full-length sequences appear to be linked to the binding selectivity of MbraPBP1 and MbraPBP2

(Maïbèche-Coisné *et al.*, 1998b), supporting the hypothesis of Du and Prestwich (Du and Prestwich, 1995) that the primary structure encodes the ligand binding specificity in pheromone-binding proteins.

In this paper we report a similar approach which allowed the characterization of soluble proteins from the proboscis of *M. brassicae* males that could be referred to as a new type of OBPs.

Materials and methods

Insects

Mamestra brassicae were reared in Le Domaine du Magneraud (INRA, France) on a semi-artificial diet (Poitout and Bues, 1974) at 20°C with a 16 h/8 h light/dark photoperiod. Males and females were sexed as pupae.

Antisera

Polyclonal antisera generated against PBP and GOBP2 of *Antheraea polyphemus* (anti-ApolPBP and anti-ApolGOBP2) (Steinbrecht *et al.*, 1992, 1995) were used in immunocytochemistry and immunoblotting.

Pheromone analogues

The synthesis of tritiated analogues of Z11–16:Ac (Maïbèche-Coisné *et al.*, 1997) and of both *cis*-11-hexadecenol (Z11–16:OH) and hexadecanyl acetate (16:Ac) have been previously reported (Bohbot *et al.*, 1998). The tritiated analogue of *cis*-11-octadecenyl acetate (vaccenyl acetate, Z11–18:Ac) was a gift of Dang Ba Pho (Université Paris XI). The specific activity of tritiated compounds was 1.7 TBq/mmol for [^{3}H]Z11–16:Ac, 1.9 TBq/mmol for [^{3}H]Z11–16:OH, 0.8 TBq/mmol for [^{3}H]Z11–18:Ac and 10.3 TBq/mmol for [^{3}H]16:Ac. For simplicity, the tritiated analogues are refered to as the cold molecules in this paper (e.g. Z11–16:Ac refers to [^{3}H]Z11–16:Ac).

Morphology and immunocytochemistry

For scanning electron microscopy, proboscis tips were fixed in modified Carnoy, dehydrated and air dried from hexamethyldisilazane (Bock, 1987). After sputtering in a Bal-Tec SCD 050 sputter coater with 6 nm of platinum, the specimens were studied in a Jeol JSM 6300F scanning electron microscope equipped with a field emission gun.

Excised proboscis tips were fixed on a thin tungsten wire and cryofixed by plunging into super-cooled propane (–180°C). Freeze-substitution was done in acetone containing 2% osmium tetroxide for morphology and in acetone containing 3% glutaraldehyde (water-free by molecular sieving) for immunocytochemistry in a home-made substitution apparatus with a programmable temperature/time protocol (Marshall and Kent, 1991). For morphology, specimens were embedded in Epon, for immunocytochemistry, the acrylic resin LR White (London Resin) was used. For details of specimen preparation see Steinbrecht and co-workers (Steinbrecht, 1993; Steinbrecht *et al.*, 1995). Ultrathin sections were cut with a diamond knife on Reichert OMU2 or Ultracut ultramicrotomes and mounted on formvar-coated single hole copper grids.

For immunocytochemistry, the following blocking solutions were used: PBS–glycine [50 mM glycine in phosphate-buffered saline (PBS)] and PBGT (PBS containing 0.05–0.2% gelatin, 0.5–1% bovine serum albumin and 0.01–0.1% Tween 20). The primary antisera, anti-ApolPBP and anti-ApolGOBP2, were used diluted 1:500 to 1:3000 in PBGT; the secondary antibody was anti-rabbit IgG coupled to 10 nm colloidal gold (Amersham Buchler, Braunschweig), diluted 1:20 in PBGT. As a control, the primary antiserum was replaced by pre-immune serum at dilutions between 1:100 and 1:1000. The labelling protocol was as described by Steinbrecht *et al.* (Steinbrecht *et al.*, 1995). Silver intensification according to Danscher (Danscher, 1981) for 15 min under reduced daylight enlarged the gold particules to ~40 nm for observation at low magnification. Sections were stained for 10 min in 2% uranyl acetate and investigated in a Zeiss EM 10A electron microscope at 60 or 80 kV.

Preparation of samples

Probosces and antennae were cut within 2 days after emergence and stored at –25°C until use. Probosces or antennae were crushed on ice by hand in 1% trifluoroacetic acid (TFA), sonicated for 10 min, then centrifuged (11 000 *g* for 15 min at 4°C). Supernatants were filtered by centrifugation (Z-spin, 0.2 µm; Gehlman Sciences), then evaporated under vacuum (Savant Speed-Vac) and stored at –70°C until use.

Protein purification

Extracts of 1400 male probosces for each purification were analyzed by reverse phase HPLC (RP-HPLC) in a Varian device (9012 pump, 9100 sampler, 9065 diode array detector). Proteins were separated on a 4.6 × 100 mm Aquapore RP-300 cartridge (Perkin Elmer), in 25 mM ammonium acetate, pH 7.2 (buffer A) and 50 mM ammonium acetate, pH 7.2 (buffer B), using an acetonitrile linear gradient (5–50% acetonitrile, flow rate 0.8 ml/min). Fractions were collected, lyophilized and analysed either by native-PAGE or by binding assay.

Electrophoresis, Western blot and binding assay

For Western blotting, proteins of total extracts were separated by native-PAGE (14%) then electrotransferred onto Immobilon P membranes, according to the semi-dry blotting procedure of Kyhse-Andersen (Kyhse-Andersen, 1984). Immobilon membranes were blocked overnight at 4°C with 5% dry milk in TBS/Tween 20 (0.1%), then incubated for 4 h with either anti-ApolPBP serum or anti-ApolGOBP2 serum at a 1:1000 dilution and room temperature. Bound antibodies were detected by goat anti-rabbit IgG coupled to horseradish peroxidase (dilution 1:10 000) for 2 h at room

temperature. The signal was revealed using an Enhanced ChemiLuminescence kit (ECL; Amersham) following the manufacturer's protocol.

After HPLC, the resulting fractions were analyzed by electrophoresis under native conditions as previously described (Nagnan-Le Meillour *et al.*, 1996) and the gels stained with a colloidal Coomassie blue R solution (Nagnan-Le Meillour *et al.*, 1996). For the binding assay, lyophilized crude extracts or purified proteins were resuspended in 15 µl electrophoresis sample buffer, then incubated with 5 µl (1 µCi) of the tritiated analogues of pheromonal compounds for 30 min on ice. The samples were subjected to native electrophoresis and electroblotted overnight (80 mA, constant current) onto ProBlott membranes (Perkin Elmer). The membranes were treated for fluorography (30 min in 7% formaldehyde, 60 min in 1 M salicylic acid), dried at room temperature and exposed to Hyperfilm MP (Amersham) for 6 days. After this first exposure, the membranes were again treated for fluorography and re-exposed for 21 days to enhance the signal. Membranes were stained with Ponceau red (0.2% in 1% acetic acid) in order to precisely associate the radioactivity with the binding protein. These bands were finally excised for N-terminal sequencing. The entire procedure (purification and binding assays) was repeated three times.

N-terminal sequences were obtained by J. d'Alayer (Institut Pasteur, France) with a gas phase microsequencer (Applied Biosystems) and reagents and methods as provided by the manufacturer.

Molecular cloning and sequencing

RNA extraction and cDNA synthesis

Total RNA was extracted from 200 probosces of adult males with the Tri-Reagent (Euromedex). Single-stranded cDNA was synthesized from 1 g of total RNA with M-MLV (US Biochemical), using the buffer and protocol supplied with the enzyme. The reaction mixture contained dNTP mix (Pharmacia), RNasin (Promega), oligo$(dT)_{18}$ with an anchor (CATGCATGCGGCCGCAAGCT_{18}VN, synthesized by Isoprim, Toulouse, France), sterile water and template RNA to a final volume of 50 µl. The mix was heated to 68°C for 5 min and then chilled on ice before adding the M-MLV (600 U), incubated for 1 h at 37°C and finally the reverse transcriptase was inactivated at 95°C for 5 min.

3′-RACE–PCR

Approximately 1 ng of cDNA was used for PCR. The sense primers were designed based on protein N-terminal sequences: GAGGACAAGTACACIGAYAAGTACGA for the protein EDKY and GAGGAGGCICAYTAYACIGAYCG for the protein EEAH. Both primers were synthesized by Isoprim. These primers were used in a pair with the primer used for cDNA synthesis [anchor–oligo$(dT)_{18}$] in order to perform the 3′-RACE. PCRs were carried out with *Taq* polymerase (1 U) (Promega) in 10 mM Tris–HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM each dNTP. Forty amplification cycles were performed with an annealing temperature of 60°C for EDKY and 55°C for EEAH.

