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Unisex Pheromone Detectors and Pheromone-binding Proteins in Scarab Beetles

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Abstract

Olfaction was studied in two species of scarab beetle, *Anomala octiescostata* and *Anomala cuprea* (Coleoptera: Scarabaeidae: Rutelinae), which are temporarily isolated and use the same sex pheromone compounds, (*R*)-buibuilactone and (*R*)-japonilure. Single sensillum recordings in *A. octiescostata* revealed highly sensitive olfactory receptor neurons (ORNs) (threshold <1 pg) that were tuned to the detection of the green leaf volatile compound (*Z*)-3-hexenyl acetate. As opposed to similar ORNs in another scarab species, *Phyllopertha diversa*, in *A. octiescostata* a diazo analogue elicited much lower neuronal responses than the natural ligand. Detectors for other floral and leaf compounds were also characterized. Extremely stereoselective ORNs tuned to sex pheromone were identified in male and female antennae. Biochemical investigations showed that, in *A. octiescostata* and *A. cuprea*, the pheromone-binding proteins (PBPs) isolated from male antennae were identical to PBPs obtained from female antennae. AoctPBP and AcupPBP had seven different amino acid residues. Binding of AoctPBP to (*R*)-japonilure is shown. PdivOBP1, which is also known to bind to (*R*)-japonilure, differed from AcupPBP in only two amino acid residues, one at the N-terminus and the other near the C-terminus. The structural features of the *Bombyx mori* PBP are compared with the sequences of eight known scarab odorant-binding proteins.

Introduction

For an insect the environment contains a myriad of chemical compounds. Most of them are physiologically irrelevant (noise), whereas others may be essential because they carry information (semiochemicals) about mate finding (sex pheromone), food sources (kairomones), oviposition sites (oviposition attractants) and many other features of the environment. While flying *en route* to a pheromone or other chemical source insects encounter intermittent signals with stimulus present in broken bursts of some tens of milliseconds in duration that occur with intervals of some hundreds of milliseconds (Murlis, 1997). This has led to the development of a remarkable selective, sensitive and dynamic olfactory system (Kaissling, 2001).

From the perspective of molecular recognition there is another complication. Information-carrying small hydrophobic ligands must reach the olfactory receptors (Clyne *et al*., 1999; Vosshall *et al*., 1999) in order to be transduced into neuronal activities (spikes). However, these olfactory receptors are located on islands (the dendrites) in the sensillar lymph and many odorants cannot 'swim' (they are water insoluble). Thus, the olfactory processing starts with odorant-binding proteins (OBPs) (Kaissling, 2001) ferrying the ligands to their receptors. Various functions have been suggested for these proteins since their discovery (Vogt and Riddiford, 1981). OBPs are postulated to solubilize semiochemicals, protect the ligands from degradation and participate in fast inactivation of chemical signals. Clearly most of the ligands need to be solubilized. It has recently been demonstrated that the *raison d'être* of these proteins is more than to solubilize ligands. An OBP named LUSH is essential for the recognition of a water-soluble semiochemical (ethanol) in *Drosophila melanogaster* (Kim and Smith, 2001). On the basis of their binding abilities, OBPs have been considered to play a significant part in the remarkable selectivity of the insect olfactory system, but the literature is also dichotomous (Campanacci *et al*., 2001) with data suggesting that some OBPs are specific, whereas others may be more 'sloppy'. Structural biology studies with the pheromone-binding protein (PBP) from the silkworm moth *Bombyx mori* have indicated that the binding pocket forms a general hydrophobic surface for binding (Sandler *et al*., 2000). Interestingly, a C-terminal sequence, which is an extended (unstructured) conformation in the pheromone–PBP complex, rearranges to form a regular α-helix that occupies the pheromone-binding pocket (Horst *et al*., 2001), thus 'ejecting' the pheromone to the receptor.

We have been studying chemical communication in scarab beetles (Coleoptera: Scarabaeidae). Identical pheromone chemistry has been characterized in species that share the same habitat in the main land of the Japanese archipelago (Leal, 1998), but are temporarily isolated. For example, the sex pheromones of *Anomala octiescostata* and *Anomala cuprea* are composed of (*R*)-buibuilactone and (*R*)-japonilure (compounds 1 and 2 respectively) (see Figure 1) (Leal, 1991; Leal *et al*., 1993, 1994a). While the sensory physiology of the latter has been investigated in detail (Leal and Mochizuki, 1993; Larsson *et al*., 1999, 2001), little is known about olfaction in the former (Leal, 1999). The purposes of this study were twofold. First, we aimed at studying the sensory physiology in *A. octiescostata* with emphasis on the olfactory receptor neurons (ORNs) involved in the detection of sex pheromones, green leaf volatiles and floral compounds. Second, we isolated PBPs from *A. octiescostata* and *A. cuprea* and cloned their cDNAs in order to compare the primary structures of the PBPs from two species that are involved in the detection of the same sex pheromone.

Materials and methods

Insects

Eggs laid by field-captured females were collected daily and transferred to wet sand in ice cream cups, which were kept at 25°C. After hatching, grubs were individually transferred to ice cream cups filled with humus and supplied with slices of sweet potato or carrot, which were renewed weekly. Diapause termination in *A. octiescostata* (adults) and *A. cuprea* (yellow stage third-instar grubs) was achieved by cold treatment (10°C for 2–3 months) and then raising the temperature to 25°C. Adults were fed on an artificial diet for the silkworm moth *B. mori* (Nosan Co., Yokohama, Japan) and kept at 25°C and 50–70% relative humidity under a 16:8 h light:dark photoregime.

Semiochemicals

(*R*,*Z*)-5-(–)-(1-Octenyl)oxacyclopentan-2-one [(*R*)-buibuilactone], (*S*)-buibuilactone, (*R*,*Z*)-5-(–)-(1-decenyl)oxacyclopentan-2-one [(*R*)-japonilure] and (*S*)-japonilure were obtained as described previously (Leal, 1999). The synthesis of (*Z*)-3-hexenyl diazoacetate has been reported elsewhere (Nikonov *et al*., 2001). (*Z*)-3-Hexenol and (*Z*)-3-hexenyl acetate were purchased from Wako Chemicals (Wako, Japan). Toyotama Koryo (Tokyo, Japan) and Koei Kogyo (Yokohama, Japan) supplied anethole and geraniol respectively.

