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Are Odorant-binding Proteins Involved in Odorant Discrimination?

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Abstract

Pheromone-sensitive sensilla trichodea of nine moth species belonging to six families and three superfamilies of Lepidoptera were immunolabelled with an antiserum against the pheromone-binding protein of *Antheraea polyphemus*. Strong immunolabelling of the sensillum lymph was observed in all long sensilla trichodea of *A. polyphemus, A. pernyi* (Saturniidae), *Bombyx mori* (Bombycidae) and *Manduca sexta* (Sphingidae). Very weak labelling was found with all sensilla trichodea of *Dendrolimus kikuchii* (Lasiocampidae) and *Lymantria dispar* (Lymantriidae). In three noctuid species, some long sensilla trichodea were labelled strongly, some only weakly and some were not labelled at all. The fraction of long sensilla trichodea that were strongly labelled was large in *Helicoverpa armigera*, but small in *Spodoptera littoralis* and *Autographa gamma*. The observed cross-reactivity was not correlated with taxonomic relatedness of the species but rather with chemical relatedness of the pheromones used by these species, as a high labelling density was consistently observed in sensilla tuned to pheromones with an alcyl chain of 16 carbon atoms. The highly divergent specificity of pheromone-receptor cells in Noctuidae appears to be mirrored by a similar diversity of the pheromone-binding proteins in the sensilla trichodea. These data support the notion that pheromone-binding proteins participate in odorant discrimination. **Chem. Senses 21: 719–727, 1996.**

Introduction

When the first vertebrate odorant-binding protein had been identified from bovine nasal mucosa, it was thought to be involved in odorant recognition (Pelosi *et al.*, 1982). Soon, however, this function was again questioned, because it became apparent that these soluble, low molecular weight proteins, which are concentrated in the mucus covering the olfactory receptor cells, display a rather low binding specificity for odorants. Even now, despite an abundance of biochemical information, the biological function of odorant-binding proteins remains indistinct; generally it is sought among perireceptor events, such as stimulus transport and/or inactivation (for review see Pelosi, 1994).

In insect antennae, proteins of similar molecular weight were detected even earlier than in vertebrates (Vogt and Riddiford, 1981). Despite a different primary structure, these proteins are also termed odorant-binding proteins, because in some cases odorant binding could be shown (reviewed in Pelosi and Maida, 1995; see also Discussion). Insect odorant-binding proteins are arranged into three major classes, based upon comparison of their amino acid sequence. 'Pheromone-binding proteins' (PBPs) (Vogt and Riddiford, 1981) are predominant in antennae of male moths and only the males have a large number of sensilla that specifically respond to the female sex attractant

pheromone. Two classes of 'general odorant-binding proteins' (GOBP1 and GOBP2) (Breer *et al.*, 1990; Vogt *et al.*, 1991a,b) are found in both male and female antennae, consistent with the distribution of the so-called general odorant receptor cells (Schneider *et al.*, 1964). Despite the low amino acid identity between these three classes, there are some characteristic common features of insect odorant-binding proteins, such as six cysteine residues at conserved positions and a similar hydropathy profile (Breer *et al.*, 1990; Krieger *et al.*, 1993).

At least one PBP and one GOBP have been found in every moth species tested, several species have one PBP and two GOBPs, and in some cases two PBPs are reported (*Antheraea pernyi*, Krieger *et al.*, 1991; *Lymantria dispar*, Vogt *et al.*, 1989). Five putative odorant-binding proteins have been revealed in *Drosophila melanogaster* (McKenna *et al.*, 1994; Pikielny *et al.*, 1994). Also in moths the number of these proteins per species has been increased by the use of refined separation methods; in *Bombyx mori*, for example, as many as four PBPs and five GOBPs have now been found which differ primarily in their isoelectric point (Maida *et al.*, 1995; R.Maida *et al.*, in preparation). Thus the urgent question, whether odorant-binding proteins are involved in olfactory specificity, is being asked again and evidence compatible with this idea is increasing (for review see Prestwich *et al.*, 1995).