Cloning and sequencing

The amplified cDNAs were purified after agarose electrophoresis using GenElute (Supelco) and ligated into the plasmid pCR-II using the TOPO cloning kit from Invitrogen. After transformation, positive clones were digested with *Eco*RI (Biolabs) to screen for presence of the insert. Recombinant plasmids were then isolated using a Plasmid Midi kit from Qiagen and were subjected to automated sequencing with vector primers (T7 and M13 promoters) by ESGS (Evry, France). For each amplification, several clones were sequenced. Database searches were performed with the BLAST program (NCBI) and sequence alignment with ClustalW (nps@ibcr).

Results

Morphology and immunolocalization

The proboscis of *M. brassicae* follows the general plan of the lepidopteran proboscis consisting of two greatly elongated and interlocked galeae forming the tube-shaped food canal. A variety of sensilla are found, particularly on the distal end of the proboscis, of which the sensilla styloconica are the largest and most conspicuous (Figure 1A). Their mean number per galea is 121 ± 20 (n = 15), with no significant differences between the sexes.

The sensilla styloconica consist of a small cone (the sensillum proper) that is located on a long stylus with conspicuous longitudinal ridges (Figure 1B,C). High resolution scanning electron microscopy revealed a complex terminal pore and additional wall pores (Figure 1D,E). Wall pores were also evident on transverse sections through the cone (Figure 3A). Three receptor neurons send their dendrites into the cone where they are surrounded by a dense dendrite sheath up to the terminal pore, while the fourth ends with a tubular body at the base of the cone (Figures 2 and 3A); the receptor cell somata are located below the base of the stylus within the proboscis. Three auxiliary cells are observed: the innermost thecogen cell continues distally with a very dense dendrite sheath surrounding the outer dendritic segments; trichogen and tormogen cells border a large sensillum lymph cavity which apically fills the sensillar cone and extends basally with two deep pouches below the base of the stylus (Figure 2). The wall of the stylus is bordered by two epidermal cells, which are not closely attached to the cuticle but leave a subcuticular space (Figures 2 and 3C); this subcuticular space increases in volume towards the stylus base.

There are also sensilla basiconica on the proboscis of *M. brassicae*, which are smaller and more dispersed than

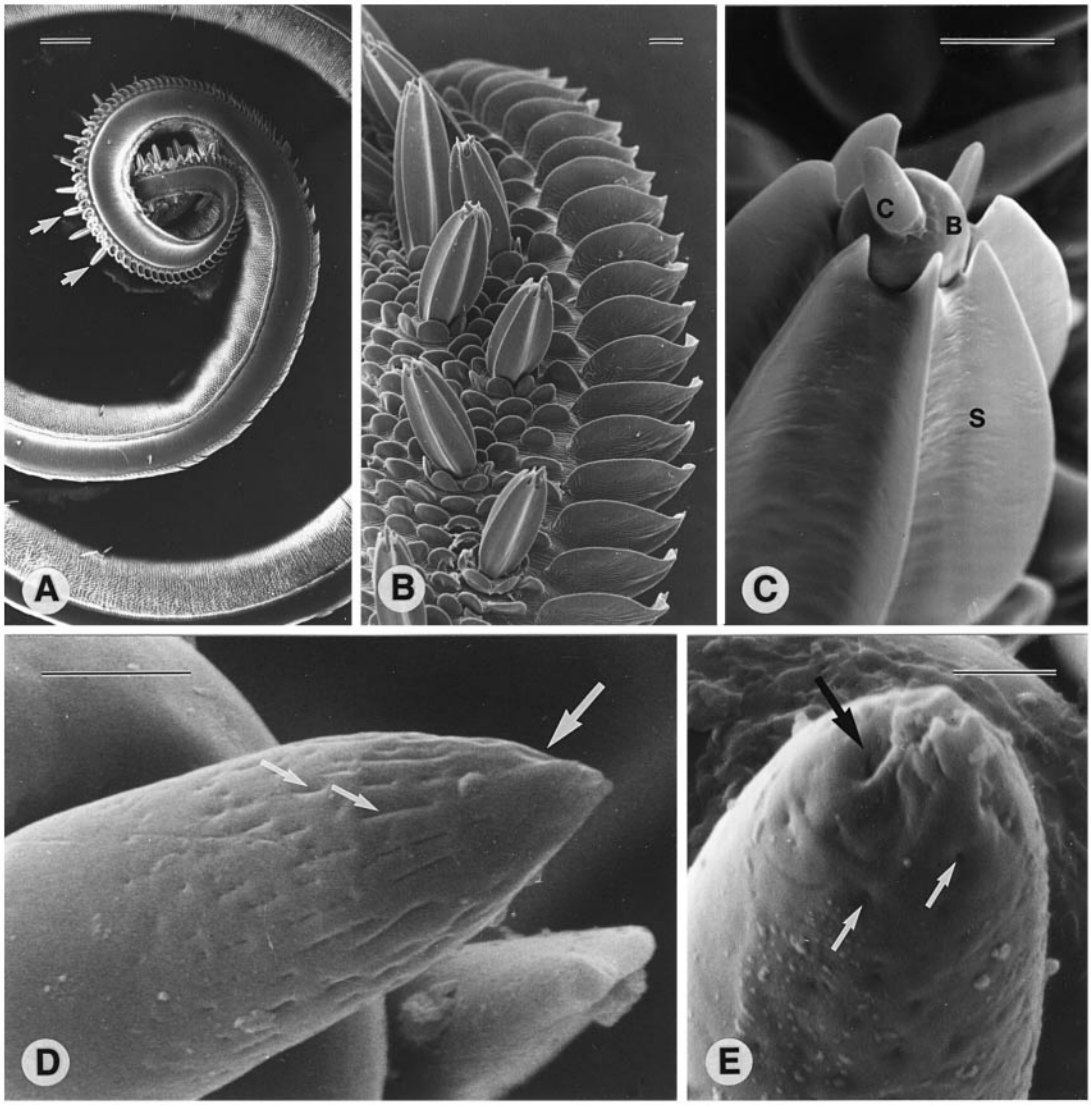

Figure 1 Proboscis of *M. brassicae* with sensilla styloconica as seen in the scanning electron microscope. **(A, B)** The sensilla styloconica are found only on the most distal parts of the galeae, where they occur in high density (arrows in A). **(C)** A single styloconic sensillum at higher magnification. The sensillum proper, the cone (*C*), emerges from a ball-shaped socket structure (*B*) on top of a large stylus (*S*) with characteristic longitudinal ridges. **(D, E)** The cone has a complex terminal pore (thick arrow) as well as round or slit-shaped wall pores (thin arrows). Scale bars 100 m in (A), 10 m in (B), 3 m in (C), 1 m in (D), 100 nm in (E). [Micrographs (A) and (C) by D. Tauban, (B), (D) and (E) by C. Bock.]

the sensilla styloconica. They were encountered too rarely in this study to permit definitive morphological classification.

Labelling with anti-ApolPBP serum was too weak to give a signal different from background anywhere on our sections, suggesting a total absence of PBPs in the proboscis. Upon labelling with anti-ApolGOBP2, gold particles were found in all sensillum lymph compartments of the sensillum, i.e. in the distal peg around the outer dendritic segments inside and outside of the dendrite sheath (Figure 3C) and in the two deep sensillum lymph cavities of the trichogen and tormogen cell at more proximal levels (Figure 3B). In addition to labelling of the sensillum lymph, we also consistently found gold particles in the subcuticular space between epidermal cells and the cuticle of the stylus (Figure 3B,C). However, gold particles at lower density were also found in other tissue compartments, e.g. cuticle, haemolymph, the cytoplasm of dendrites and various cells (Figure 3B). These with all likehood represent non-specific background (see Discussion).