Single sensillum recordings

Beetles were immobilized in 1.5 ml microfuge tubes that were cut at the bottom. Their heads were sticking out of the hole and their antennae were fixed by dental wax. The three lamellae of the antennae were separated and held immobile with a thin metal pin. The indifferent (ground) electrode was a thin silver wire inserted in the abdomen. Extracellular contacts with antennal ORNs were established under a stereomicroscope with up to ×300 magnification by inserting a tungsten electrode (sharpened electrolytically in a KNO_2 solution) through the cuticle adjacent to a sensillum placodeum (Hubel, 1957). The antennal preparation was continuously flushed by a charcoal-filtered and moistened airstream flowing at 0.5 m/s through an 8 mm (inner diameter) glass tube ending 10 mm before the preparation. Each test compound was applied to a piece of filter paper (0.5 cm × 1 cm), which was dried for at least 10 min and inserted into a Pasteur pipette. Stimulus was performed by inserting the tip of the test pipette into a hole 6 cm from the outlet of the glass tube and air was blown through the pipette for 300 ms by a stimulus controller (CD-02/E, Syntech, Hilversum, The Netherlands). The signal was amplified using a Nihon Kohden MEZ-8300 amplifier (Tokyo, Japan). Antennal responses were stored using Axotape 2.0.2 (Axon Instruments, Inc., Foster City, CA) and visualized on a Nihon Kohden memory oscilloscope VC-11 (Tokyo, Japan). Spikes were counted manually from the computer screen (a time bin of 250 ms). We analyzed neuronal activity in the period 1 s before and 1 s after the onset of stimulation. Positive responses were higher than two standard deviations of the background activity (1 s period before stimulus onset). Net spikes were calculated as the number of spikes during 1 s after (300 ms) stimulation minus the number 1 s before stimulation. The results obtained were statistically processed (Zacks, 1971) and represented as dose–response plots.

Protein extracts

The beetles were anaesthetized on ice and their antennae and legs were collected, frozen in liquid nitrogen and lyophilized. Homogenization was performed in an ice-cold glass Dounce tissue grinder in 10 mM Tris–HCl, pH 8. Homogenized samples were centrifuged twice at 12 000 r.p.m. (4°C for 5 min). The supernatants were concentrated by centrifugation under vacuum and analyzed by native polyacrylamide gel electrophoresis (PAGE). In order to perform sequencing, proteins were separated by native PAGE, transferred to polyvinyl difluoride (PVDF) membranes using electroblotting and bands were cut out and analyzed for microsequencing. For molecular mass measurements, bands were cut out directly from gels, the proteins were electroeluted in a microelectroeluter, concentrated by centrifugation in a Centricon 3 (Millipore Corporation, Bedford, MA) and injected onto an on-line liquid chromatograph–mass spectrometer (LC-MS). Binding assays followed a previously reported protocol (Wojtasek *et al*., 1998). Radiolabelled (*R*)-buibuilactone and (*R*)-japonilure were prepared by Amersham Biosciences K. K. (Tokyo, Japan) by catalytic reduction (with tritium gas) of the alkyne precursors following a protocol for deuteration of these lactones (Wojtasek *et al*., 1998). Native PAGE binding

assays were performed as described previously (Wojtasek *et al.*, 1998).

Analytical procedures

Native PAGE (15%) analyses were performed on a Mini-PROTEAN II (Bio-Rad, Hercules, CA). Gels were stained with Coomassie blue R-250. N-terminal amino acid sequences were obtained on a Hewlett-Packard Protein Sequencer model 241 with phenylthiohydantoin (PTH) derivatives separated on a Hewlett-Packard (Agilent, Palo Alto, CA) series 1100 high-performance liquid chromatography (HPLC) system. Electrospray ionization mass spectra (ESI-MS) were obtained with a Hewlett-Packard on-line chromatograph–mass spectrometer equipped with an electrospray interface (LC/ESI-MS). HPLC separations were achieved on a Zorbax 300 CB-C8 column (150 mm × 2.1 mm and 5 μm) by using water and acetonitrile with 2% acetic acid as modifier in both solvents and a gradient from 20 to 80% acetonitrile in 15 min at a flow rate of 0.2 ml/min. The MSD parameters were as follows: skimmer collision-induced dissociation voltage (fragmentor) 120 V, drying gas flow 6 l/min, nebulizer pressure 310 KPa, nebulizer temperature 300°C and capillary voltage 3500 V. DNA sequences were obtained with an automated ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, CA). DNA and protein sequences were analyzed by using the MacVector software (Accelrys, Cambridge, UK).

cDNA cloning and sequencing

Total RNA was extracted (100 antennae) by using TRIzol™ Regent (Invitrogen Life Technologies, Carlsbad, CA) and mRNA was purified with the polyATract mRNA Isolation System (Promega, Madison, WI). First strand cDNA was synthesized in the presence of an oligo(dT) primer by using SuperScript II™ (Invitrogen). Two degenerate primers were designed on the basis of the first six residues of the N-terminal sequences of the isolated antenna-specific proteins from *A. octiescosta* and *A. cuprea*: ATG*AG*-(*C*/*T*)GA(A/G)GA(A/G)ATGGA and ATG*TC*(*A*/*C*/*G*/*T*)-GA(A/G)GA(A/G)ATGGA. The two primers differed only in the codons for serine (italics). Polymerase chain reactions (PCRs) were carried out in a MiniCycler (Model 150, MJ Research) by using Taq DNA polymerase (Roche Molecular Biochemicals, Indianapolis, IN) with an annealing temperature at 46°C. PCR fragments were cloned into pCR2.1-TOPO (Invitrogen) and transformed into OneShot™ Top10F′ competent cells (Invitrogen). Positive insertions were identified by PCR (with M13 forward and M13 reverse primers). Sequence reactions were carried out using the Dye Terminator Cycle Sequence FS Ready Reaction Kit (Applied Biosystems) according to the instruction manual. Nucleotide sequences were determined from both strands from at least five independent clones.