The morphology of insect olfactory organs fits well to the diversity of odorant-binding proteins, as the receptor cells of insects are compartmentalized in the so-called sensilla with separate sensillum–lymph spaces, in contrast to vertebrates where olfactory receptor cells are arranged in a mucosa covered by a common mucus layer. But even in the vertebrate nasal mucus, multiple domains and substantial microchemical heterogeneity have been revealed by topochemical methods (Menco and Farbman, 1992; Getchell *et al.*, 1993). Insect sensilla can be morphologically classified into different types, and by electrophysiological recording the specificity of their receptor cells can be recognized (for review see Keil and Steinbrecht, 1984; Kaissling, 1987).

Immunocytochemistry allows the gap between the biochemical, electrophysiological and morphological data to be bridged. Using a polyclonal antiserum against the PBP of *Antheraea polyphemus*, it was possible to prove that this protein is found predominantly in the pheromone-sensitive sensilla trichodea (s. trichodea) of the male moth (Steinbrecht *et al.*, 1992). Another antiserum against the GOBP of the same species primarily labels sensilla basiconica (Laue *et al.*, 1994; Steinbrecht *et al.*, 1995) which respond to a wide variety of plant and other non-pheromonal odours, thus justifying the term 'general odorant protein'.

Steinbrecht *et al.* (1992) observed that the antiserum against the PBP of *A. polyphemus* cross-reacted also with the sensillum lymph of other species, in the pheromone-sensitive s. trichodea of male *Bombyx mori* and in a subset of the s. trichodea of male *Autographa gamma* (Noctuidae). Taking advantage of this cross-reactivity, a comparative immunocytochemical study was started which now comprises nine species from six different lepidopteran families. Here, the first results are reported which reveal that species which show strong cross-reactivity also use similar pheromones.

Materials and methods

Antheraea polyphemus Cramer, *A. pernyi* Guerin- Meneville, and *B. mori* L. were obtained as pupae from various commercial breeders; *Manduca sexta* L. were bred and kindly supplied by Professor Wieczorek, University of Munich, *Spodoptera littoralis* Boisd. by Dr Bill Hansson, University of Lund, *Helicoverpa armigera* Hb. by Dr Elke Hartlieb, Seewiesen; *Dendrolimus kikuchii* Matsumura were collected during an outbreak in Yunnan Province, China, by Mr Zhang Shan-gan, Academia Sinica, Beijing; *Lymantria dispar* L. were collected during an outbreak in Upper Bavaria in spring 1994. *Autographa gamma* L. were caught with a light trap during the summer months in Seewiesen. Except for *A. gamma*, where the age of moths was unknown, moths of the other species were killed 2–7 days after emergence from the pupae.

Antennae were cryofixed and freeze-substituted as described in detail by Steinbrecht (1993), embedded in LR White resin and sectioned with a diamond knive. For immunocytochemistry a polyclonal rabbit antiserum against purified PBP of *A. polyphemus* [anti-PBP(Apo)] was used in dilutions of 1:3000–1:30 000 and labelled by a secondary antibody [goat-anti-rabbit IgG conjugated with 10 nm colloidal gold (GAR10)] in a dilution of 1:20. Silver intensification according to Danscher (1981) enlarged the grains to some 40 nm. Sections were stained with uranyl acetate. Details of antibody preparation and immunocytochemical protocols are given in Steinbrecht *et al.* (1992, 1995).