Compared to the labelling of sensilla basiconica of *Antheraea polyphemus* with anti-ApolGOBP2, which resulted in grain densities of 100 particles/m^2 with a serum dilution of 1:9000 (Laue *et al.*, 1994) (unpublished data), sensilla styloconica on the proboscis of *M. brassicae* showed on average only 3 ± 1.5 gold particles/m^2 (n = 17) at a serum dilution of 1:3000 (Figure 3C). With higher serum

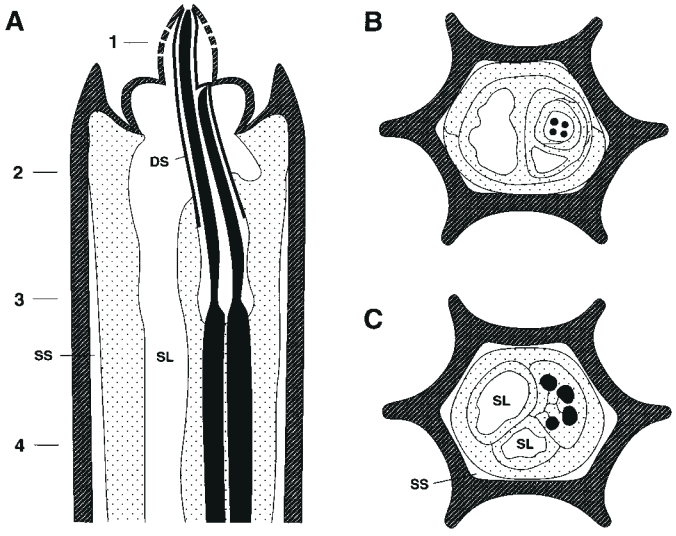

Figure 2 Schematic drawing of sensilla styloconica on the proboscis of *M. brassicae*. Sensory dendrites are drawn in black, auxilliary and epidermal cells are stippled and the cuticle hatched. **(A)** A longitudinal section of the distal stylus with a porous sensory cone. Only two of the four sensory dendrites are drawn; one ends at the base of the cone, the other terminating below the terminal pore; all are surrounded by a dense dendrite sheath (DS). Cell membranes between auxilliary cells and epidermal cells are omitted [for details see (B) and (C)]. Note extensive sensillum lymph cavity (SL) and narrow cuticular space (SS). Numbers indicate cross-sectional levels: 1, level of Figure 3A; 2, level of Figure 3C; 3, level of Figure 2B; 4, level of Figures 2C and 3B. **(B, C)** Cross-sections at the level of the ciliary and inner dendritic segments, respectively. Note the complex arrangement of the three auxilliary cells surrounding the dendrites, which in turn are surrounded by a ring of epidermal cells. The two sensillum lymph cavities of tormogen and trichogen cells merge at the distal end of the stylus (Figure 3C), in the proximal direction they continue below the basis of the stylus where the perikarya of all sensillar cells are located (not shown).

concentrations the labelling density increased, but at the same time the background also increased.

Sensilla basiconica were encountered too rarely to decide whether they were specifically labelled. No compartment in sensilla or elsewhere on the proboscis tip was found that labelled stronger with anti-ApolGOBP2 than sensillum lymph or the subcuticular space in sensilla styloconica.

Identification of proboscis soluble proteins

Proboscis homogenates of *M. brassicae* were electrophoretically compared with homogenates of male antennae and legs. The electrophoretic pattern of proboscis extracts after separation of proteins by native-PAGE was comparable with that of antennal extracts, while the more acidic bands were missing in legs (see arrows in Figure 4A). In western blots using anti-ApolGOBP2 serum, two bands were labelled in antennae and proboscis extracts and one band in the leg extract (Figure 4B). Only one band corresponding to the MbraPBPs in antennal extract (Bohbot *et al.*, 1998) was labelled by anti-ApolPBP serum (Figure 4C). The bands labelled with anti-ApolGOBP2 serum were submitted to N-terminal sequencing. In antennae, proboscis and legs, the upper band contains the major sequence EDKY mixed with minor sequences. In antennae, the lower band contains the TAEVM sequence of MbraGOBP2, as previously determined (Nagnan-Le Meillour *et al.*, 1996; Bohbot *et al.*, 1998). In the proboscis, the lower band contains a mixture of two sequences, DAPAASPLQDIEKHA (82%) and SEEDKAAFEEAAEPI (18%).

We tested the affinity between homogenates of proboscis and different tritium-labelled compounds in binding assays. The upper band detected with anti-ApolGOBP2 serum (EDKY sequence) was labelled by the four tritiated analogues of pheromones (Figure 4D). To better determine the binding specificity and assignment of the different bands we purified the proteins from the proboscis homogenates.

Purification of proboscis soluble proteins and their affinity for pheromone analogues

An homogenate of 1400 probosces was split into seven samples corresponding to 200 proboscis equivalents. The chromatograms obtained with the seven samples were identical with respect to peak retention times. The fractions corresponding to the same retention time were pooled for further analysis. Fractions between 0 and 35 min and 55 and 80 min were checked by native-PAGE and binding assay; they did not contain proteins of interest (data not shown). Figure 5 shows the chromatogram of fractions from 36 to 54 min, numbered 1–19, and the corresponding Coomassie stained native gel.

The proteins collected in the 19 fractions were tested in binding assays with the four pheromone analogues as above (Figure 6). Positive binding was observed not only in fractions 13 and 14, containing the EDKY sequence (band D), as already found in total extracts, but also in fractions 1–3, 6 and 7. In fraction 1, band A has strong affinity for 16:Ac (Figure 6B), Z11–16:OH (Figure 6C) and Z11–18:Ac (Figure 6D), but has no affinity for Z11–16:Ac (Figure 6A). Band A in fraction 2 binds all four pheromone analogues. The same band A in fraction 3 has strong affinity for both 16:Ac (Figure 6B) and Z11–18:Ac (Figure 6D). Band B in fraction 6 has affinity for 16:Ac, Z11–16:OH and Z11–18:Ac, but no affinity for Z11–16:Ac. Band B in fraction 7 binds 16:Ac and Z11–18:Ac. Band C and an additional band (B′) just above band B contained in the same fraction bind only Z11–18:Ac.

Identification of proboscis soluble proteins by N-terminal sequencing

Bands B and C from fractions 6 and 7 contain the same N-terminal sequence, EEAHYTDRYDNVDLDEILGN. Band D collected in fractions 13 and 14 contains the pure N-terminal sequence EDKYTDKYDNINLDEILANK. The chromatogram (Figure 5) indicates that in each case (fractions 6, 7, 13 and 14) the two sequences could correspond either to isoforms sharing the same N-terminus or to the same protein eluted in two fractions. In addition,