Results

Green leaf volatile detectors

Ninety-seven ORNs in the male antennae responded to the green leaf volatile compound (*Z*)-3-hexenyl acetate (Figure 1.8) (for chemical structures, see Figure 1) in a dose-dependent manner (Figure 2). These ORNs are the most sensitive detectors for semiochemicals investigated thus far in scarab beetles (Leal and Mochizuki, 1993; Hansson *et al.*, 1999; Larsson *et al.*, 1999, 2001; Nikonov *et al.*, 2001) and showed a threshold lower than 1 pg (Figure 3). They were remarkably silent when challenged with the related alcohol (*Z*)-3-hexenol (Figure 1.9) even with doses five orders of magnitude higher than the threshold to the specific stimulus (Figure 3). Neuronal activity was always recorded from the sensilla containing the (*Z*)-3-hexenyl acetate ORNs when the preparations were stimulated with anethole (Figure 1.11). Although the threshold was almost four orders of

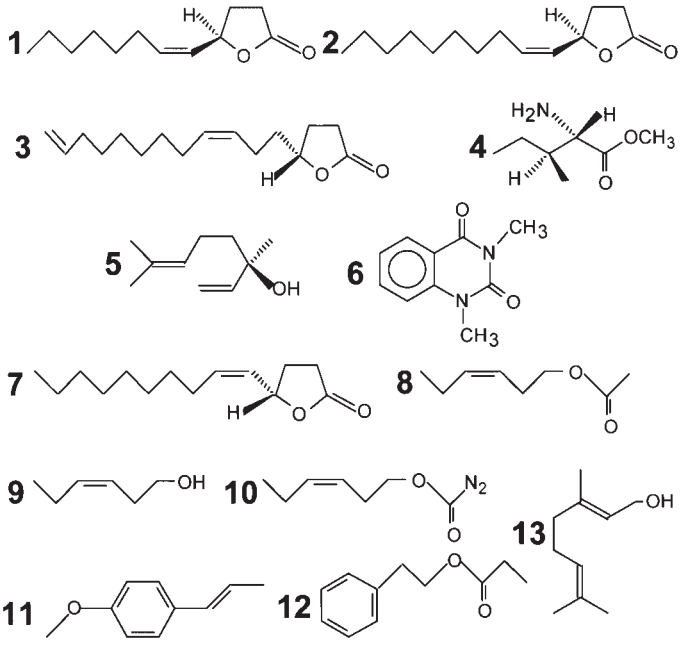

Figure 1 Chemical structures of the pheromones and other semiochemicals involved in the chemical communication of scarab beetles. (1) (*R*,*Z*)-5-(–)-(1-Octenyl)oxacyclopentan-2-one [(*R*)-buibuilactone], the major component of the sex pheromone system in *A. octiescostata* and *A. cuprea*. (2) (*R*,*Z*)-5-(–)-(1-Decenyl)oxacyclopentan-2-one [(*R*)-japonilure], the sex pheromone of *P. japonica* and a secondary component of the sex pheromone of *A. cuprea* and *A. octiescostata*. (3) (*R*,*Z*)-7,15-Hexadecadien-4-olide, the sex pheromone of *Heptophylla picea*. (4) L-Isoleucine methyl ester (LIME) and (5) *R*-(–)-linalool, the major and minor components of the sex pheromone of *Holotrichia parallela*. (6) 1,3-Dimethyl-2,4-(1H,3H)-quinazolinedione (medicinal alkaloid), the sex pheromone of *P. diversa*. (7) (*S*,*Z*)-5-(+)-(1-Decenyl)oxacyclopentan-2-one ((*S*)-japonilure), the sex pheromone of *A. osakana*. (8) (*Z*)-3-Hexenyl acetate and (9) (*Z*)-3-hexenol, green leaf volatile compounds. (10) (*Z*)-3-Hexenyl diazoacetate, a photoaffinity-labelled green leaf volatile. (11) Anethole, (12) phenylethyl propionate and (13) geraniol, floral compounds that attract some species of scarab beetles.

magnitude higher, the detectors responded in a dose-dependent manner (Figure 2). The (*Z*)-3-hexenyl acetate ORNs in *A. octiescostata* responded in a dose-dependent manner to a photoaffinity-labelled compound (*Z*)-3-hexenyl diazoacetate (Figure 1.10) (Figure 2). In contrast to the same type of ORNs previously identified in another scarab beetle *Phyllopertha diversa* (Nikonov *et al.*, 2001), the threshold of the ORNs in *A. octiescostata* to the diazo compound was three orders of magnitude higher than the threshold for the natural compound. ORNs tuned to (*Z*)-3-hexenyl acetate were found also in 61 sensilla in female antennae, but they had a 10 000-fold higher threshold (10 ng), which is a very large difference. In addition, 38 sensilla housing ORNs sensitive and selective to (*Z*)-3-hexenol were found in both male and female antennae (data not shown).

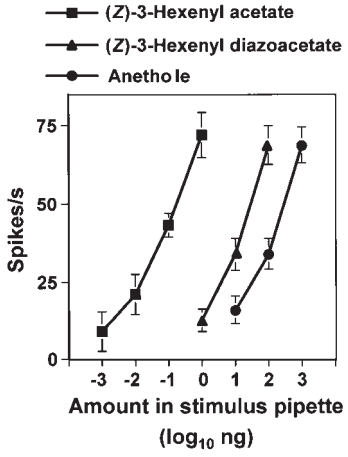

Figure 2 The dose–response curves from ORNs identified on the antennae of *A. octiescostata* male beetles stimulated by (*Z*)-3-hexenyl acetate (Figure 1.8) in a dose-dependent manner. The sensitivity of these ORNs to a related photoaffinity-labelled green leaf volatile (Figure 1.10) was remarkably lower (three orders of magnitude). Either the (*Z*)-3-hexenyl acetate ORNs or a different neuron in the same sensilla responded to anethole (Figure 1.11) at higher doses.

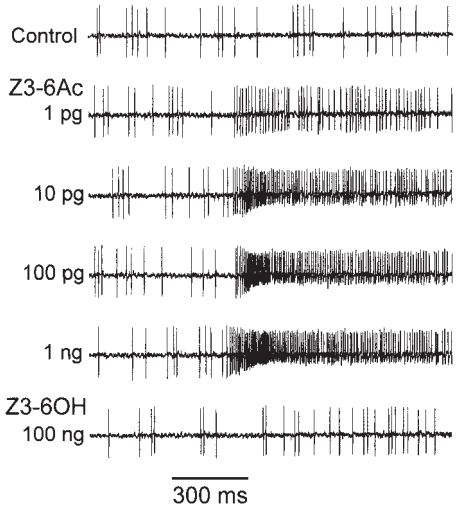

Figure 3 Single sensillum recordings on the male antennae of *A. octiescostata* showing the responses of an ORN tuned to (*Z*)-3-hexenyl acetate (Figure 1.8). The ORN was silent to (*Z*)-3-hexenol (Figure 1.9), even when challenged with 100 000 times the threshold to the related acetate.

Detectors for floral compounds

Thirty-three sensilla in males had a combination of ORNs, with one responding to phenylethyl propionate (Figure 1.12) and geraniol (Figure 1.13). The responses to these two stimuli showed dose dependence, with a higher threshold to geraniol (Figure 4). Again, it was not possible to determine accurately whether the spikes were generated by the same or different ORNs. This type of combination neurons was also found in female antennae (26), with similar threshold and dose–response curves (data not shown).