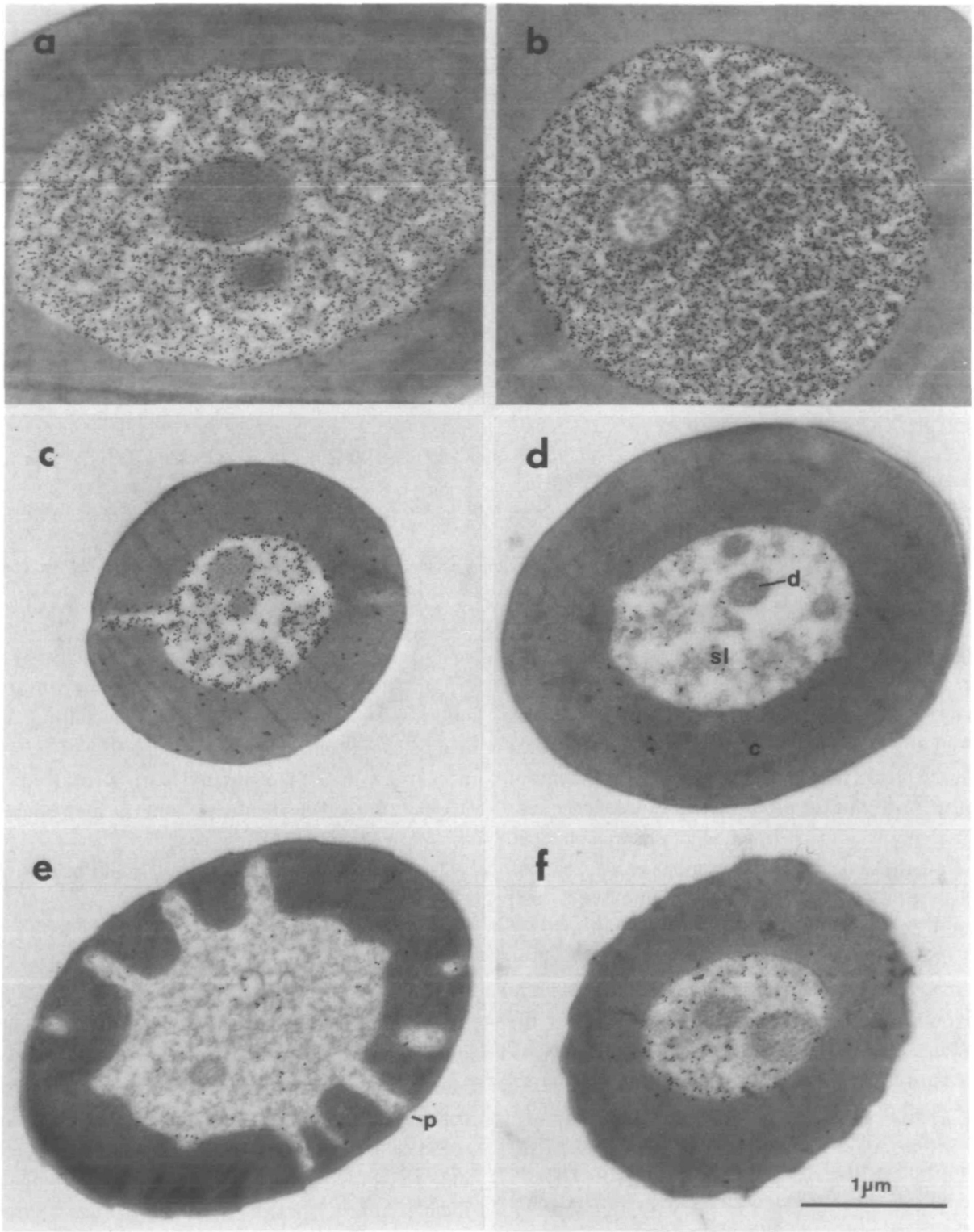

Figure 1 Cross sections of male pheromone-sensitive long sensilla trichodea of six moth species belonging to six different families after immunolabelling with anti-PBP(Apo) as shown by 10 nm gold grains. All electronmicrographs are from the same labelling experiment (dilution of primary antibody 1:3000). In *A. polyphemus* (**a**), *M. sexta* (**b**), *B. mori* (**c**) labelling of the sensillum lymph (sl) was always very strong, whereas in *D. kikuchii* (**d**) and *L. dispar* (**e**) it was always very weak. In *A. gamma* (**f**), as in the other noctuid moths, labelling density differed among the s. trichodea; the micrograph shows a B-type sensillum with intermediate labelling density. The dendrites (d) are not labelled; on the cuticle (c) some unspecific background staining is visible. Cuticular pores (p) are not encountered on every section, in particular close to the hair bases.

Table 1 Quantitative immunocytochemistry of pheromone-binding proteins in the sensillum lymph of long sensilla trichodea of nine moth species

Species[a]	Hair subtype[b]	*n*	Total area (μm^2)	Mean label density (gold grains/μm^2 ± SD)
1 *A. polyphemus*		16	94	596 ± 46
2 *A. pernyi*		15	98	596 ± 58
3 *B. mori*		19	22	619 ± 62
4 *D. kikuchii*		20	38	38 ± 12
5 *M. sexta*		13	73	648 ± 66
6 *L. dispar*		20	49	28 ± 9
7 *H. armigera*	A	13	6	501 ± 61
8 *S. littoralis*	A	14	6	431 ± 69
9 *A. gamma*	A	14	11	660 ± 63
	B	8	7	157 ± 52
	C	4	4	58 ± 16
Haemolymph	–	10	148	1.7 ± 0.8

n, number of sensilla; total area, sum of evaluated section profiles per species (in *A. gamma*, *n* does not represent the frequency of subtypes).

