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Expression of Pheromone Binding Proteins During Antennal Development in the Gypsy Moth *Lymantria dispar*

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We have identified 2 olfactory specific proteins in the gypsy moth *Lymantria dispar* that are uniquely associated with the male antennae, the principal olfactory organs of this animal. These proteins were the major soluble protein components of the olfactory sensilla, present in equivalent amounts. Both proteins comigrated on SDS-PAGE, showing an apparent molecular mass of 15,000 Da but migrated separately on non-SDS-PAGE, indicating differences in net charge. *N*-terminal amino acid sequence analysis showed that the 2 proteins share 50% identity, indicating that they are genetically distinct homologs. Both proteins bound the *L. dispar* sex-pheromone, associated with antisera prepared against the previously identified pheromone-binding protein (PBP) of the moth *Antheraea polyphemus*, and shared sequence identity with the *A. polyphemus* PBP. These 2 proteins are therefore identified as *L. dispar* PBPs and are termed PBP_1 and PBP_2 based on their migration differences on non-SDS-PAGE. It is estimated that PBP_1 and PBP_2 are present in the sensilla lumen at a combined concentration of 13.4 mM.

The expression of the *L. dispar* PBPs was examined during the 11 d development of the adult antenna. PBP_1 and PBP_2 were first detected by non-SDS-PAGE analysis and Coomassie blue staining 3 d before adult eclosion, on day A-3. Levels increased, reaching a plateau on day A-1 that continued into adult life. *In vivo* labeling studies indicated that the rate of PBP synthesis increased from A-3 to a plateau on A-2, where it remained into adult life. *In vitro* translations of antennal mRNAs indicated that translatable PBP mRNA was available at a very low level on day A-4, increased slightly on A-3 and dramatically on A-2, and remained at a high level into adult life. PBP mRNA represented the major translatable mRNA in the antenna during this period. It was estimated that the PBPs undergo a combined steady-state turnover of 8×10^7 molecules/hr/sensillum. Cursory *in vivo* and *in vitro* translation studies of antennal mRNA from *A. polyphemus* and *Manduca sexta* showed similar temporal patterns of PBP expression, suggesting that the *L. dispar* observations are general.

Terrestrial animals detect odorants with sensory dendrites bathed in an aqueous medium. However, most airborne odorants are hydrophobic molecules and thus do not rapidly partition into an aqueous medium. Both invertebrate and vertebrate animals appear to have adapted to this situation by evolving odorant binding proteins to aid in odorant capture. Odorant binding proteins have been isolated and characterized from the olfactory organs of insects (Vogt and Riddiford, 1981a, b; Gyorgyi et al., 1988), cows (Pelosi et al., 1982; Pevsner et al., 1985), rats (Pevsner et al., 1986), and frogs (Lee et al., 1987). A potentially analogous proteinaceous fluid has also been described in a land snail (Chase and Tolloczko, 1985; Chase, 1986). Odorant binding proteins are (1) small, water soluble, and extracellular proteins that (2) are located in the fluid surrounding the sensory dendrite and (3) bind odorants (Vogt and Riddiford, 1981b; Pelosi et al., 1982; Pevsner et al., 1985). The odorant binding proteins are thought to enhance the capture rate of hydrophobic odorants by (1) helping to solubilize these odor molecules, thus enhancing their partitioning into the aqueous fluid surrounding the dendrites and (2) then aiding in the transport of the odorants to receptor proteins located in the dendrite membrane (Vogt et al., 1985; Vogt, 1987; Pevsner et al., 1988a).

A pheromone binding protein (PBP) was first described from the male antennae of the wild silkmoth *Antheraea polyphemus* (Vogt and Riddiford, 1981a, b). This 15,000 Da protein was both sex and tissue specific in its location. The protein was extracellular and was located in the sensillum lumen surrounding the sensory dendrites (Fig. 1) at an estimated concentration of 10 mM (Vogt and Riddiford, 1981b; Klein, 1987). There is evidence that this PBP may exist *in vivo* as a 30,000 Da dimer (de Kramer and Hemberger, 1987). Sex pheromone was shown to associate with the *A. polyphemus* PBP (Vogt and Riddiford, 1981b; Vogt et al., 1988), and although this association appeared to be weak (Vogt and Riddiford, 1986; Vogt, 1987), it also appeared to be relatively specific in nature (de Kramer and Hemberger, 1987). Recently, an antennal protein from the tobacco hawk moth *Manduca sexta* has been identified as a PBP based on its (1) sequence homology to the *A. polyphemus* PBP, (2) tissue specificity, and (3) association with the olfactory sensilla (Gyorgyi et al., 1988). A full-length amino acid sequence of the *M. sexta* PBP was indirectly obtained through cDNA cloning techniques and DNA sequencing (Gyorgyi et al., 1988). The *M. sexta* PBP was first expressed late in adult development. However, unlike the *A. polyphemus* PBP, the *M. sexta* PBP was

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expressed in female as well as male antennae, but at relatively low levels (Gyorgyi et al., 1988).