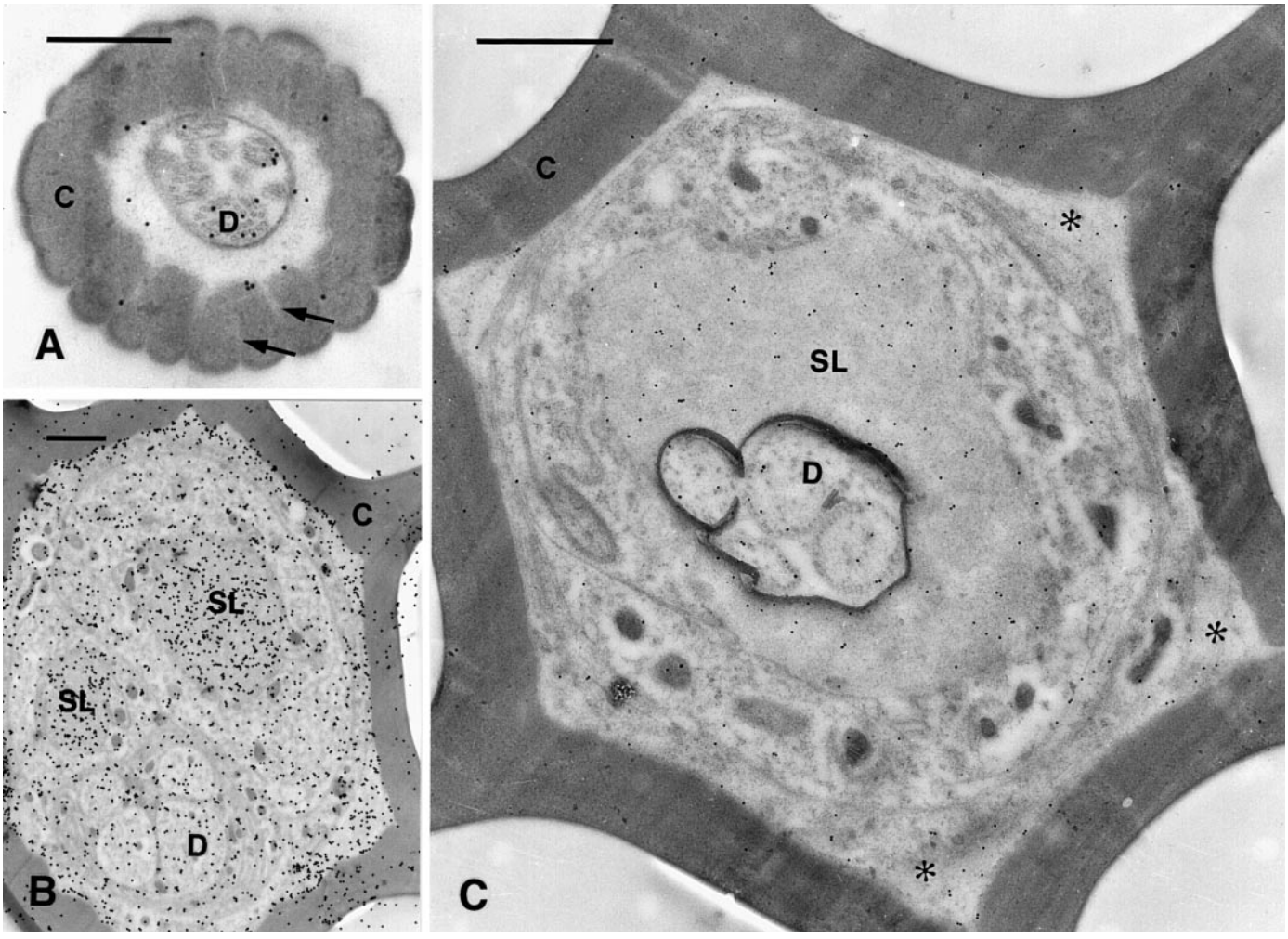

Figure 3 Transmission electron micrographs of immunolabelled sections of sensilla styloconica of *M. brassicae*. **(A)** Cross-section through cone close to tip. Several dendritic branches (*D*) are surrounded by a dendrite sheath. The cuticular wall (*C*) of the cone exhibits wall pores (arrows). This section was incubated with pre-immune serum (1:100); only a few gold particules are visible, mostly on the cuticule or dendrites. **(B, C)** Cross-sections through a stylus labelled with anti-ApolGOBP2. (B) is taken at a more proximal level than (C) (cf. Figure 2A), the dendrites (*D*) are surrounded by auxilliary cells and there are two sensillum lymph cavities (*SL*), while in (C) the dendrites are surrounded by a dense dendrite sheath and a common sensillum lymph space. At high serum concentrations [(B) 1:300], a high non-specific background prevails, nevertheless, the sensillum lymph (*SL*) and the space immediately below the cuticle (*C*) exhibit higher labelling density. At low serum concentrations [(C) 1:3000] the labelling of sensillum lymph (*SL*) and subcuticular spaces (asterisks) mostly remains, albeit at low density. In (B) the gold particules are enlarged by silver intensification. Scale bars 0.5 m in (A), 1 m in (C).

binding between fraction 7 and Z11–18:AC resulted in one more labelled band (B′) above band B, corresponding to the same N-terminus EEAHY (Figure 6D). In fraction 7, no differences in the three primary sequences of bands B, C and B′ could be observed in the 20 first N-terminal residues (but see Discussion).

N-terminal sequencing of the band A in fractions 1–3 gave the pure sequence AKLTTEELQMLEAFD, which was not immunodetected by anti-ApolGOBP2 serum. The other proteins contained in HPLC fractions gave negative binding and were not further studied here.

Cloning and cDNA sequencing

3′-RACE–PCR amplifications were performed using degenerate primers corresponding to the determined N-terminal sequences of the proteins together with the anchor–oligo(dT). Products of 650 and 480 bp were obtained using the EDKY primer and the EEAH primer, respectively. After cloning and sequencing, we obtained seven different sequences, five of which encoded 112 amino acids with the N-terminal sequence EDKY, two of which encoded 108 amino acids with the N-terminal sequence EEAH. These proteins were named CSP-*Mbra*A1–CSP-*Mbra*A5 (EDKY N-terminal sequence) and CSP-*Mbra*B1 and CSP-*Mbra*B2 (EEAH N-terminal sequence), for chemosensory proteins, according to Angeli *et al.* (Angeli *et al.*, 1999).

Nucleotide sequences have been deposited in the GenBank database with accession nos AF211177–AF211183 for CSP-*Mbra*A1–CSP-*Mbra*A5 and CSP-*Mbra*B2 and CSP-*Mbra*B2. Figure 7 shows the alignment of amino acid

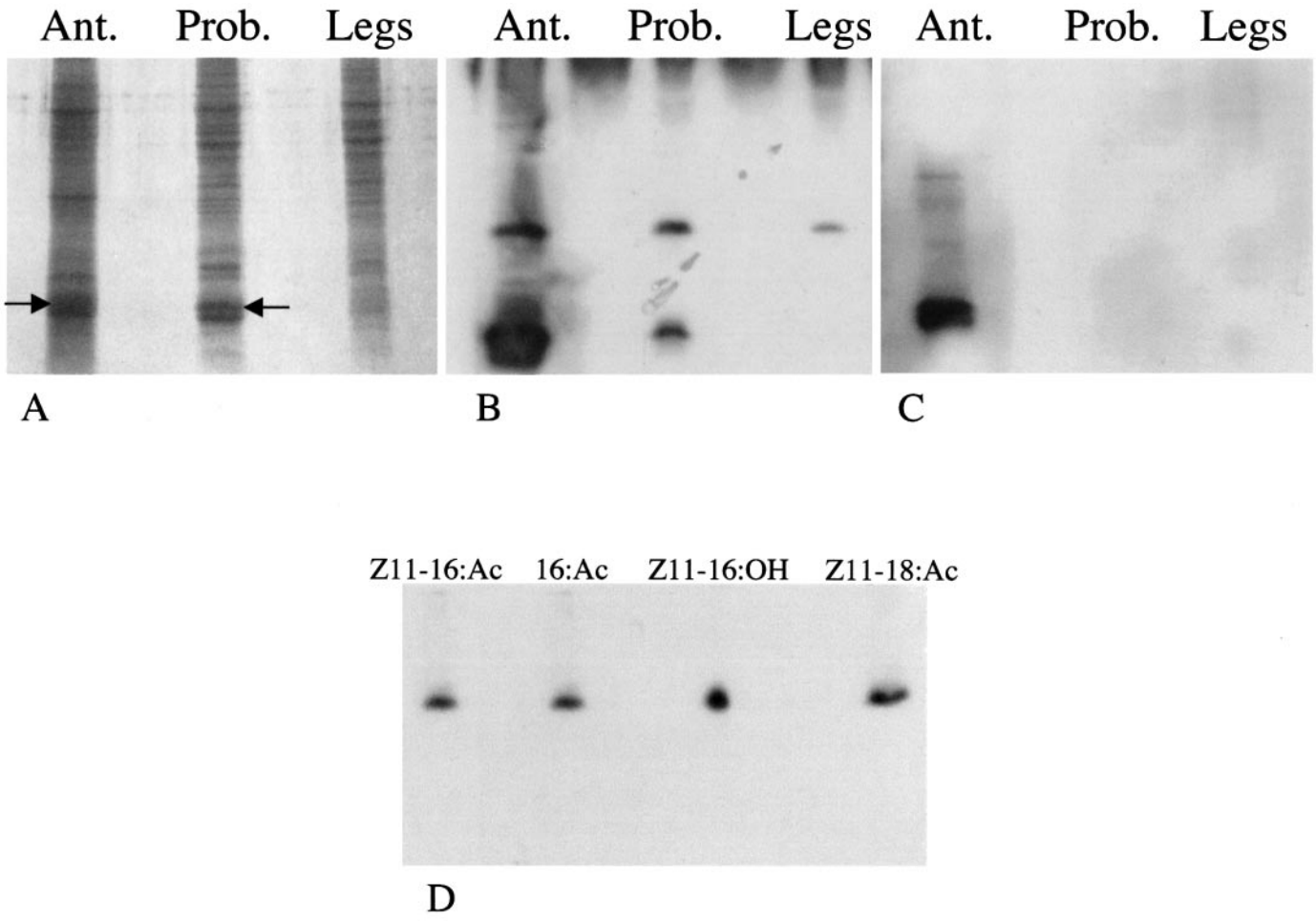

Figure 4 Immunodetection of soluble proteins and binding. **(A)** Coomassie blue staining of extracts of antennae (15 antennae equivalent), probosces (30 males equivalent) and legs (3 legs equivalent). **(B)** ECL detection (10 min exposure) of proteins using antisera raised against ApolGOBP2. **(C)** ECL detection (10 min exposure) of proteins using ApolPBP. **(D)** Binding assay: each well contains an extract of 100 male proboscis equivalents and 1 Ci of one of the four tritiated analogues (Z11–16:Ac, 16:Ac, Z11–16:OH or Z11–18:Ac); 7 days exposure at –70°C.

sequences deduced from the seven clones. Theoretical molecular masses and isoelectric points were calculated using MWCALC (Infobiogen). They range between 12 665 and 13 115 Da for molecular masses and between 5.34 and 6.66 for pI. The full-length sequences revealed four cysteines in conserved positions in the seven protein sequences. These could be involved in two disulphide bridges, as has been shown for the CSP of *Schistocerca gregaria* (Cys57–Cys60 and Cys29–Cys38) (Angeli *et al.*, 1999). The five deduced amino acid sequences CSP-*Mbra*A matched the first 20 amino acids of these proteins as determined by Edman degradation. They differed from each other by only 1–3 residues (99–97% identity). CSP-*Mbra*B2 differed from the sequence obtained by Edman degradation by 1 residue, whereas CSP-*Mbra*B1 matched totally with the N-terminal sequence. CSP-*Mbra*B1 and CSP-*Mbra*B2 differed from each other by 18 residues (83.3% identity). Identity percentages between the different CSP-*Mbra*A and CSP-*Mbra*B varied around 50%.