Label density is expressed in numbers of gold grains per μm^2 of compartment profile ± SD. Haemolymph data of the different species were pooled and represent non-specific background.

[a]Species nos. 1–4 belong to the superfamily Bombycoidea, no. 5 to Sphingoidea, nos. 6–9 to Noctuoidea. All data are derived from the same labelling experiment.

[b]Labelling of long s. trichodea was uniform in species nos. 1–6, but in the three noctuid species (nos. 7–9), morphologically similar s. trichodea displayed different labelling densities and were categorized into subtypes A, B, C as shown for *A gamma*. In *S. littoralis* and *A. gamma*, the majority of s. trichodea remained unlabelled (cf. Figure 3).

For qualitative comparison, at least 100 sensilla in at least two antennae were observed in each species. For quantitative evaluation of labelling density, antennal sections of all species were labelled together in the same immunocytochemical experiment; the blocks had been prepared following identical cryofixation and substitution protocols (substitution medium: acetone containing 3% glutaraldehyde) and, wherever possible, were taken from the same freeze-substitution process. Sections were labelled with anti-PBP(Apo) in a dilution of 1:3000 overnight at 4°C, and with GAR10 in a dilution of 1:20 for 90 min at room temperature without subsequent silver intensification. For each species, at least 12 labelled sensilla were chosen at random, photographed at 20 000:1, and the negatives scanned with a flatbed scanner (UMAX Powerlook II) connected to an Apple Power PC (8500/120). Image analysis (i.e. measurement of area and number of particles) was done with a customized version of NIH Image program (version 1.58, developped at the US National Institutes of Health; customized by Steve Barrett, Surface Science Research Centre, University of Liverpool, and self-made macros).

Results

The long s. trichodea that in male moths carry the pheromone-sensitive receptor cells can be easily discriminated from other antennal olfactory sensilla by the great length, diameter and wall thickness of the sensory hair. In addition, s. trichodea have essentially unbranched outer dendritic segments and a smaller number of pores per unit of surface area as compared with s. basiconica, which display branched dendrites and a high pore density (Steinbrecht, 1973).

The level of non-specific background was estimated by evaluating the labelling density on haemolymph which is a similar extracellular compartment but devoid of PBP as shown by gel electrophoresis and Western blots (G.Ziegelberger, unpublished data). Haemolymph never had more than 4 gold grains/μm^2 (Table 1). Cuticle in some species showed higher labelling (e.g. Figure 1c), but this was also observed in controls using pre-immune serum and therefore has to be considered non-specific. Whenever a sensillum was specifically labelled by anti-PBP(Apo), the grain density was uniform throughout the sensillum–lymph compartment in the hair lumen. In cases of inhomogeneous electron density of the sensillum–lymph, most of the grains were found on the denser parts, indicating that most of the binding protein is present in or actually is represented by the dense substance (Figure 1c,d).

All long s. trichodea displayed identical labelling properties in the following species: *A. polyphemus*, *A. pernyi*, *B. mori*, *M. sexta*, *D. kikuchii* and *L. dispar*. However, while

Table 2 Percentage of identical amino acids in fully sequenced pheromone-binding proteins of six moth species[a]

PBP	Apol	Aper-1	Aper-2	Bmor	Msex	Ldis-2	Hvir
Apol	–						
Aper-1	90	–					
Aper-2	87	85	–				
Bmor	67	64	66	–			
Msex	68	64	70	73	–		
Ldis-2	47	45	44	49	44	–	
Hvir	59	57	60	68	68	51	–

[a]Apol, PBP1 of *Antheraea polyphemus* (Raming *et al.*, 1989); Aper-1, PBP1 of *Antheraea pernyi* (Raming *et al.*, 1990); Aper-2, PBP2 of *Antheraea pernyi* (Krieger *et al.*, 1991); Bmor, PBP1 of *Bombyx mori* (Krieger *et al.*, 1996); Msex, PBP1 of *Manduca sexta* (Györgyi *et al.*, 1988); Ldis-2, PBP2 of *Lymantria dispar* (Prestwich *et al.*, 1995); Hvir, PBP1 of *Heliothis virescens* (Krieger *et al.*, 1993).