This paper describes the pheromone binding proteins of the gypsy moth *Lymantria dispar*. Since *L. dispar* was accidentally introduced to North America in 1869 at Medford, Massachusetts (Forbush and Fernald, 1896), it has proven a very tenacious pest, defying almost all attempts to control it in the Northeastern region of the United States. The females are too heavy to fly due to their production of a very large mass of eggs and have very reduced antennae. In contrast, the males are very active fliers and possess 2 large antennae each with about 22,000 pheromone-sensitive sensilla measuring ca. 200 μm in length and 5 μm in diameter (Scheffler, 1975; Schneider et al., 1977). The sensilla are essentially identical repeats, each containing 2 sensory neurons (Fig. 1) (Schneider et al., 1977). The females attract males with a single-component pheromone (7R,8S)-7,8-epoxy-2-methyloctadecane, commonly termed (+)disparlure (Bierl et al., 1970; Prestwich et al., 1989). However, only one of the pair of sensory neurons responds specifically to this (+)optical enantiomer (Hansen, 1984). The second neuron has been shown to respond specifically to the corresponding (−) optical enantiomer, although this (−) enantiomer is not a component of the *L. dispar* pheromone (Hansen, 1984). However, the (−) enantiomer is released by the sibling species *Lymantria monacha* (Hansen, 1984), which cohabits with *L. dispar* (South, 1907), and this compound has been shown to modify *L. dispar* behavior in a specific manner (Preiss and Kramer, 1983).

In this paper we identify 2 *L. dispar* proteins as PBPs and describe their expression during the development of the male antenna. We also compare *L. dispar* PBP expression with PBP expression in *A. polyphemus* and *M. sexta*. Although PBPs were originally described in *A. polyphemus, L. dispar* was chosen for these studies because this species is much easier to raise in the laboratory and is thus more amenable to developmental studies. Furthermore, the high degree of sensory specificity of the male *L. dispar* antenna makes this tissue ideal for the study of biochemical and molecular biological mechanisms that underlie olfactory function.

Materials and Methods

Animals and tissue collection. L. dispar (Lepidoptera: Lymantriidae) were obtained as larvae already on artificial diet from APHIS, USDA Otis Air Force Base, MA, courtesy of Dr. C. P. Schwalbe. Animals were then raised at 27°C on a 16:8 L:D cycle. Lights off was at 2200 hr. *A. polyphemus* (Lepidoptera, Saturniidae) were obtained as pupae from either H. W. Hartman (Elkhorn, IN) or D. Bantz (Caledonia, WI) and stored at 4°C until use. To initiate development, *A. polyphemus* pupae were incubated at 27°C on a 16:8 L:D cycle. *Manduca sexta* (Lepidoptera: Sphingidae) male antennae were provided frozen courtesy of Drs. T. Gyorgyi and M. R. Lerner. All tissues were frozen in 1.5 ml microcentrifuge tubes in liquid nitrogen (LN) immediately following dissection and subsequently stored at −90°C until use.

Animals used in developmental studies were selected as prepupae by a characteristic immobility and a dorsal to ventral bending. Selection was done between 1700 and 2200 hr. Greater than 95% of those animals selected as prepupae during these times were fully tanned pupae 24 hr later. Individuals not fully tanned at this time were discarded. At the appropriate age, antennae were dissected, frozen, and stored. Individuals were staged at the time of dissection according to visual criteria described in Figure 5.

Tissue preparation. Tissues used for all electrophoretic studies were first lyophilized and then homogenized by hand in 10 mM Tris-HCl (pH 7.0) in a small ground glass homogenizer held in ice/water. Homogenates were centrifuged (5 min, 12,000 × *g*, 4°C), and the supernatants were removed, transferred to microcentrifuge tubes, and frozen in LN. These were then lyophilized, dissolved in an appropriate volume of npH_2O (NanoPure water filtration system, Barnstead) followed by an equal volume of appropriate 2× electrophoresis sample buffer. Sample buffer (2×) for non-SDS gels was 100 mM Tris-HCl (pH 6.8), 20% glycerol, 0.005% bromphenol blue (BPB). Sample buffer (2×) for SDS gels was 125 mM Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 10% mercaptoethanol, 0.005% BPB.