Discussion

Morphology of proboscis sensilla and localization of proboscis soluble proteins

The sensilla styloconica on the proboscis of *M. brassicae* in their fine structure closely resemble those on the proboscis of *R. bubo* (Arctiidae) (Altner and Altner, 1986), *Choristoneura fumiferana* (Tortricidae) (Walters *et al.*, 1998) and *Vanessa cardui* (Nymphalidae) (Krenn, 1998). Common features are the prominent stylus, the terminal pore at the tip of the cone, which is innervated by dendrites from three receptor cells, and the existence of a fourth mechanoreceptive neuron ending with a tubular body at the base of the cone. Thus, the general morphology of these proboscis sensilla in Lepidoptera apparently follows a common plan.

There are, however, a few striking differences in sensillar number and fine structure. While the counts of sensilla styloconica/galea were only 35 and 28 in *Choristoneura* and *Vanessa*, respectively, these were 102 in *Rhodogastria* and

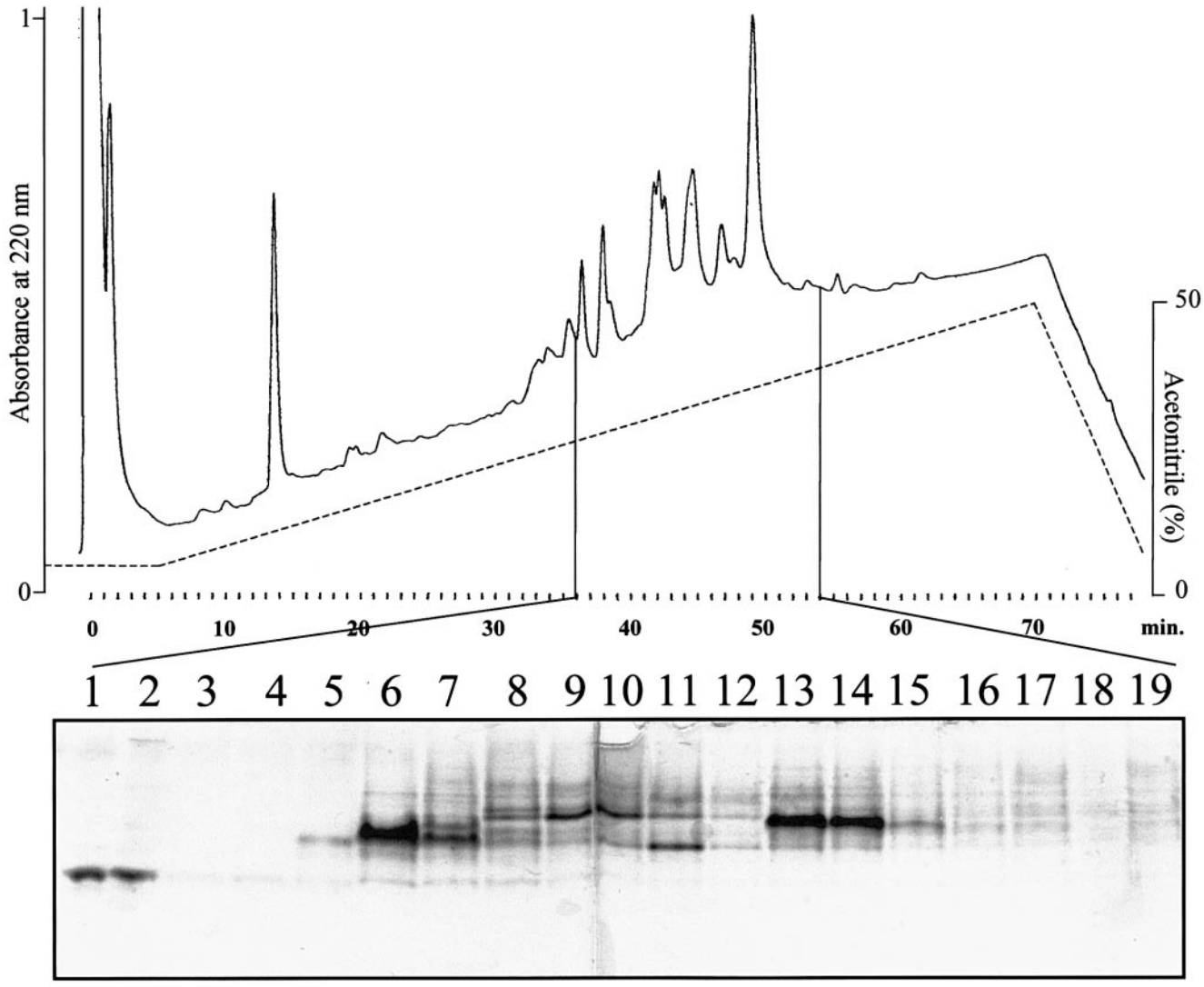

Figure 5 Purification of proteins from the proboscis by RP-HPLC. **(Top)** Chromatogram of proboscis extract (200 male equivalent) (see Materials and methods). **(Bottom)** Coomassie stain of native-PAGE of the 19 fractions collected between 36 and 54 min after RP-HPLC. The linear gradient of acetonitrile is indicated by the hatched line.

121 in *Mamestra*. Moreover, a subset of sensilla styloconica in *Rhodogastria* was reported to display wall pores in addition to the terminal pore. Such wall pores were also shown in the present study but were not found in *Choristoneura* or *Vanessa*. In *Mamestra*, as in *Rhodogastria*, two subtypes of sensilla styloconica might occur (Figure 1D,E), however, our scanning electron micrographs indicate wall pores in both subtypes (Figure 1D,E). The presence of wall pores and the tendency of one of the outer dendritic segments to form branches were discussed by Altner and Altner (Altner and Altner, 1986) as hinting at an olfactory function of these proboscis sensilla in addition to contact chemoreception. Unfortunately, to our knowledge this was never confirmed by electrophysiological experiments in this or any other lepidopteran species.

The weak labelling of proboscis sensilla of *Mamestra* with antisera against OBPs of *A.polyphemus* was expected from the western blots, which necessitated long exposure times and extremely sensitive detection methods. Anti-ApolPBP did not produce even weak labelling, while anti-ApolGOBP2 produced a signal in the sensillum lymph of sensilla styloconica of *Mamestra* that hardly exceeded the background. It should be mentioned that the antisera used were not previously affinity purified, as such purification often results in loss of the most strongly binding antibodies of the serum. Such crude sera are very specific, if used at high dilutions (1:>1000). At low dilutions, they may become rather non-specific and the distinction between specific signal and non-specific background is difficult. Pre-immune sera can give helpful information about non-specific binding only if applied at much higher concentrations than the antisera, in order to compensate for their lower titer. The