Percentage values according to Krieger *et al.* (1996) and S. LaForest and J. Krieger (personal communication).

the first four species displayed very strong cross-reactivity, the grain densities on the sensillum lymph of long s. trichodea of *D. kikuchii* and *L. dispar* was always extremely low (Table 1, Figure 1). After mild osmium fixation at –70°C during the freeze-substitution, the cross-reaction in *Dendrolimus* was totally abolished ($n > 200$ hairs in two antennae), while in *Bombyx* the same schedule reduced the cross-reactivity only by 37% (data not shown).

In the three noctuid species, *H. armigera, S. littoralis* and *A. gamma*, only a fraction of the long s. trichodea was labelled. In addition, different sensilla displayed different grain densities, as has been quantitatively evaluated in *A. gamma* (Table 1). Only some s. trichodea showed a high labelling density comparable to that in *Antheraea;* these are designated as type-A hairs in this study (B- and C-type hairs have moderate and low labelling density, respectively). The fraction of type-A hairs was large in *H. armigera,* but small in *S. littoralis* and *A. gamma* (Figure 3). Morphological differences between the type-A hairs and the other subtypes of s. trichodea were either absent or minimal. In *S. littoralis* and *A. gamma*, the type-A hairs were usually somewhat shorter than the other hairs and were located more towards the centre of the sensillum field. In *A. gamma*, all type-A hairs had three sensory cells, whereas the other subtypes of s. trichodea had two or three sensory cells.

Discussion

Pheromone-sensitive sensilla are particularly suitable for a study of specificity relationships because, other than in most generalist olfactory sensilla, the natural stimulus is precisely known. Moreover, there are good reasons to assume that in these sensilla the specificity and sensitivity of olfaction has evolved to a maximum (Schneider, 1992). Kaissling and Priesner (1970) have shown that a single pheromone molecule may be able to trigger an action potential. They already mentioned that, in order to perform at this high sensitivity, the stimulus transport system from the adsorption on the cuticular surface of the sensillum to the receptor membrane must work with extreme efficiency. The evolution of a specific class of pheromone-binding proteins should be seen in this context.

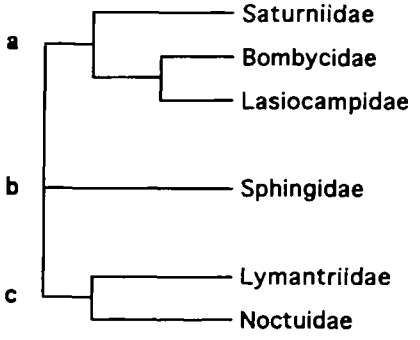

Figure 2 Cladogram illustrating the taxonomic relationships between the six moth families studied (other groups omitted). The branches a, b and c correspond to the superfamilies Bombycoidea, Sphingoidea and Noctuoidea of Richards and Davies (1977).

Whereas both classes of GOBPs have fairly high amino acid identity within each class (84–100%; Pelosi and Maida, 1995), a much larger variability of sequences is found with the PBPs ranging from 44 to 90% (Table 2). The PBP2 of *Lymantria dispar*, which has the lowest amino acid identity with the PBP of *A. polyphemus* (47%), also shows an extremely low cross-reactivity with anti-PBP(Apo). It is

more surprising that the PBPs of *B. mori* and *M. sexta* show no reduction of cross-reactivity despite a considerably reduced amino acid identity (67 and 68%). However, the three-dimensional structures of different PBPs might be even more similar than their amino acid sequences, because six cysteine residues are conserved among all sequenced odorant-binding proteins. In addition, Western blots of native gels showed a stronger cross-reaction with anti-PBP(Apo) than did gels under denaturing conditions (Steinbrecht *et al.*, 1995; R.Maida and G.Ziegelberger, unpublished data). The tissue preparation and labelling protocol of the immunocytochemical experiments are probably closer to the situation of the native protein than of the denatured and reduced protein; this may explain that some PBPs with fairly low sequence identities nevertheless show good labelling with anti-PBP(Apo).