Sex pheromone-specific detectors in males and females

ORNs tuned to the major constituent of the pheromone system (*R*)-buibuilactone were found in both male (115) and female antennae (43). Although the pheromone detectors in males and females showed similar dose–response curves (Figure 5), female ORNs showed a 10 times higher threshold. These detectors were not stimulated by the antipode of the major sex pheromone component even when challenged with 10 000 ng of (*S*)-buibuilactone, in agreement with the suggested enantiomeric anosmia in *A. octiescostata* (Leal, 1999). ORNs responding to the second pheromone component (*R*)-japonilure (Figure 1.2) were found in both male (53) and female (25) antennae, but they showed a higher threshold (100 ng) (data not shown). The (*R*)-japonilure detectors were silent when challenged with 10 000 ng of (*S*)-japonilure.

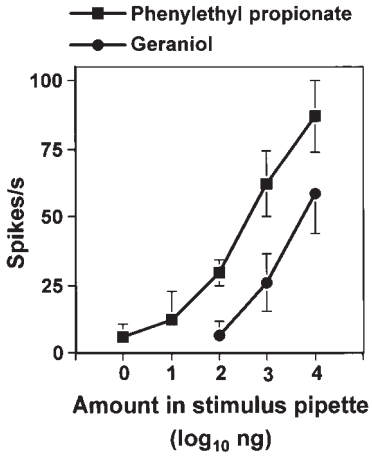

Figure 4 The dose–response relationships from ORNs identified on the antennae of *A. octiescostata* male beetles and sensitive to the floral compound phenylethyl propionate (Figure 1.12). Either the same ORNs or other neurons (with a similar spike amplitude) were activated by higher concentrations of geraniol.

Identification of pheromone-binding proteins

The native PAGE analyses of olfactory and control tissues from the two species of scarab beetles *A. octiescostata* and *A. cuprea* showed antenna-specific bands in these two species (Figure 6). The antenna-specific proteins showed mobility similar to the OBPs identified to date in insects, in particular OBPs from other scarab beetles (Wojtasek *et al*., 1998, 1999; Peng and Leal, 2001; Deyu and Leal, 2002). The antenna-specific band in *A. octiescostata* showed a slightly faster mobility than its counterpart in *A. cuprea.* In agreement with the observation that males and females of *A. octiescostata* (this paper) detect the female-produced sex pheromone, the antenna-specific bands were detected in both sexes (Figure 6). This was also observed in *A. cuprea* (data not shown) which also possess pheromone-detecting ORNs in the male and female antennae (Larsson *et al*., 1999).

The native PAGE binding assays showed that AoctPBP binds to (*R*)-japonilure (Figure 7). However, we were not able to demonstrate association of the protein with the major pheromone constituent (*R*)-buibuilactone. On the other hand, assays with crude antennal extracts showed a considerable amount of radioactivity associated with slow

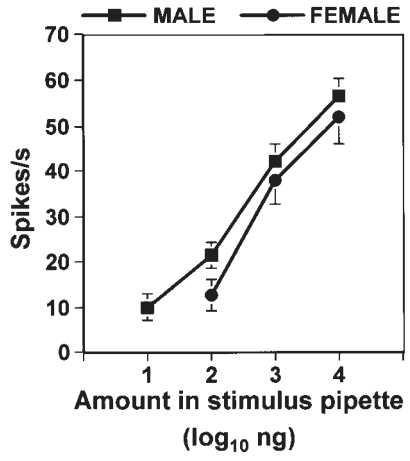

Figure 5 The dose–response relationships from ORNs in the antennae of male and female *A. octiescostata* tuned to the major pheromone constituent (*R*)-buibuilactone (Figure 1.1). Enatiomeric anosmia was confirmed by challenging these ORNs with higher doses of (*S*)-buibuilactone.

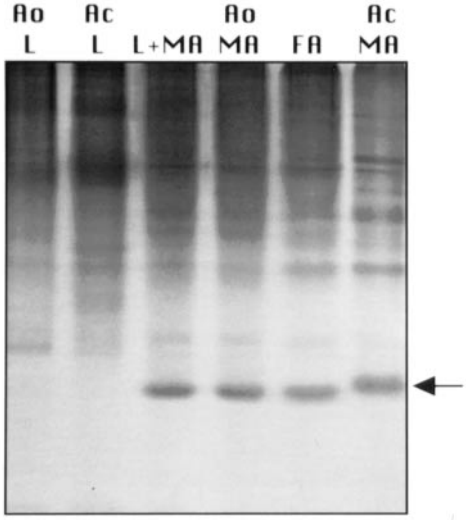

Figure 6 Soluble proteins from *A. octiescostata* (Ao) and *A. cuprea* (Ac) separated on 15% native PAGE. L, legs (three leg-equivalent); MA, male antennae; FA, female antennae (15 antennae-equivalent); L + MA, leg and male antennae of *A. cuprea.* The arrow indicates an antenna-specific protein in *A. cuprea* later identified as AcupPBP. The counterpart protein in *A. octiescostata* migrated slightly faster than AcupPBP.

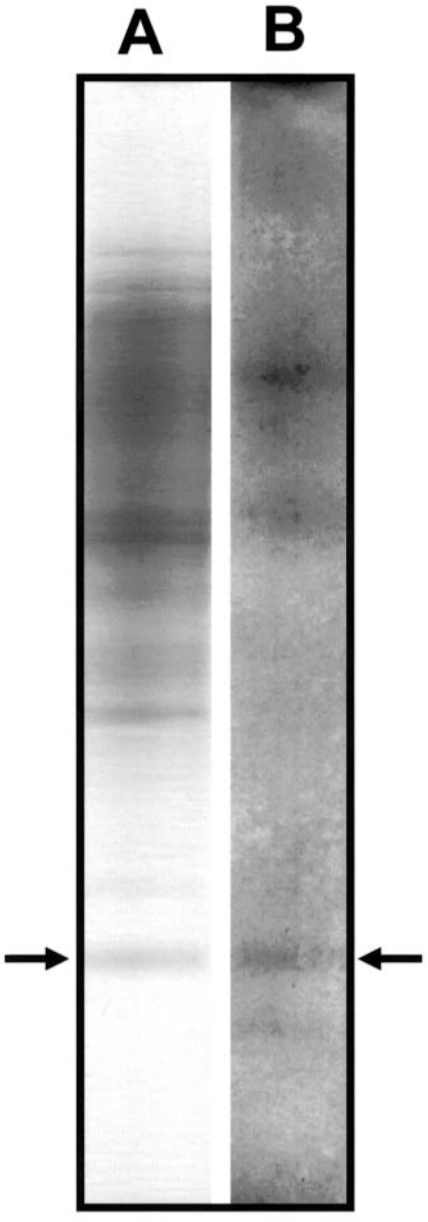

Figure 7 Binding of AoctPBP to (*R*)-japonilure (Figure 1.2) as analyzed by the native PAGE assay. Aliquots of a crude antennal extract were incubated with radiolabelled pheromone. No binding was detected with (*R*)-buibuilactone (data not shown). **(A)** Coomassie blue-stained gel. **(B)** Fluorogram.

migrating proteins in the gel (Figure 7), probably due to non-specific binding.