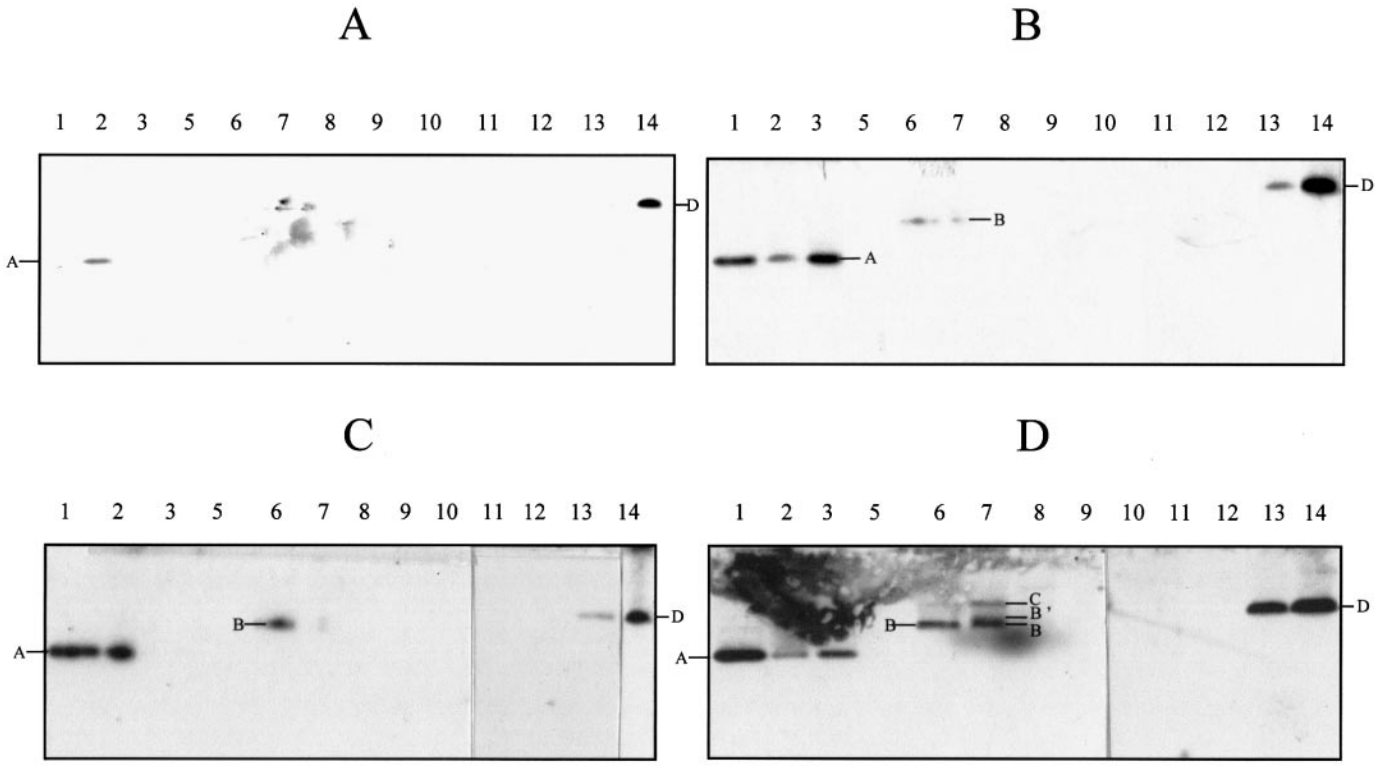

Figure 6 Binding assay between fractions obtained after RP-HPLC separation and tritiated analogues of pheromones. **(A)** Z11–16:Ac; **(B)** 16:Ac; **(C)** Z11–16:OH; **(D)** Z11–18:Ac. The quantity of radioactive compounds used is the same as in Figure 4. (D) shows a radioactive contamination of the film, not co-localized with the labelled bands. Seven days exposure at –70°C.

```
                        10        20        30        40        50        60
                        |         |         |         |         |         |
CSP-MbraA1   -EDKYTDKYDNINLDEILANKRLLVAYVNCVMERGKCSPEGKELKEHLQDAIENGCKKCA
CSP-MbraA2   -EDKYTDKYDNINLDEILANKRLLVAYVNCVMERGKCSPEGKELKEHLQDAIENGCKKCA
CSP-MbraA3   -EDKYTDKYDNINLDEILANKRLLVAYVNCVMERGKCSPEGKELKEHLQDAIENGCKKCT
CSP-MbraA4   -EDKYTDKYDNINLDEILANKRLLVAYVNCVMERGKCSPEGKELKEHLQDAIENGCKKCT
CSP-MbraA5   -EDKYTDKYDNINLDEILANKRLLVAYVNCVMERGKCSPEGKELKEHLQDAIENGCKKCT
CSP-MbraB1   EEAHYTDRYDNVDLDEILGNRRLMVPYIKCILDQGKCAPDAKELKEHIREALENECGKCT
CSP-MbraB2   EEAHYTDRYDSVDLDEILGNRRLLVPYVKCILDQGKCAPDGKELKEHIKEALENECGKCT

                        70        80        90        100       110
                        |         |         |         |         |
                                                                  MW        pI
CSP-MbraA1   ENQEKGAYRVIEHLIKNEIEIWRELTAKYDPTGNWRKKYEDRAKAAGIVIPEE  13042.78  5.77
CSP-MbraA2   ENQEKGAYRVIEHLIENEIEIWRELTAKYDPTGNWRKKYEDRAKAAGIVIPEE  13043.72  5.34
CSP-MbraA3   ENQEKGAYRVIEHLIKNEIEIWRELTAKYDPTGNWRKKYEDRAKAAGIVIPEE  13072.81  5.77
CSP-MbraA4   ENQEKGAYRVIEHLIKNEIEIWRELTAKYDPTVNWRKKYEDRAKAAGIVIPEE  13114.89  5.77
CSP-MbraA5   ENQEKGAYRAIEHLIKNEIEIWRELTAKYDPTGNWRKKYEDRAKAAGIVIPEE  13044.76  5.77
CSP-MbraB1   ETQKNGTRRVIGHLINHEDAYWKELTAKYDPQSKFTAKYEKELKEIKH-----  12722.35  6.66
CSP-MbraB2   DAQKKGTRRVIAHLINHEEDFWNELTAKFDPERKFTAKYEKELKDIKE-----  12665.28  5.75
```

Figure 7 Alignment of the seven deduced amino acid sequences of proboscis proteins. The four conserved cysteines are in bold. Calculated molecular masses (Da) and isoelectic points are indicated for each isoform.

possibility, that the antiserum contained in addition antibodies against OS-D-like proteins is rather improbable, since in native gels these proteins have highly different mobilities to GOBP2 (see Figure 4) and, therefore, should not have electroeluted together. The cross-reactivity is most probably due to common epitopes borne by proteins that could have a similar function (see below).

An immunolabelling signal as low as in the present case cannot provide convincing information about the localization of an antigen. Nevertheless, the weak cross-reactivity of proboscis chemosensory proteins with antisera against moth OBPs led to the characterization and sequencing of new chemosensory proteins, and binding studies with radioactive compounds indeed showed their odorant-binding

CSP-*Mbra*A1							
50	CSP-*Mbra*B1						
76	48	CLP-1					
51	50	48	CSP-*sg*1				
43	42	41	49	CSP-*ec*1			
47	44	46	59	46	P10		
45	42	45	47	41	53	OS-D	
34	29	42	52	33	57	47	EBSP

Figure 8 Comparison between the deduced amino acid sequences of OS-D-like proteins (% identity). For species having several isoforms, one sequence only was used. CSP-*Mbra*A1 and CSP-*Mbra*B1, this study; CLP-1, *C. cactorum* (Maleszka and Stange, 1997); CSP-*sg*1, *S. gregaria* (Angeli *et al.*, 1999); CSP-*ec*1, *Eurycantha calcarata* (Marchese *et al.*, GenBank accession no. AF139196); P10, *P. americana* (Kitabayashi *et al.*, GenBank accession no. AF030340); OS-D, *D. melanogaster* (McKenna *et al.*, 1994; Pikielny *et al.*, 1994); EBSPIII, *D. melanogaster* (Dyanov and Dzitoeva, GenBank accession no. U08281).

capacity (see below). Furthermore, almost all insect OBPs and OBP-related proteins identified so far were localized in the sensillum lymph of various olfactory and gustatory sensilla, therefore, the labelling of the sensillum lymph of sensilla styloconica, which morphologically feature mixed traits of olfactory and gustatory function, is in line with the general localization of OBPs. Even the labelling of the subcuticular space is not an entirely new finding, but has been observed with anti-PBPRP2 in *Drosophila* (Park *et al.*, 2000). Thus, the sensilla styloconica are the most likely place where the observed proboscis chemosensory proteins are expressed.

Immunodetection

In western blot experiments, proteins from the proboscis are immunoreactive with antisera raised against ApolGOBP2 but not with antisera raised against ApolPBP (Figure 4B,C). A relatively long time of exposure (10 min) was necessary to obtain labelling, compared with the antennal proteins, that give a signal in <1 min (Figure 4B). This suggests that the proboscis proteins have poor homology with the PBP and GOBP classes previously defined by Vogt *et al.* (Vogt *et al.*, 1991). Indeed, MbraPBPs and MbraGOBP2 are absent from the proboscis, as antisense RNA probes deduced from these sequences gave no signal on probosces in *in situ* hybridization experiments (E. Jacquin-Joly, unpublished results).

Binding with tritiated pheromone analogues

The four tritiated pheromone analogues were chosen because they are either produced in the female gland (Z11–16:Ac, 92% of the pheromonal blend; 16:Ac, 7%) (Descoins *et al.*, 1978) or are specifically detected by conspecific males as a behavioural inhibitor (Z11–16:OH) (Farine *et al.*, 1981). Z11–18:Ac (vaccenyl acetate) was recently identified as a minor component of the *M. brassicae* female pheromonal secretion (P. Nagnan-Le Meillour and E. Jacquin-Joly, unpublished results). In addition, they are the only specific tritiated ligands available for such a study.

In total extracts, the four tritiated pheromones are only bound in band D, containing the sequence EDKY. After purification, binding with pheromone analogues revealed additional labelling, at the level of bands A–C. The different results obtained with total extracts and purified fractions could be explained either by protein enrichment in a given fraction or by the fact that in total extracts proteins are in competition to bind pheromones. In this latter case, one can assume that the EDKY protein in band D has the highest affinity for the four ligands. In the purified fractions, there is no competition and other proteins (EEAH and AKLT) with less affinity for the ligands can bind them. It is worth noting that the four coumpounds used in this study are structurally close and could be in competition for the binding site of *Mamestra* OBPs. In addition, we have previously shown that components of the *M. brassicae* pheromone, not detected by species such as *Bombyx mori* or *Antheraea pernyi*, gave negative binding with antennal extracts of these species (Bohbot *et al.*, 1998). Moreover, in antennal extracts, vaccenyl acetate is only bound by proteins of the EDKY sequence and not by the antennal-specific MbraPBPs and MbraGOBP2 (Bohbot *et al.*, 1998), which were not detected in proboscis extracts. Thus, the proboscis proteins have a strong affinity for pheromonal compounds, but they show remarkably little selectivity for the different odorants tested, which was expected from the binding results obtained with antennal extracts, where similar proteins bind the four pheromone analogues (Bohbot *et al.*, 1998).