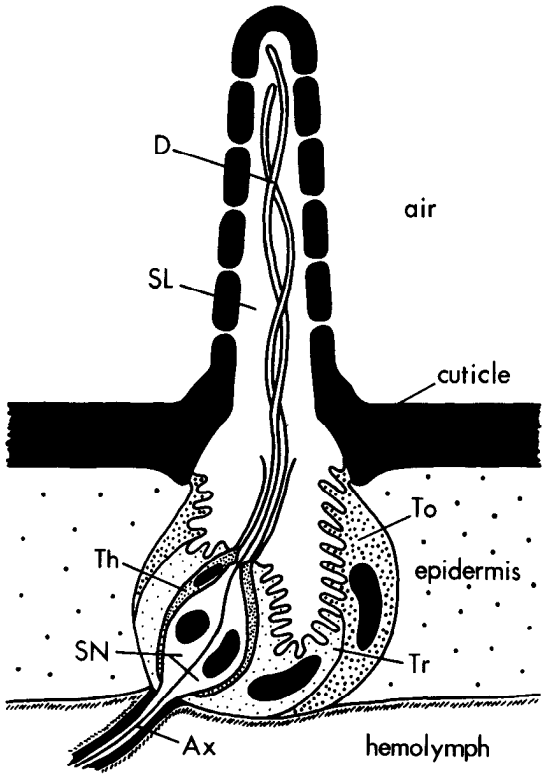

Figure 1. Schematic of a pheromone-sensitive sensillum. The cell bodies of the sensory neurons (*SN*) reside at the base of the sensillum, surrounded concentrically by the thecogen (*Th*), trichogen (*Tr*), and tormogen (*To*) accessory cells. Dendrites (*D*) project into the sensillum lumen (*SL*), which is filled with fluid containing PBP. Axons (*Ax*) project to the brain. (Redrawn after Steinbrecht, 1987.)

Sensilla isolation. Sensilla were isolated from adult male antennae as described previously (Vogt, 1987), modified after Klein and Keil (1984). Branches of 40 antennae were combined with 4 ml of glass beads (0.1–0.5 mm diameter) in a 15 ml Corex tube, chilled in LN, and then shaken violently for about 3 sec on a vortex mixer. The tube and contents were lyophilized, and the contents were transferred to a glass petri dish. The sensilla adhered to the glass surface while the rest was shaken away. The sensilla were scrapped from the glass and transferred to a microcentrifuge tube for storage at −90°C. This procedure yielded about 0.5 mg lyophilized sensilla per 100 antennae. Sensilla homogenates were prepared by homogenizing lyophilized sensilla in a ground glass homogenizer, as described above under Tissue Preparation. Following centrifugation at 12,000 × *g* for 5 min, the resulting supernatant was used for protein determination and electrophoretic studies.

Determination of sensilla protein concentration. The total protein content of the supernatant of isolated sensory hair was measured using the BCA assay (Pierce Chemical Company, Rockford, IL) with BSA as the protein standard.

Electrophoresis. All protein separations were done using non-SDS (nondenaturing) or SDS (denaturing)-PAGE. Recipes were after Laemmli (1970) and Ames (1974). For non-SDS gels, the SDS was replaced with npH_2O. Non-SDS gels were run in a 4°C cold cabinet. SDS gels were run at room temperature. Molecular-weight markers for SDS gels were either BioRad low-molecular-weight markers or Amersham ^{14}C-labeled Rainbow markers. For general protein visualization, gels were stained in 0.25% Coomassie blue R-250 in 50% methanol (MeOH), 10% acetic acid.

For fluorography of ^{35}S-methionine-labeled proteins, gels were soaked 15 min in 100% glacial acetic acid, 60 min in 10% PPO (Sigma) in glacial acetic acid, 15 min in npH_2O, and dried onto paper under heat

and vacuum. Dried gels were exposed to preflashed XAR5 X-Ray film (Kodak) at −90°C for an appropriate time.

For immunoblots, proteins were transferred from non-SDS polyacrylamide gels to nitrocellulose (NC) (BioRad) in 25 mM Tris base, 192 mM glycine in 20% MeOH at 70 V, 4°C for up to 12 hr (Towbin et al., 1979). Following transfer, NC was blocked 12 hr at room temperature in 10% nonfat dry milk (NFDM) in PBS-T (2.5 mM NaH_2PO_4, 7.9 mM Na_2HPO_4, 150 mM NaCl containing 0.05% Tween 20 detergent). Blocked NC was incubated with primary antisera in PBS-T at indicated dilution. Bound primary antibody was visualized using the Vectastain ABC peroxidase kit (Vector Laboratories). The peroxidase reaction was carried out in a solution containing 2 ml 4-chloro-1-naphthol (3 mg/ml in MeOH) and 5 μl H_2O_2 (30%) per 10 ml PBS-T.

N-terminal amino acid sequencing. Proteins were electrophoretically purified by non-SDS-PAGE and transferred directly to modified glass fiber filters (Whatman GF/C, soaked in neat trifluoroacetic acid 60 min and air-dried) following a procedure modified after Aebersold et al. (1986) in which NP40 was omitted from all steps. Transfer was in 1% glacial acetic acid in npH_2O at 70 V, 4°C for 12 hr. Proteins were visualized using Coomassie blue staining (Aebersold et al., 1986). The blots were dried under vacuum, and the protein bands of interest cut out and stored in sealed microcentrifuge tubes at −90°C until sequencing.