In the case of *D. kikuchii* and *L. dispar,* the extremely low cross-reactivity of anti-PBP(Apo) could have qualitative and/or quantitative reasons. The s. trichodea in these species might either contain a highly different PBP or a very low concentration of PBP. The latter is unlikely, as wherever PBP concentration in sensory hairs has been estimated, it was found extremely high (of the order of 10 mM, Vogt and Riddiford, 1981, Klein, 1987); for *Lymantria dispar,* Vogt *et al.* (1989) computed the total concentration of PBP in the long s. trichodea as 13.4 mM.

Obviously there is no good correlation of the cross-reactivity of anti-PBP(Apo) with taxonomic relatedness. The species studied in this paper belong to three different superfamilies (Figure 2). Strong cross-reactivity and, therefore, similar PBPs are observed in certain species of all three superfamilies examined while, at the same time, other species, even within the same superfamily as *Antheraea,* display very low cross-reactivity and hence express a PBP greatly different from that of *A. polyphemus.*

In Figure 3, the results of our immunolabelling study are supplemented by data on the pheromone components used by these species. In all cases, where there is strong cross-reaction with anti-PBP(Apo), the pheromones have the same alcyl-chain length (C-16) as in *A. polyphemus. Lymantria* and *Dendrolimus,* on the contrary, show a very low cross-reactivity; they also use rather different sex pheromones. The pheromone of *D. kikuchii* is still not known, but all those *Dendrolimus* species (*D. pini, D. punctatus, D. spectabilis, D. tabulaeformis*) whose pheromones have been identified use the same C-12 alcyl chains only with different terminal functional groups (Arn *et al.*, 1992). Thus, cross-reactivity of sensilla with anti-PBP(Apo) is correlated with the pheromone-specificity of these sensilla and is particularly strong in sensilla which respond to pheromones of the same alcyl-chain length as in *A. polyphemus.*