Characterization of proboscis soluble proteins

Proteins corresponding to three different N-terminal sequences were able to bind pheromones *in vitro*. For each of the N-terminal sequences, EDKY and EEAH, several

isoforms were identified, migrating at distinct positions in native-PAGE, reflecting different isoelectric points. To confirm the presence of several isoforms, several clones were sequenced after the amplification of cDNAs from probosces. Different protein sequences were obtained, sharing high identities, with isoelectric points ranging from 5.34 to 6.66, which explains the different positions in native-PAGE. Molecular cloning demonstrated the presence of isoforms in two protein classes, CSP-*Mbra*A and CSP-*Mbra*B, confirming the observation of several bands sharing the same N-terminal sequence on the gels. As the PCR amplifications and biochemical studies were carried out on a pool of probosces, this could reflect either expression of several alleles in each individual or expression of alleles in the population. However, many CSP isoforms have been found in individual *S. gregaria* legs (Angeli *et al.*, 1999), reflecting a real microdiversity in the same animal, although we do not know if this is the case in *M. brassicae*.

A search for sequence similarity in databases showed remarkable homologies between *M. brassicae* proboscis proteins and other proteins previously characterized in several orders of insects (Figure 8). These proteins are called OS-D like (Vogt *et al.*, 1999), because the first member of this family was the OS-D gene product, discovered in *D. melanogaster* (McKenna *et al.*, 1994; Pikielny *et al.*, 1994). The proboscis proteins CSP-*Mbra*A (EDKY N-terminus) show 99% similarity in the N-terminus with the antennal isoform MbraAOBP2 (Bohbot *et al.*, 1998) (E. Jacquin-Joly, unpublished results). The amino acid sequence similarity in the OS-D-like family of proteins has a constant value around 50%, irrespective of phylogenetic relationship. In addition, the N-terminal deduced amino acid sequences of the proboscis proteins also share a high percentage identity with partial amino acid sequences of a purified protein from the bee (ASP3) (Danty *et al.*, 1998) as well as proteins from the Phasmatodea (Mameli *et al.*, 1996; Tuccini *et al.*, 1996).

The last sequence corresponding to positive binding in the proboscis is AKLT. Searches in databases using BLASTp (National Center for Biotechnology Information, NCBI) revealed no homologies with known proteins. This protein does not belong to the class mentioned above.

The OS-D-like class differs from the PBPs and GOBPs, the two OBP classes primarily defined by Vogt *et al.* (Vogt *et al.*, 1991) based on homology criteria, in several aspects. (i) There is no sequence motif conserved between OBPs and OS-D-like proteins, suggesting that there is no recent ancestral gene from which these two groups emerged through a gene duplication event, as was shown for the PBP class (Merrit *et al.*, 1998; Vogt *et al.*, 1999). (ii) OBPs are highly divergent within and between species (Vogt *et al.*, 1999) while the OS-D-like proteins are highly conserved within species but homogeneously divergent between species (~50%). (iii) While OBPs are antenna-specific (Vogt and Riddiford, 1981), the OS-D-like proteins are commonly observed to be expressed in diverse organs [proboscis, antennae and legs in *M. brassicae* (this study); legs and antennae in *S. gregaria* (Angeli *et al.*, 1999), *Periplaneta americana* (Kitabayashi *et al.*, 1998); maxillary palps in *Cactoblastis cactorum* (Maleszka and Stange, 1997); the ejaculatory bulb (Dyanov and Dzitoeva, 1995); the third antennal segment in *Drosophila* (McKenna *et al.*, 1994; Pikielny *et al.*, 1994)]. (iv) OBPs have consistently been shown to discriminate between odorants (Vogt *et al.*, 1989; Du and Prestwich, 1995; Maïbèche-Coisné *et al.*, 1997; Wojtasek *et al.*, 1999) while we demonstrate in this report that proboscis proteins have little selectivity for the odorants tested.

Function of the proboscis chemosensory proteins

The characterization of OBP-related proteins in organs typically devoted to taste functions has already been reported in *D. melanogaster* for PBPRP-2 (Pikielny *et al.*, 1994; Ozaki *et al.*, 1995) and in *Phormia regina* for CRLBP (Ozaki *et al.*, 1995). In *P. regina*, electrophysiological studies have shown that the responses of the fifth cell to fragrant components of apple juice are depressed in the presence of antibodies raised against CRLBP. The authors suggested that these proteins could be involved in the recognition of volatile, more generally lipophilic signals from plants, either in the antennae or in the taste organs.

Furthermore, a general body sensitivity to vapours, after removal of all known olfactory receptors, was early reported by McIndoo (McIndoo, 1934). The eventuality that insects could possess a common chemical sensitivity to vapours has been the subject of discussion (Dethier, 1972). The characterization of proteins able to bind pheromones in the proboscis reactualizes such a possibility. Several roles were proposed for this class of proteins, without supporting functional data. In *C. cactorum*, the protein is expressed in maxillary palps, involved in CO_2 detection (Maleszka and Stange, 1997) but binding experiments between *S. gregaria* CSPs and CO_2 was negative (Angeli *et al.*, 1999). The p10 protein is expressed in regenerating legs of *Periplaneta americana* and was proposed to be involved in the regeneration process (Kitabayashi *et al.*, 1998). In *D. melanogaster*, EBSPIII protein could be involved in the transmission of vaccenyl acetate from the male to the female during copulation (Dyanov and Dzitoeva, 1995). Our data support a role in binding of lipophilic molecules, such as odorants. Considering the available data, we propose distinguishing between the PBP and GOBP families (OBP-Type 1) and chemosensory proteins related to the OS-D gene family (OBP-Type 2), as recently suggested by Vogt *et al.* (Vogt *et al.*, 1999).

These findings lead to the hypothesis that the same proteins could be involved in different physiological process according to their localization. Their ability to bind pheromones could be a biochemical property leading to different physiological functions depending on their localization and, thus, the cellular environment. Proteins of the CSP-*Mbra* subclass could be involved in either olfactory

transduction when expressed in antennae or in other mechanisms (transport, deactivation) when expresssed in other organs.

The question of whether the binding with pheromonal compounds is specific or not remains and can only be solved by electrophysiological studies, which are strongly needed.

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References

Altner, H. and **Altner, I.** (1986) *Sensilla with both terminal pore and wall pores on the proboscis of the moth,* Rhodogastria bubo *Walker (Lepidoptera: Arctiidae)*. Zool. Anz., 216, 129–150.

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

Bock, C. (1987) *A quick and simple method for preparing soft insect tissues for scanning electron microscopy using Carnoy and hexamethyldisilazane*. Beitr. Zurelek. Direktabb. Oberflächen, 20, 209–214.

Bohbot, J., Sobrio, F., Lucas, P. and **Nagnan-Le Meillour, P.** (1998) *Functional characterisation of a new class of odorant-binding proteins in the moth* Mamestra brassicae. Biochem. Biophys. Res. Commun., 253, 489–494.

Büttiker, W., Krenn, H.W. and **Putterill, J.F.** (1996) *The proboscis of eye-frequenting and piercing Lepidoptera (Insecta).* Zoomorphology, 116, 77–83.

Danscher, G. (1981) *Localization of gold in biological tissue—a photochemical method for light and electron microscopy*. Histochemistry, 71, 81–88.

Danty, E., Arnold, G., Huet, J.C., Huet, D., Masson, C. and **Pernollet, J.C.** (1998) *Separation, characterization and sexual heterogeneity of multiple putative odorant-binding proteins in the honeybee* Apis mellifera *L. (Hymenoptera: Apidea)*. Chem. Senses, 23, 83–91.

Descoins, C., Priesner, E., Gallois, M., Arn, H. and **Martin, G.** (1978) *Sur la sécrétion phéromonale des femelles de* Mamestra brassicae *L. et de* Mamestra oleracea *L. (Lepidoptera: Noctuidae, Hadeninae)*. C. R. Acad. Sci., 286, 77–80.

Dethier, V.G. (1972) *Sensitivity of the contact chemoreceptors of the blowfly to vapors*. Proc. Natl Acad. Sci. USA, 69, 2189–2192.

Dyanov, H.M. and **Dzitoeva, S.G.** (1995) *Method for attachment of microscopic preparartions on glass for* in situ *hybridization, PRINS and* in situ *PCR studies*. Biotechniques, 18, 822–824.