Amino terminal sequences were obtained directly from protein bound to the glass fiber filters. Sequencing was performed by Mr. Thomas Fisher, using an Applied Biosystems 470A protein sequencer at the State University of New York at Stony Brook Center for Analysis and Synthesis of Macromolecules. An earlier version of the *A. polyphemus* sequence appeared in Vogt and Prestwich (1987), and a partial sequence of one of the *L. dispar* proteins appeared in Vogt (1987). The sequences presented here are based on 6 independent samples of the *A. polyphemus* PBP and on 2 independent samples of each of the *L. dispar* PBPs. The *M. sexta* sequence presented in Figure 4 was kindly provided by Drs. T. Gyorgyi and M. R. Lerner (Gyorgyi et al., 1988).

Radiolabeled pheromone. Both enantiomers of disparlure were prepared at high specific activity (58 Ci/mmol) by homogeneous tritiation of the optically active alkenyl oxiranes, as described elsewhere (Prestwich et al., 1989). Storage solutions were maintained in 1:1 heptane:toluene at −20°C (Prestwich, 1987). Ethanol (EtOH) stock solutions were prepared by drying an aliquot of the heptane–toluene solution under N_2, then dissolving in EtOH, and finally diluting in EtOH to 10^{-4} and 10^{-5} M stocks, respectively.

^{3}H-disparlure binding study. The binding of labeled disparlure to PBP was examined by non-SDS-PAGE of a sensilla extract solution containing ^{3}H-disparlure. Lyophilized sensory hairs (0.65 mg) were homogenized in 300 μl of non-SDS 1× electrophoresis sample buffer and centrifuged (5 min, 12,000 × *g*). This preparation was shown not to degrade either (+) or (−)disparlure over the time course of 1 hr, when analyzed by thin-layer chromatography (R. G. Vogt, unpublished observations). Sensilla supernatant was distributed to 6 borosilicate tubes (10 × 75 mm), 45 μl/tube, with the tubes held in crushed ice. Each tube contained 5 μg total protein. At t_0, 1 μl of either (+) or (−)^{3}H-disparlure from EtOH stocks (see above) was added to each tube. Tubes were mixed briefly on a vortex mixer and returned to the ice. After 5 min incubation, 30 μl of each incubation was loaded onto a 15% non-SDS gel and electrophoresed. Immediately following electrophoresis, the gel was fixed in 7% formaldehyde (30 min), soaked in 1 M salicylic acid (Na^+-salt) for 1 hr (Chamberlain, 1979), and air-dried (room temp.) overnight between 2 sheets of cellophane clamped to a glass plate. The gel was then exposed to preflashed XAR5 X-Ray film (Kodak) at −90°C.

Production of polyclonal antisera. Antisera to the PBP of *A. polyphemus* was prepared as previously described (Vogt, 1984). A centrifuged homogenate of 100 male antennae (10 mM Tris-HCl, pH 7.0) was concentrated to 5 ml by vacuum dialysis (Pro-di-Con, Bio Molecular Dynamics). PBP was purified from this using preparative non-SDS-PAGE. The edges of the gel were sliced and stained to localize the PBP region, which was then removed and homogenized by passage through syringe needles (23 gauge), using Tris-HCl (10 mM, pH 7.0) as a lubricant. The gel slurry was mixed 1:1 with Freund's complete adjuvant (Sigma, 20 ml final volume) and stored in 1 ml aliquots at −20°C. Two milliliters were injected subcutaneously into the backs of 2 Rex rabbits on a biweekly schedule for 8 weeks. Serum was collected from a peripheral ear vein.

Antisera were prepared separately against both PBP_1 and PBP_2 of *L. dispar*. Two thousand male antennae were collected from newly emerged adults, frozen in LN, and stored at −90°C. These were lyophilized and then homogenized (BioSpec) in 50 ml Tris-HCl (10 mM, pH 7.0) in 15 ml Corex tubes in ice/water. The centrifuged supernatant (10,000 rpm, Beckman SW50 rotor, 4°C, 10 min) was concentration by lyophilization, redissolved in 6 ml of non-SDS sample buffer (1×), and electrophoresed through a 7.5% non-SDS preparative polyacrylamide gel (4.2 mm × 10 cm cross section, 100 mA constant current, 4°C). The acrylamide at the dye front, containing the PBPs, was excised and laid onto a 15% non-SDS preparative gel (5 mm × 10 cm cross section). Following electrophoresis (100 mA constant current, 4°C), the edges of this gel were removed and stained with Coomassie blue to locate PBP_1 and PBP_2. The corresponding gel sections were excised and homogenized separately by passage through syringe needles (23 gauge) in npH_2O to a final volume of 10 ml each, and stored at −90°C. The protein purity was assessed by non-SDS-PAGE (15%), which showed that each PBP was contaminated by about a 10% level by the other PBP, but was otherwise electrophoretically pure. PBP_1 and PBP_2 gel slurries were respectively injected subcutaneously into the backs of 2 New Zealand White rabbits on a 10 d schedule. The first injections were 2 ml each, consisting of 50% Freund's complete adjuvant (Sigma). Subsequent injections were 2 ml of gel slurry only. Serum was collected by cardiac puncture.