The N-terminal sequences of the antenna-specific proteins in *A. octiescostata* males (MSEEMEELAKQL-HND-VAQT) and females (MSEEMEELAKQLHND) were indistinguishable. In addition, the amino acid sequences of the protein specific to *A. cuprea* antennae (male and female) gave the sequence MSEEMEELAKQLHND-VAQT.

Characterization of *A. octiescostata* and *A. cuprea* pheromone-binding proteins

Two types of PCR products were amplified from *A. octiescostata* and *A. cuprea* with ~500 and 550 bp, yet the open reading frames for these cDNAs were identical and both coded for proteins with 116 amino acids. Sequences of several clones from *A. octiescostata* (eight from males and seven from females) showed that the male and female PBPs are identical (Figure 8). AoctPBP showed six well-conserved cysteine residues at positions 16, 44, 48, 86, 95 and 114. Based on the encoded sequence, the protein has a p*I* of 4.4 and a (calculated) molecular mass of 12 940 Da (12 934 Da when considering the formation of three disulphide bonds). The mass spectral data of the proteins isolated from the male and female antennae were indistinguishable. The charge envelope was deconvoluted to give a molecular mass of 12 934 Da (Figure 9), indicating that indeed male and female proteins are identical.

Although the two species of scarab beetles *A. octiescostata* and *A. cuprea* use the same sex pheromone components [(*R*)-buibuilactone and (*R*)-japonilure] their PBPs are not identical. Their amino acid sequences had seven

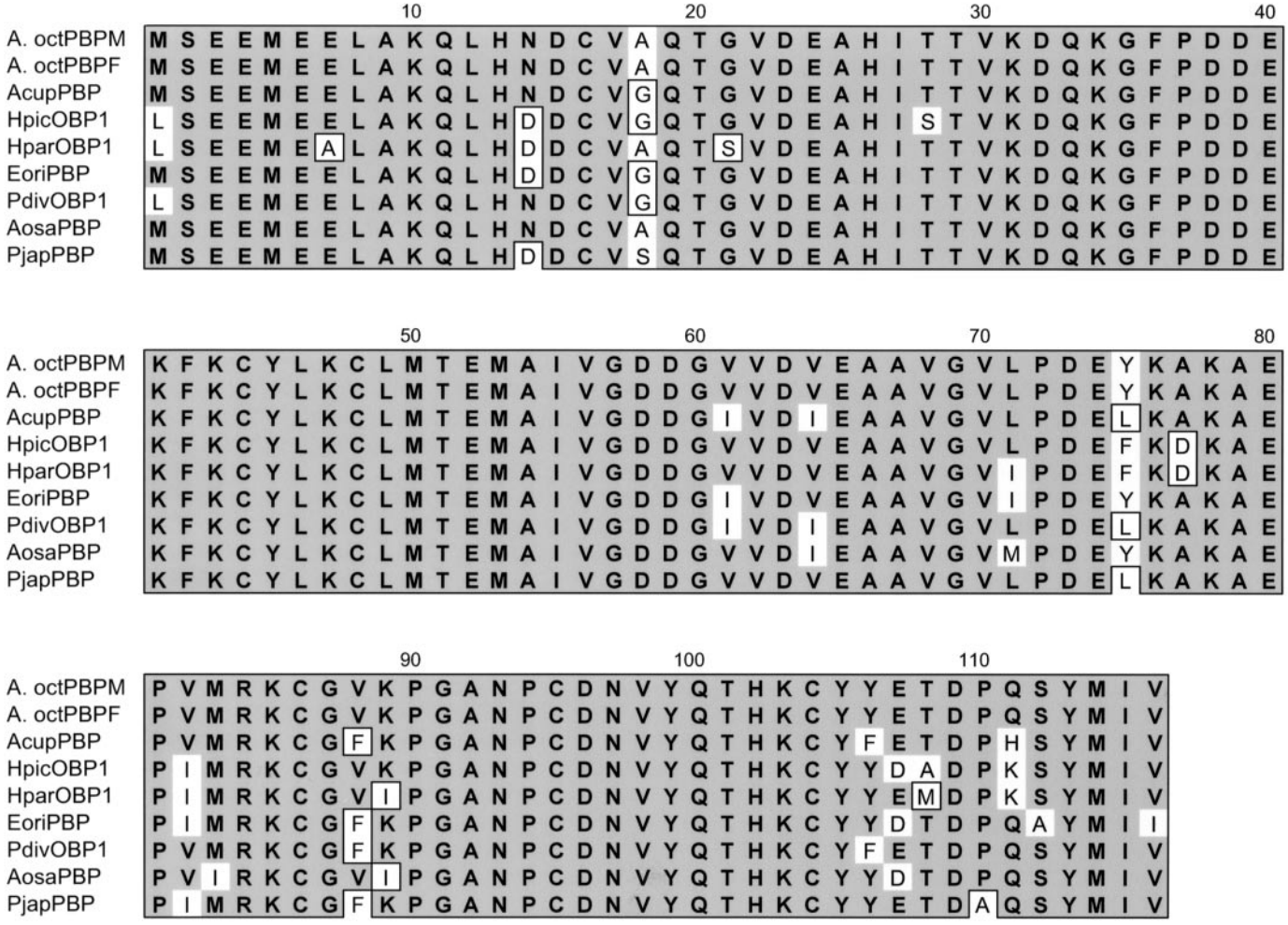

Figure 8 Sequence alignments of the PBPs and OBPs from various species of scarab beetle using the ClustalW algorithm. AoctPBPM, *A. octiescostata* PBP encoded by a male cDNA. AoctPBPF, *A. octiescostata* PBP encoded by a female cDNA. AcupPBP, *A. cuprea* PBP. HpicOBP1, OBP from *H. picea*. HparOBP1, OBP from *H. parallela*. EoriPBP, *Exomala orientalis* PBP. PdivOBP1, OBP from *P. diversa*. AosaPBP, *A. osakana* PBP. PjapPBP, *P. japonica* PBP. The structures of the pheromone constituents of these species are shown in Figure 1. The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases under accession numbers AB040143 for AoctPBP, AB040141 for AcupPBP and AB040981 and AB040980 for the signal peptide sequences for *A. octiescostata* and *A. cuprea* respectively.