Du, G. and **Prestwich, G.D.** (1995) *Protein structure encodes the ligand binding specificity in pheromone binding proteins*. Biochemistry, 34, 8726–8732.

Eastham, L.E.S. and **Eassa Y.E.E.** (1955*) The feeding mechanism of the butterfly* Pieris brassicae *L*. Phil. Trans. R. Soc. Lond. Ser. B, 239, 1–43.

Farine, J.P., Frérot, B. and **Isart, J.** (1981) *Facteurs d'isolement chimique dans la sécrétion phéromonale de deux noctuelles Hadeninae:* Mamestra brassicae *(L.) and* Pseudaletia unipuncta *(Haw.).* C.R. Acad. Sci. Paris III, 292, 101–104.

Faucheux, M.J. (1991) *Morphology and distribution of sensilla on the cephalic appendages, tarsi and ovipositor of the European sunflower moth,* Homoeosoma nebulella *Den. and Schiff. (Lepidoptera: Pyralidae)*. Int. J. Morphol. Embryol., 20, 291–307.

Kitabayashi, A.N., Arai, T., Kubo, T. and **Natori, S.** (1998) *Molecular cloning of cDNA for p10, a novel protein that increases in the regenerating legs of* Periplaneta americana *(American cockroach).* Insect Biochem. Mol. Biol., 28, 785–790.

Krenn, H.W. (1998) *Proboscis sensilla in* Vanessa cardui *(Nymphalidae, Lepidoptera): functional morphology and significance in flower-probing*. Zoomorphology, 118, 23–30.

Kyhse-Andersen, J. (1984) *Electroblotting of multiple gels: a simple apparatus without tank buffer for rapid transfer of proteins from polyacrylamide to nitrocellulose*. J. Biochem. Biophys. Methods, 10, 203–209.

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

Maïbèche-Coisné, M., Sobrio, F., Delaunay, T., Lettéré, M., Dubroca, J., Jacquin-Joly, E. and **Nagnan-Le Meillour, P.** (1997) *Pheromone-binding proteins of the moth* Mamestra brassicae*: specificity of ligand binding*. Insect Biochem. Mol. Biol., 27, 213–221.

Maïbèche-Coisné, M., Longhi, S., Tegoni, M., Jacquin-Joly, E., Gastinel, L., Cambillau, C. and **Nagnan-Le Meillour, P.** (1998a) *Molecular cloning and bacterial expression of a General Odorant Binding Protein in the cabbage armyworm* Mamestra brassicae. Eur. J. Biochem., 258, 768–774.

Maïbèche-Coisné, M., Jacquin-Joly, E., François, M.C. and **Nagnan-Le Meillour, P.** (1998b) *Molecular cloning of two pheromone-binding proteins in the cabbage armyworm* Mamestra brassicae. Insect Biochem. Mol. Biol., 28, 815–818.

Malezska, R. and **Stange, G.** (1997) *Molecular cloning, by a novel approach, of a cDNA encoding a putative olfactory protein in the labial palps of the moth* Cactoblastis cactorum. Gene, 202, 39–43.

Mameli, M., Tuccini, A., Mazza, M., Petacchi, R. and **Pelosi, P.** (1996) *Soluble proteins in chemosensory organs of phasmids*. Insect Biochem. Mol. Biol., 26, 875–882.

Marshall, A.T. and **Kent, M.** (1991) *A device for freeze-substituting a large number of samples under controlled conditions*. J. Microsc., 162, 335–340.

McIndoo, N.E. (1934) J. Morphol., 56, 445–475.

McKenna, M.P., Hekmat-Scafe, D., Gaines, P. and **Carlson, J.R.** (1994) *Putative* Drosophila *pheromone-binding proteins expressed in a subregion of the olfactory system*. J. Biol. Chem., 10, 16340–16347.

Merrit, T. J. S., LaForest, S., Prestwich, G. D., Quattro, J. M. and **Vogt, R.G.** (1998) *Patterns of gene duplication in lepidopteran pheromone binding proteins*. J. Mol. Evol., 46, 272–276.

Nagnan-Le Meillour, P., Huet, J.C., Maïbèche, M., Pernollet, J.C. and **Descoins, C.** (1996) *Purification and characterization of multiple forms of odorant/pheromone binding proteins in the antennae of* Mamestra brassicae *(Noctuidae)*. Insect Biochem. Mol. Biol., 26, 59–67.

Ozaki, M., Morisaki, K., Idei, W., Ozaki, K. and **Tokunaga, F.** (1995) *A putative lipophilic stimulant carrier protein commonly found in the taste and olfactory systems. A unique member of the pheromone-binding protein superfamily*. Eur. J. Biochem., 230, 298–308.

Park, S.-K., Shanbhag, S.R., Wang, Q., Hasan, G., Steinbrecht, R.A. and **Pikielny, C.W.** (2000) *Different expression patterns of two putative*

odorant-binding proteins in the olfactory organs of Drosophila *have different implications for their functions*. Cell Tissue Res., 300, 181–192.

Paulus, H.F. and **Krenn, H.W.** (1996) *Vergleichende Morphologie des Schmetterlingsrüssels und seiner Sensillen—Ein Beitrag zur phylogenetischen Systematik der Papilionoidea (Insecta, Lepidoptera)*. J. Zool. Syst. Evol. Res., 34, 203–216.

Pikielny, C., Hasan, G., Rouyer, F. and **Rosbach, M.** (1994) *Members of a family of* Drosophila *putative odorant binding proteins are expressed in different subsets of olfactory hairs*. Neuron, 12, 35–49.

Poitout, S. and **Bues, R.** (1974) *Elevage de 28 espèces de lépidoptères Noctuidae et de deux espèces d'Arctiidae sur milieu artificiel simplifié. Particularités selon les espèces*. Ann. Zool. Ecol. Anim., 6, 431–441.

Sellier, R. (1975) *Etude ultrastructurale en microscopie électronique par balayage des organes sensoriels de la trompe des Lépidoptères Rhopalocères*. Alexanor Rev. Lepidop. Fr., 9, 9–15.

Städler, E. and **Hanson, F.E.** (1975) *Olfactory capabilities of the 'gustatory' chemoreceptors of the tobacco hornworm larvae*. J. Comp. Physiol., 104, 97–102.

Städler, E. and **Seabrook, W.D.** (1975) *Chemoreceptors on the proboscis of the female eastern spruce budworm: electrophysiological study*. Entomol. Exp. Appl., 18, 153–160.

Städler, E., Städler-Steinbrüchel, M. and **Seabrook, W.D.** (1974) *Chemoreceptors on the proboscis of the female eastern spruce budworm.* Mitt. Schweiz. Entomol. Ges., 47, 63–68.

Steinbrecht, R.A. (1993) *Freeze-substitution for morphological and immunocytochemical studies in insects*. Microsc. Res. Tech., 24, 488–504.

Steinbrecht, R.A., Ozaki, M. and **Ziegelberger, G.** (1992) *Immunocytochemical localization of pheromone-binding protein in moth antennae*. Cell Tissue Res., 270, 287–302.

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

Tuccini, A., Maida, R., Rovero, P., Mazza, M. and **Pelosi, P.** (1996) *Putative odorant-binding protein in antennae and legs of* Carausius morosus *Insecta, Phasmatodea*. Insect Biochem. Mol. Biol., 26, 19–24.

Vogt, R.G. and **Riddiford, L.M.** (1981) *Pheromone-binding and inactivation in moth antennae*. Nature, 293, 161–163.

Vogt, R.G., Köhne, A.C., Dubnau, J.T. and **Prestwich, G.D.** (1989) *Expression of pheromone-binding proteins during antennal development in the gypsy moth* Lymantria dispar. J. Neurosci., 9, 3332–3346.

Vogt, R.G., Prestwich, G.D. and **Lerner, M.R.** (1991) *Odorant-binding protein subfamilies associate with distinct classes of olfactory receptor neurons in insects*. J. Neurobiol., 22, 74–84.

Vogt, R.G., Callahan, F.E., Rogers, M.E. and **Dickens, J.C.** (1999) *Odorant Binding Protein diversity and distribution among the insect orders, as indicated by LAP, an OBP-related protein of the true bug* Lygus lineolaris *(Hemiptera, Heteroptera)*. Chem. Senses, 24, 481–495.

Walters, B.D., Albert, P.J. and **Zacharuk, R.Y.** (1998) *Morphology and ultrastrucuture of sensilla on the proboscis of the adult spruce budworm,* Choristoneura fumiferana *(Clem.) (Lepidoptera: Tortricidae)*. Can. J. Zool., 76, 466–479.

Wojtasek, H., Picimbon, J.-F. and **Leal, W.S.** (1999) *Identification and cloning of Odorant Binding Proteins from the scarab beetle* Phyllopertha diversa. Biochem. Biophys. Res. Commun., 263, 832–837.

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