In vivo *labeling of antennal proteins.* ^{35}S-methionine (Tran^{35}S-Label, ICN) was diluted 1:2 with insect saline (5.2 mM NaCl, 26.8 mM KCl, 12.9 mM $MgCl_2$, 1.25 mM $KHCO_3$, 1.25 mM KH_2PO_4, 1.35 mM $CaCl_2$, 166 mM glucose). Ten microliters (33 μCi) was injected into the heads of pupae and adults using a Hamilton syringe (50 μl, 30 gauge needle). The injection was made between and just forward of the antennal attachment sites, and the injection hole did not require sealing. Tissues were prepared as described above.

Purification of total antennal RNA. RNA purification was modified from a protocol previously used to isolate RNA from insect integument (Riddiford, 1982). Antennae were frozen in LN immediately following removal from the animal and stored at −90°C. Frozen antennae were homogenized in a lysis buffer containing 6 M urea, 69 mM SDS, 35 mM NaCl, 0.1 mM EDTA, 1 mM Tris-HCl, pH 8.0. Following phenol/chloroform extraction, the RNA was collected by CsCl density-gradient centrifugation (Maniatis et al., 1982). Following EtOH precipitation, the RNA was stored in either 70% EtOH or npH_2O at −90°C until use. This procedure yielded ca. 150 μg total RNA (1 OD_{260} unit = 40 μg total RNA) per 100 adult male *L. dispar* antennae (90 mg wet tissue). The quality of the RNA (ca. 5 μg) was routinely assessed at the end of each isolation by 1% nondenaturing agarose gel electrophoresis in the presence of ethidium bromide (Maniatis et al., 1982) and by *in vitro* translation (IVT).

IVTs and immunoprecipitations. IVTs of total RNA were performed using a rabbit reticulocyte lysate kit (ProMega) in the presence of RNasin (placental, ProMega) and ^{35}S-methionine (Tran^{35}S-Label, ICN), following protocols supplied (15–45 μg total RNA per reaction). Total reaction volume was 50 μl. At the end of the reaction (1 hr, 30°C) 10 μl was mixed with 20 μl npH_2O and 20 μl 2× SDS-sample buffer, boiled 5 min, and stored at −90°C for electrophoretic analysis of the total IVT mixture.

Immunoprecipitations were done using Protein A-Sepharose (PA-S) (Pharmacia) immediately following the translation reaction. PA-S (1 mg per sample to be precipitated) was suspended in 1 ml PBSE-NP40 (25 mM K_2HPO_4, 100 mM NaCl, 100 mM EDTA, pH 7.6, 0.25% NP-40 detergent). Following 30 min incubation on ice, the PA-S was washed several times until the milk was visibly gone, and the final volume was adjusted to 20 μl/mg PA-S with PBSE-NP40.

Immunoprecipitations were initiated by combining 20 μl of an IVT sample with 80 μl PBSE-NP40 and 50 μl of a 1:20 dilution of desired antisera. Following 30 min incubation on ice, 20 μl PA-S (1 mg/ml) was added. Following an additional 30 min incubation on ice, the mixture was centrifuged (3 min, 12,000 × *g*, 4°C), and the pellet washed 4 times with PBSE-NP40. The final pellet was frozen, lyophilized, and dissolved in 40 μl npH_2O followed by addition of 40 μl of 2× SDS-sample buffer. This was boiled 5 min and stored at −90°C. Total translated and immunoprecipitated proteins were examined on 15% SDS polyacrylamide slab gels which were subsequently fluorographed as described above.

Results

Identification of L. dispar *PBPs*

The *L. dispar* sex pheromone is detected by sensillae (Fig. 1) arrayed along the branches of the male antennae. Non-SDS