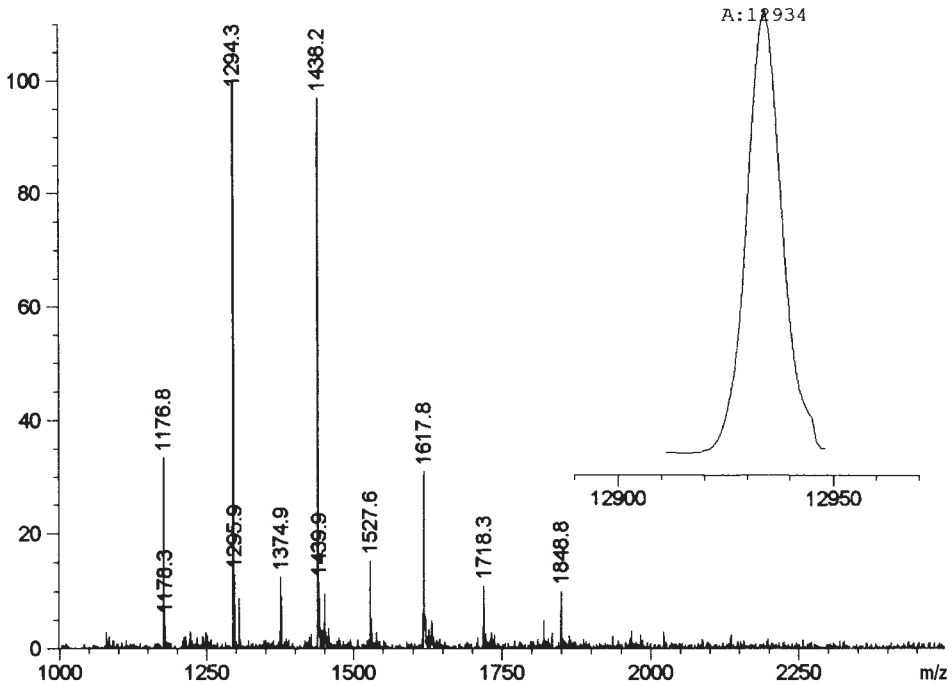

Figure 9 Mass spectrometry data of the antenna-specific protein in male *A. octiescostata* isolated directly from a native polyacrylamide gel. The charge envelope was deconvoluted to give a molecular mass of 12 934 Da (inset). The female antenna-specific protein was indistinguishable from the male protein in retention time (11.8 min), mass spectrometry profile and molecular mass (12 934 Da).

different amino acid residues (Figure 8). AcupPBP differed slightly in molecular mass (12 944 Da or 12 938 Da with disulphide bridges) and p*I* (calculated as 4.49). Scarab PBPs/OBPs show high amino acid identities (86–98%) (Figure 8), with the proteins from *A. cuprea* and *P. diversa* (PdivOPB1) differing in only two amino acid residues, one at the N-terminus (Met-1 versus Leu-1) and the other near the C-terminal sequence (His-101 versus Gln-101).

Discussion

An interesting characteristic of the sensory physiology of scarab beetles is the existence of highly sensitive and selective ORNs that are tuned to (*Z*)-3-hexenyl acetate. The threshold of these ORNs to the green leaf volatile acetate far exceeds the sensitivity of the sex pheromone detectors. The green leaf volatile ORNs in *A. octiescostata* showed even lower threshold responses (<1 pg) than the ORNs previously identified in *P. diversa* (Hansson *et al.*, 1999; Nikonov *et al.*, 2001) and *A. cuprea* (Larsson *et al.*, 2001). The high selectivity of these neurons is demonstrated in the lack of responses to the related alcohol (*Z*)-3-hexenol and other green leaf compounds, such as (*E*)-2-hexenyl acetate, (*E*)-2-hexenol and (*E*)-2-hexenal. Another feature of the sensilla placodea housing the ORNs tuned to (*Z*)-3-hexenyl acetate is the response to anethole as the second best stimulant. The threshold responses to anethole in *A. octiescostata* were four orders of magnitude higher than the response to (*Z*)-3-hexenyl acetate (Figure 2). The amplitudes of the spikes generated by the two stimuli are indistinguishable, but two lines of evidence have suggested that different ORNs (housed in the same sensilla) are activated. First, the (*Z*)-3-hexenyl acetate ORNs did not respond even to other structurally related green leaf volatile compounds. Second, sensilla placodea in scarab beetles in general contain more than one ORN (Meinecke, 1975; Kim and Leal, 2000).

In the field, adults of *A. octiescostata* are attracted to and voraciously feed on dandelion (*Taraxacum officinale*). Field experiments have demonstrated that the attraction is semiochemically mediated, with (*Z*)-3-hexenyl acetate being one of the attractants (Leal *et al.*, 1994b).

The occurrence of ORNs specific to (*Z*)-3-hexenyl acetate in *P. diversa* and *A. cuprea* led us to suggest that these ORNs might carry homologous receptor proteins in these two species of scarab beetle (Larsson *et al.*, 1999). Despite the remarkable selectivity of these ORNs, the photoaffinity-labelled (*Z*)-3-hexenyl diazoacetate (Figure 1.10) mimics the natural ligand so perfectly that the whole detecting machinery in *P. diversa* was tricked by the synthetic ligand. The green leaf acetate receptors responded to the diazo compound with even higher neural activity than that elicited by (*Z*)-3-hexenyl acetate (Nikonov *et al.*, 2001). Interestingly, (*Z*)-3-hexenyl diazoacetate could not trick the olfactory system in *A. octiescostata* (Figure 2). The responses elicited by the diazo compound were three orders of magnitude lower than the response to the natural acetate. These results suggest that, although tuned to the same natural ligand, namely (*Z*)-3-hexenyl acetate, the specific olfactory receptor proteins in *P. diversa* and *A. octiescostata*

are different. Olfactory receptors have been identified in *D. melanogaster* (Clyne *et al*., 1999; Vosshall *et al*., 1999). It has been observed (L.B. Vosshall, personal communication) that, although *D. melanogaster* and *Caenorhabditis elegans* 'smell' diacetyl (2,3-butanedione), there is no receptor protein in flies with significant amino acid similarity to the nematode diacetyl receptor odr10 (Zhang *et al*., 1997). This is consistent with the hypothesis that *A. octiescostata* and *P. diversa* detect (*Z*)-3-hexenyl acetate with different olfactory receptor proteins.