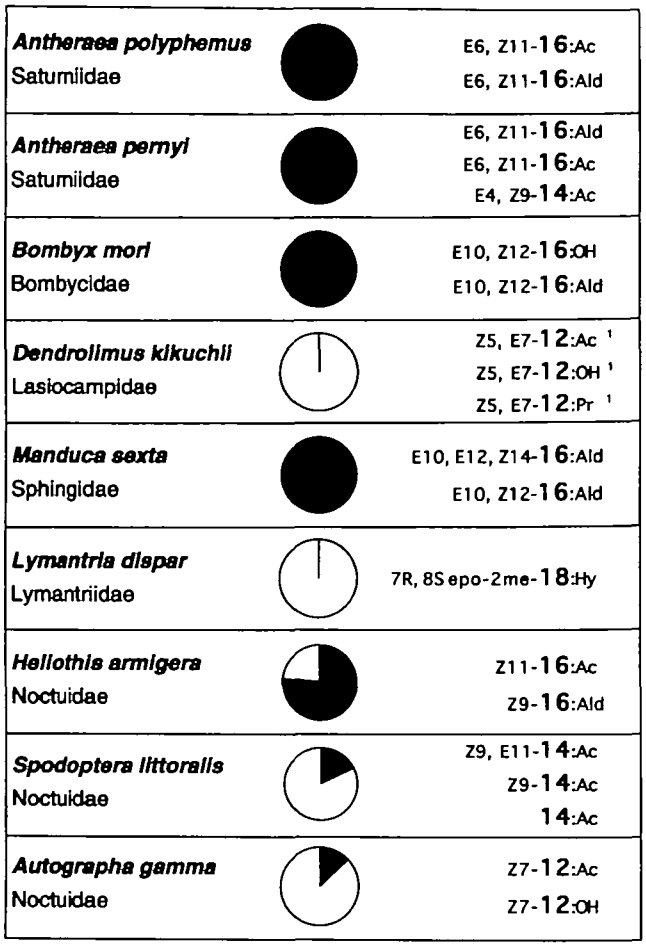

Figure 3 The nine moth species of this study arranged according to taxonomic relatedness and their main pheromone components in short annotation [the large number gives the number of C-atoms of the alcyl chain; to the left of it, the position and type of double bonds and side chains, to the right of it, the type of functional group at C_1 is indicated (Ac, acetate; Ald, aldehyde; Hy, hydrocarbon without functional group; OH, alcohol; Pr, propionate)—see Arn *et al.* (1992) for further details]; e.g. E6,Z11–16:Ac means E6,Z11-hexadecenyl-1-acetate and 7R,8Sepo-2me-18:Hy means 7R,8S-epoxy-2-methyl-octadecane. The pie charts indicate the percentage of long s. trichodea on male antennae that strongly cross-react with anti-PBP(Apo), i.e. with grain densities >350/mm^2 (A-type sensilla). Cross reaction is strong in species using pheromone components with an alcyl-chain length of 16 carbon atoms. In Noctuidae, the functional specificity of labelled sensilla is not known; probably the cross-reacting sensilla in *H. armigera,* but not the few cross-reacting sensilla in *S. littoralis* and *A. gamma* are tuned to the main pheromone components of these species. [1]As the pheromone components in *D. kikuchii* are not yet known, those of the sibling species *D. punctatus* are given.

The Noctuidae, finally, are well known for using complex pheromone blends of several pheromone components (Arn *et al.*, 1992; Hansson, 1995). Other than in the families referred to above, in noctuids the long s. trichodea are not a functionally homogenous population. Different long s. trichodea may be tuned to different components of their own pheromone or of that of sibling species (Priesner, 1979, 1980). Such interspecific pheromone communication is widespread in this family to avoid interbreeding of closely related species (Hansson, 1995). Usually, functional subtypes of long s. trichodea cannot be discriminated by mere morphological criteria. This paper shows that in all three noctuid species studied, morphologically similar s. trichodea may differ as to their odorant-binding proteins. The quota of strongly labelled sensilla (type-A s. trichodea; Table 1) are different in the three species. In *Helicoverpa,* where the main pheromone components have C-16 alcyl chains, the majority of long s. trichodea is strongly labelled. In *Spodoptera* and *Autographa*, only a minority of the s. trichodea is strongly labelled and these sensilla probably do not contain receptor cells for the main pheromone components, which have C-14 and C-12 alcyl chains respectively (Figure 3). The functional diversity of noctuid pheromone-sensitive sensilla thus appears to be reflected by a diversity of the PBPs they contain.

Although the number of species studied, and, in particular, the number of specific antisera used, is far too small to give a conclusive answer, our findings support the idea that the structure of PBPs is related to the structure of their ligands, which would mean that the PBPs could be involved in pheromone discrimination. Direct proof of such ligand specificity can be obtained only by binding studies which, however, are very difficult and capricious with these extremely hydrophobic ligands. Nevertheless, in 1987, data were published showing that the PBP of *A. polyphemus* has a much stronger affinity to its pheromone than to related analogues (De Kramer and Hemberger, 1987). Recently, Du and Prestwich (1995) studied the ligand specificities and binding affinities of the two recombinant PBPs of *A. pernyi* (Aper-1 and Aper-2) and found opposite binding specificities for two pheromone components. Aper-1 showed a 15-fold higher affinity for 6E,11Z-16:Ac than for 4E,9Z-14:Ac, while Aper-2 showed a 3.5-fold preference for binding the compound with the shorter chain. This fits well with the proposed binding site of these PBPs as mapped by photoaffinity labelling (Du *et al.*, 1994). Hence at least in one case there is direct evidence that odorant specificity is encoded into an odorant-binding protein and that chain length is an important discriminator.

The functional significance of the odorant-binding proteins obviously has many facets. In the case of the PBP of *A. polyphemus*, there is good evidence for a role in stimulus transport (Van den Berg and Ziegelberger, 1991). In this species, two redox-states of the PBP are observed and it is suggested that stimulus transport is performed by the reduced form of PBP, whereas rapid deactivation is accomplished by a redox shift of the complex to its oxidized form, probably during interaction with the receptor molecule (Ziegelberger, 1995). By preferentially binding a particular class of ligands, stimulus transport can contribute to odour discrimination, an adaptation which could have particularly evolved with the pheromone recognition system but to some extent may also serve as filter for general odorants. The final recognition of the stimulus and the triggering of the second-messenger cascade that leads to the electrical signals will most probably remain the task of membrane-bound receptor proteins, which in insects, however, have not yet been discovered.

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