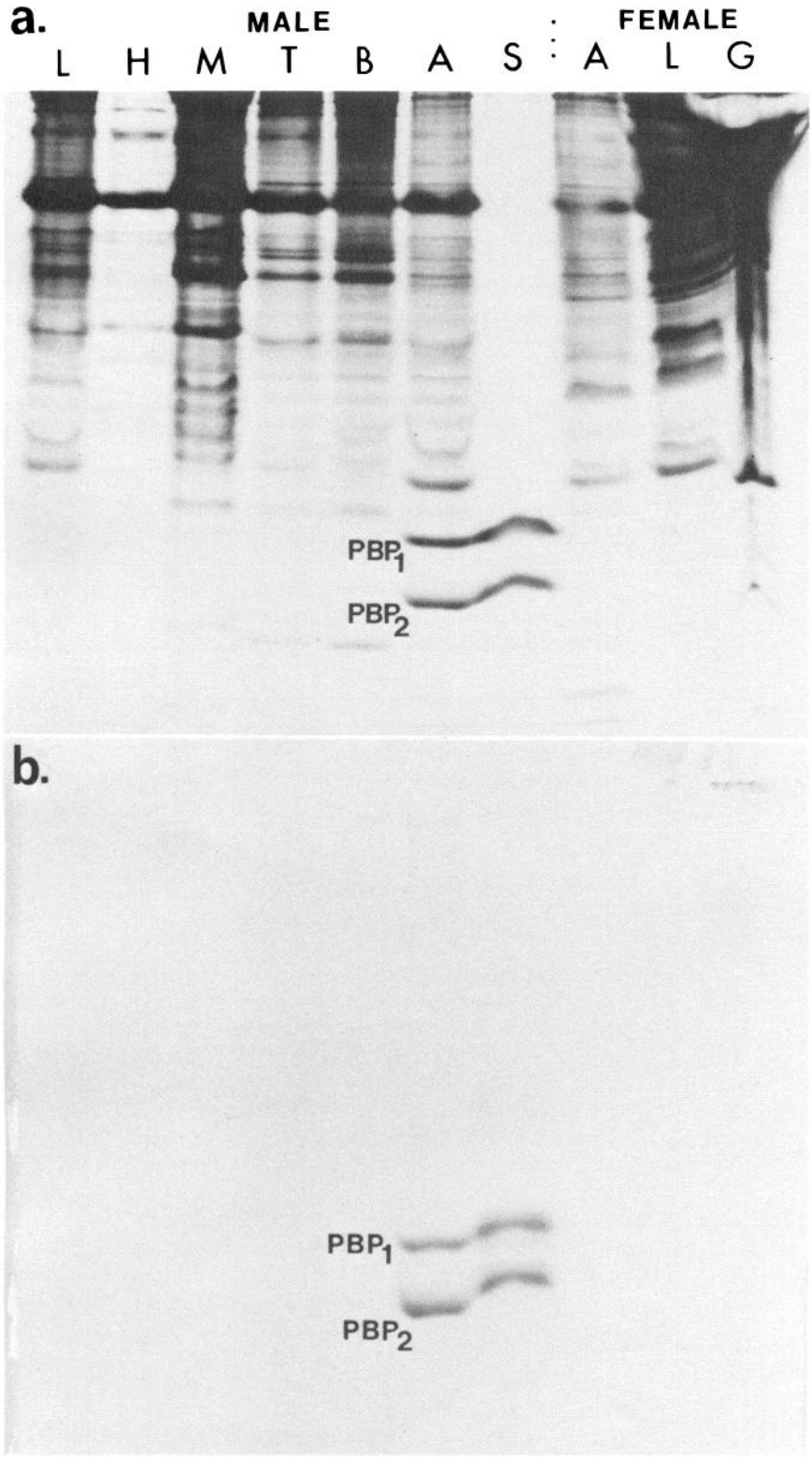

Figure 2. Electrophoretic comparison of male and female *L. dispar* tissues, with proteins visualized by Coomassie blue staining (*a*) and immunostaining (*b*). Tissue homogenates were divided between 2 gels, which received 75% (*a*) and 25% (*b*) of each sample, respectively. Antiserum used in the immunoblot (*b*) was generated against the *A. polyphemus* PBP and used at a dilution of 1:200. Tissues and amounts represented in the total homogenates were, from left to right: *L*, 11 male legs; *H*, 2 μl of male hemolymph; *M*, dorsal longitudinal muscles from 5 males; *T*, 8.5 male thoracic ganglia; *B*, 8 male brains; *A*, 5 male antennae; *S*, 0.25 mg male sensilla; *A*, 18.5 female antennae; *G*, 6 female abdominal tips which include the pheromone-producing glands. The apparent difference in mobility in the PBP bands in *lanes A* and *S* was due to different amounts of total protein in the respective lanes and not to differences in the proteins, indicated by the fact that the bands join at the lane boundaries. Protein separation was by non-SDS-PAGE (15%).

(nondenaturing)-PAGE analysis of sensilla extracts revealed 2 prominent protein bands that were uniquely associated with male antennae (Fig. 2*a*). Both proteins reacted strongly with antisera prepared against the PBP of *A. polyphemus* (Fig. 2*b*). SDS-PAGE analysis of sensilla extracts showed only a single major protein band with an apparent molecular mass of 15,000 Da (data not shown, but see Fig. 8). Furthermore, both (+) and (−)disparlure associated with both proteins, remaining so through electrophoretic separation, and showed a higher degree of association with the faster-migrating (lower) protein (Fig. 3). Thus, the 2 major sensilla proteins satisfy the criteria used to identify the *A. polyphemus* PBP (Vogt and Riddiford, 1981b). They (1)