In addition to the green leaf volatile detectors, *A. octiescostata* possesses ORNs for the detection of floral compounds (Figure 4). All sensilla placodea housing ORNs responding to phenylethyl propionate also responded to geraniol. These compounds are biologically relevant for *A. octiescostata* as a mixture of anethole, geraniol and phenyl propionate increases the trap catches of female beetles (Leal *et al*., 1994b). Given that the spike amplitudes in response to the two semiochemicals are identical, it was not possible to determine accurately whether the neuronal activities were generated by the same or different ORNs co-localized in the same sensilla. Considering that the chemical structures of the two ligands are unrelated (Figure 1) and taking into consideration that each sensillum generally contains more than one ORN (Meinecke, 1975; Kim and Leal, 2000), it is tempting to conclude that phenylethyl propionate and geraniol stimulate different ORNs co-localized in the same sensilla.

As has been observed in *A. cuprea* (Leal and Mochizuki, 1993; Larsson *et al*., 1999), the pheromone-detecting ORNs in *A. octiescostata* also showed remarkable enantiomeric specificity. The pheromone detectors were silent even when challenged with (*S*)-buibuilactone at ×10 000 higher concentrations than the threshold to the major pheromone component (*R*)-buibuilactone. Although the Japanese beetle *Popillia japonica* and the Osaka beetle *Anomala osakana* detect both enantiomers of japonilure, one as a sex pheromone and the other as a behavioural antagonist (Wojtasek *et al*., 1998), it has been suggested that these are particular cases, with the inability to detect non-natural stereoisomers of pheromones (enantiomeric anosmia) being a common feature in the sensory physiology of scarab beetles (Leal, 1999).

Pheromone detectors have been found in the male and female antennae of *A. octiescostata*, consistent with the observation that both male and female beetles are attracted to the female-released sex pheromone (Leal *et al*., 1994a). As in the Japanese beetle (Kim and Leal, 2000), the number of pheromone-detecting sensilla placodea in female antennae is lower than their counterparts in male beetles. In moths, female antennae of most species are apparently anosmic to their own pheromone while autodetection of female pheromones is a less observed phenomenon (Schneider *et al*., 1998) (see also references therein).

The lack of dimorphism in scarab beetles is also observed in the pheromone-detecting biochemical machinery. PBPs are found in the male and female antennae in *A. octiescostata* and *A. cuprea* (Figure 6). The identical amino acid sequence in the male and female PBPs was demonstrated by cloning the cDNAs of male and female AoctPBP (Figure 8), as well as by the measurement of the molecular masses of the native proteins (Figure 7). That *A. octiescostata* and *A. cuprea* PBPs are 'unisex' is consistent with the electrophysiological data.

Despite the fact that *A. octiescostata* and *A. cuprea* use the same sex pheromone and, consequently, their sensory systems are involved in the detection of identical compounds, AoctPBP and AcupPBP differed in seven residues at positions 18, 61, 64, 75, 88, 106 and 111. Even OBPs from scarab beetles of different subfamilies can interact with the same ligand. As shown here (Figure 7) the PBP from *A. octiescostata*, which is a ruteline species, binds to (*R*)-japonilure. OBP1 from *P. diversa*, which is a melolonthine species, binds to the same ligand (Wojtasek *et al*., 1999). However, the amino acid sequences of the two proteins (AoctPBP and PdivOPB1) showed only 93% identity (Figure 8). It would be interesting to determine whether the different amino acid residues of these OBPs are involved in forming the binding cavities of these proteins.

We have previously observed a pH-dependent conformational change in the PBP of the silkworm moth *B. mori* (Wojtasek *et al*., 1998), which leads to the formation of the acid and basic forms of the protein, i.e. $BmPBP^{A}$ and $BmPBP^{B}$ respectively (Damberger *et al*., 2000). This conformational change was hypothesized as a mechanism for the release of pheromone to the receptors upon interaction with negatively charged membranes at the surface of dendrites (Leal, 2000). Based on structural data, it was proposed that three histidine residues at positions 69, 70 and 95, which are strictly conserved across all known lepidopteran PBPs and OBPs, might contribute to the conformational change (Sandler *et al*., 2000). Comparison of $BmPBP^{A}$ with the protein conformation in the bound complex revealed that the histidine side-chains are indeed more widely separated in $BmPBP^{A}$, which may be the result of charge repulsion by protonation at a slightly acidic pH value (Horst *et al*., 2001). Interestingly, scarab PBPs (Figure 8) possess three strictly conserved histidine residues (His-13, His-26 and His-102).

Of the residues involved in binding to bombykol in $BmPBP^{B}$, the most variable in other lepidopteran PBPs (Met-61, Leu-62, Ile-91 and Val-114) are either valine, leucine, isoleucine or methionine. These variations are suggested to be specificity determinants, with the binding cavity being most sensitive to the length of the pheromone (Sandler *et al*., 2000). Hitherto, there is no structural data on scarab OBPs for determining which residues are involved in pheromone binding. However, it is worth mentioning that the four variable residues at positions 61, 64, 71 and 82 (Figure 8) are either valine, leucine, isoleucine or methionine. Interestingly, in two proteins that bind to (*R*)-japonilure,

AoctPBP and PdivOBP1, these four residues are identical. On the other hand, AcupPBP is involved in the detection of the same pheromone system as AoctPBP, but the two proteins have a pair of different residues at two of the positions, i.e. Val-61 and Val-64 in AoctPBP and Ile-61 and Ile-64 in AcupPBP.

In summary, research has been conducted in order to obtain a better insight into the sensory physiology of scarab beetles. ORNs specific to the detection of ecologically significant compounds have been identified in *A. octiescostata*. In addition to the remarkably sensitive detectors for (*Z*)-3-hexenyl acetate, other sensilla placodea housing ORNs tuned to floral compounds were characterized. Pheromone detectors were identified in male and female antennae. The PBPs isolated from male antennae were identical to those isolated from female antennae. The PBPs from the two species investigated, namely *A. cuprea* and *A. octiescostata*, showed only 93% amino acid similarity. Comparison of the sequences of the scarab OBPs identified to date with the structure of BmPBP shows some interesting features of scarab PBPs, which might be related to the pH-dependent conformational change and specificity binding determinants.

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References

Campanacci, V., Krieger, J., Bette, S., Sturgis, J.N., Lartigue, A., Cambillau, C., Breer, H. and **Tegoni, M.** (2001) *Revisiting the specificity of* Mamestra brassicae *and* Antheraea polyphemus *pheromone-binding proteins with a fluorescence binding assay*. J. Biol. Chem., 276, 20 078–20 084.

Clyne, P.J., Warr, C.G., Freeman, M.R., Lessing, D., Kim, J. and **Carlson, J.R.** (1999) *A novel family of divergent seven-transmembrane proteins: candidate odorant receptors in* Drosophila. Neuron, 22, 327–338.

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

Deyu, Z. and **Leal, W.S.** (2002) *Conformational isomers of insect odorant-binding proteins*. Arch. Biochem. Biophys., 397, 99–105.

Hansson, B.S., Larsson, M.C. and **Leal, W.S.** (1999) *Green leaf volatile-detecting olfactory receptor neurones display very high sensitivity and specificity in a scarab beetle*. Physiol. Entomol., 24, 121–126.

Horst, R., Damberger, F., Luginbuhl, P., Guntert, P., Peng, G., Nikonova, L., Leal, W.S. and **Wuthrich, K.** (2001) *NMR structure reveals intramolecular regulation mechanism for pheromone binding and release*. Proc. Natl Acad. Sci. USA, 98, 14 374–14 379.

Hubel, D.H. (1957) *Tungsten microelectrode for recording from single units.* Science, 125, 549–550.

Kaissling, K.-E. (2001) *Olfactory perireceptor and receptor events in moths: akinetic model*. Chem. Senses, 26, 125–150.

Kim, J.-Y. and **Leal, W.S.** (2000) *Ultrastructure of pheromone-detecting sensillum placodeum of the Japanese beetle,* Popillia japonica *Newmann (Coleoptera: Scarabaeidae)*. Arthropod Struct. Dev., 29, 121–128.

Kim, M.-S. and **Smith, D.P.** (2001) *The invertebrate odorant-binding protein LUSH is required for normal olfactory behavior in* Drosophila. Chem. Senses, 26, 195–199.

Larsson, M.C., Leal, W.S. and **Hansson, B.S.** (1999) *Olfactory receptor neurons specific to chiral sex pheromone components in male and female* Anomala cuprea *beetles (Coleoptera: Scarabaeidae)*. J. Comp. Physiol. A, 184, 353–359.

Larsson, M.C., Leal, W.S. and **Hansson, B.S.** (2001) *Olfactory receptor neurons detecting plant odours and male volatiles in* Anomala cuprea *beetles (Coleoptera: Scarabeidae)*. J. Insect Physiol., 47, 1065–1076.

Leal, W.S. (1991) *(R,Z)-5-(–)-(Oct-1-enyl)oxacyclopentan-2-one, the sex pheromone of the scarab beetle* Anomala cuprea. Naturwissenschaften, 78, 521–523.

Leal, W.S. (1998) *Chemical ecology of phytophagous scarab beetles*. Annu. Rev. Entomol., 43, 39–61.

Leal, W.S. (1999) *Enantiomeric anosmia in scarab beetles*. J. Chem. Ecol., 25, 1055–1066.

Leal, W.S. (2000) *Duality monomer–dimer of the pheromone-binding protein from* Bombyx mori. Biochem. Biophys. Res. Commun., 268, 521–529.

Leal, W.S. and **Mochizuki, F.** (1993) *Sex pheromone reception in the scarab beetle* Anomala cuprea*: enantiomeric discrimination by sensilla placodea*. Naturwissenschaften, 80, 278–281.

Leal, W.S., Sawada, M. and **Hasegawa, M.** (1993) *The scarab beetle* Anomala cuprea *utilizes the sex pheromone of* Popillia japonica *as a minor component*. J. Chem. Ecol., 19, 1303–1313.

Leal, W.S., Hasegawa, M., Sawada, M., Ono, M. and **Ueda, Y.** (1994a) *Identification and field evaluation of* Anomala octiescostata *(Coleoptera: Scarabaeidae) sex pheromone*. J. Chem. Ecol., 20, 1643–1655.

Leal, W.S., Ono, M., Hasegawa, M. and **Sawada, M.** (1994b) *Kairomone from dandelion,* Taraxacum officinale*, attractant for scarab beetle* Anomala octiescostata. J. Chem. Ecol., 20, 1697–1704.

Meinecke, C.-C. (1975) *Riechsensillen und Systematik der Lamellicornia (Insecta, Coleoptera)*. Zoomorphologie, 82, 1–42.

Murlis, J. (1997) *Odor plumes and the signal they provide*. In Cardé, R.T. and Minks, A.K. (eds), Insect Pheromone Research: New Directions. Chapman & Hall, New York, pp. 221–247.

Nikonov, A.A., Valiyaveettil, J.T. and **Leal, W.S.** (2001) *A photoaffinity-labeled green leaf volatile compound 'tricks' highly selective and sensitive insect olfactory receptor neurons*. Chem. Senses, 26, 49–54.

Peng, G. and **Leal, W.S.** (2001) *Identification and cloning of a pheromone-binding protein from the Oriental beetle,* Exomala orientalis. J. Chem. Ecol., 27, 2183–2192.

Sandler, B.H., Nikonova, L., Leal, W.S. and **Clardy, J.** (2000) *Sexual*

attraction in the silkworm moth: structure of the pheromone-binding protein–bombykol complex. Chem. Biol., 7, 143–151.

Schneider, D., Schultz, S., Priesner, E., Ziesmann, J. and **Francke, W.** (1998) *Autodetection and chemistry of female and male pheromone in both sexes of the tiger moth* Panaxia quadripunctaria. J. Comp. Physiol. A, 182, 153–161.

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

Vosshall, L.B., Amrein, H., Morozov, P.S., Rzhetsky, A. and **Axel, R.** (1999) *A spatial map of olfactory receptor expression in the* Drosophila *antenna*. Cell, 96, 725–736.

Wojtasek, H., Hansson, B.S. and **Leal, W.S.** (1998) *Attracted or repelled: a matter of two neurons, one pheromone binding protein, and a chiral center*. Biochem. Biophys. Res. Commun., 250, 217–222.

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.

Zacks, S. (1971) The Theory of Statistical Interference. John Wiley, New York.

Zhang, Y., Chou, J.H., Bradley, J., Bargmann, C.I. and **Zinn, K.** (1997) *The* Caenorhabditis elegans *seven-transmembrane protein ODR-10 functions as an odorant receptor in mammalian cells.* Proc. Natl Acad. Sci. USA, 94, 12 162–12 167.

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