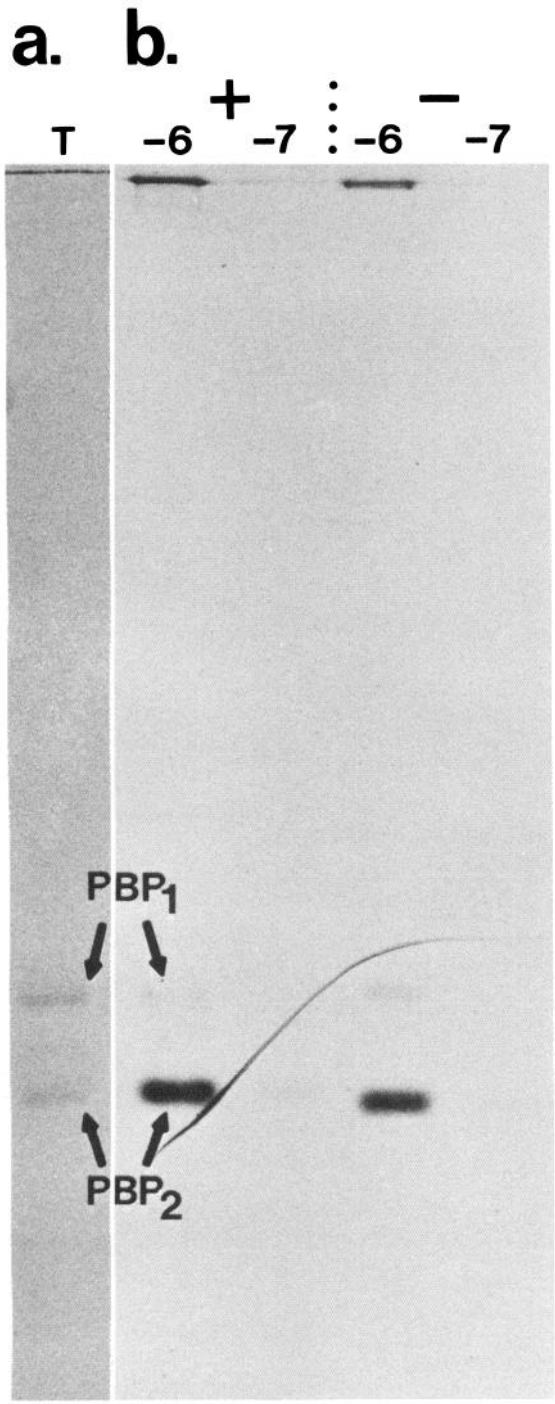

Figure 3. Electrophoretic demonstration of ^{3}H-disparlure binding to PBPs. Homogenates of *L. dispar* sensilla were incubated with the (+) and (−)enantiomers of ^{3}H-disparlure, and then electrophoresed. *a* (*T*), Coomassie blue-stained lane of one of these samples, showing the relative amounts and positions of PBP_1 and PBP_2. *b*, Fluorograph showing the (+) and (−)enantiomers of ^{3}H-disparlure associating with PBP_1 and PBP_2. The disparlure concentrations were 10^{-6} M (−6) and 10^{-7} M (−7). The PBP concentration was 7×10^{-6} M throughout. Amounts of radioactivity loaded per sample from left to right were 0.73, 0.05, 0.43, and 0.04 μCi, respectively, based on measurement of a 3 μl aliquot by liquid-scintillation counting. The amount of bound radioactivity was not measured. Protein separation was by non-SDS-PAGE (15%).

are uniquely associated with the male antennae, (2) are abundant soluble components of the pheromone sensitive sensilla, and (3) bind pheromones. We therefore identify these new proteins as PBPs, with the slower (upper) protein identified as PBP_1 and the faster (lower) protein identified as PBP_2, as characterized by non-SDS-PAGE. The concentration of total protein in sensilla extracts was measured to be 45 μg/mg lyophilized sensilla. Based on the Coomassie blue staining pattern of sensilla homogenate in Figure 2*a*, this measurement represents the combined PBP concentration in *L. dispar* sensilla.

N-terminal amino acid sequences were obtained for PBP_1 and PBP_2 of *L. dispar* and for the PBP of *A. polyphemus*. The first 30 *N*-terminal amino acids are compared in Figure 4, along with the same region of the PBP of *M. sexta* (Gyorgyi et al., 1988). The regions shown represent ca. 20% of the full length of these proteins. Like the *L. dispar* PBPs, the PBPs of *A. polyphemus* and *M. sexta* have molecular masses of ca. 15,000 Da, and the *M. sexta* PBP has a predicted size of 142 amino acids based on its cDNA-derived full-length sequence (Vogt and Riddiford, 1981b; Gyorgyi et al., 1988). Table 1 summarizes the degree of identity between these 4 PBPs. Positions 1, 5, 12, 14, 22, and 27 are conserved in all 4 proteins. An amino acid appears at least twice within the group at every other position except positions 9 and 18, and the majority of the amino acid differences represent conservative changes on the basis of property (e.g., hydrophobicity). The 2 *L. dispar* proteins, while not the same gene product, sharing only 50% identity, are nevertheless clearly homologs, as is the entire group (Doolittle, 1986). Thus, in stark contrast to *A. polyphemus* and *M. sexta*, which possess only a single PBP species, respectively, *L. dispar* possesses 2 distinctly different PBPs.

Developmental staging of L. dispar

In order to examine the time course of PBP expression, it was first necessary to establish criteria for staging the animals in their development. Adult development occurs during the pupal stage. Larval animals were initially selected as prepupae by a characteristic immobility and a dorsal to ventral bending. Greater than 95% of these animals were fully tanned pupae 24 hr later. The day of pupation was counted as pupal day 0, or P0. The day of prepupal selection was counted as P-1, and the days following pupation were P1, P2, P3, etc. Of a group of 237 males selected as prepupae, 38% eclosed as adults on P10, 60% on P11, and 2% on P12.

This asynchrony in development relative to P0 was not acceptable for determining the time of events occurring late in adult development. Instead, we utilized changing pigmentation patterns in wings and antennae to stage developing adults relative to the day of adult eclosion (A0). The staging criteria are illustrated in Figure 5. Similar criteria have been used to accurately stage preadult *M. sexta* (Schwartz and Truman, 1983). Days preceding adult eclosion were counted as A-1, A-2, A-3, etc., and the days following were A1, A2, A3, etc. In the following studies we examined tissues ranging from stage A-5 to A1.

Developmental expression of L. dispar *PBPs*

PBP_1 and PBP_2 were first detectable in homogenates of A-3 antennae analyzed by both Coomassie blue staining and immunoreactivity following non-SDS-PAGE (Fig. 6). The amounts of both proteins had increased to adult levels 2 d later, on A-1. PBP_2 appeared fainter than PBP_1 in this immunoblot (Fig.

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                    10                  20                  30
LdisPBP1N-Ser Lys Glu Val Met Lys Gln Met Thr Ile Asn Phe Ala Lys Pro Met Glu Ala Cys Lys Gln Glu Leu Asn Val Pro Asp Ala Val Val
LdisPBP2N-Ser Lys Asp Val Met His Gln Met Ala Leu Lys Phe Gly Lys Pro Ile Lys Leu Leu Gln Gln Glu Leu Gly Ala Asp Asp Ser Val Val
ApolPBP N-Ser Pro Glu Ile Met Lys Asn Leu Ser Asn Asn Phe Gly Lys Ala Met Asp Gln Ser Lys Asp Glu Leu Ser Leu Pro Asp Ser Val Val
MsexPBP N-Ser Pro Asp Val Met Lys Asn Leu Cys Leu Asn Phe Gly Lys Ala Leu Asp Glu Cys Lys Ala Glu Met Asn Leu Ser Asp Ser Ile Lys
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Figure 4. *N*-terminal sequences of *L. dispar* PBP_1 (*Ldis1*) and PBP_2 (*Ldis2*), *A. polyphemus* PBP (*Apol*), and *M. sexta* PBP (*Msex*).

6*b*). This may reflect the fact that the antisera used in this blot were generated against the *A. polyphemus* PBP, which shares less identity with PBP_2 than with PBP_1 (Table 1).

In vivo labeling studies showed that, while the level of both PBPs plateaued by A-1, the rate of PBP synthesis plateaued a day earlier on A-2, and remained at this level into the adult stage (Fig. 7). These features were indicated by both a constant level of Coomassie blue staining (Fig. 7*a*) and a constant level of ^{35}S-methionine incorporation (Fig. 7*b*) in the PBPs on and following days A-1 and A-2, respectively. A constant amount under constant synthesis suggests that the PBPs are under a constant rate of turnover. Change in the overall antennal protein pattern was slight and gradual from A-5 through A-1, with the notable exception of the PBPs. However, at adult eclosion (A0), several new protein bands appeared to be expressed. None of these represented a change in state of the PBPs, however, as the immunoblot pattern did not change at this pupal–adult transition.

Immunoprecipitations of *in vitro* translated mRNAs isolated from different stages showed that translatable PBP mRNA was present at very low levels on A-4, increasing slightly on A-3, and then increasing dramatically on A-2 (Fig. 8). Translatable PBP mRNA remained abundant on A-1 and A0. Furthermore, translated PBP was clearly observed in the total translated mixtures from A-3 to A0 (Fig. 8), suggesting that PBP mRNA is present in high abundance relative to the background of other mRNAs at these stages and that the turnover of PBP is occurring at a high rate. There was a difference in apparent molecular masses between the *in vitro* translated PBPs and metabolically *in vivo* labeled PBPs (Fig. 8). This difference in mass was around 2000 Da, which could correspond to the removal of a ca. 18 amino acid leader sequence during extracellular export of the proteins. A deduced leader sequence of 25 amino acids was observed in the cDNA-derived sequence of the *M. sexta* PBP (Gyorgyi et al., 1988).

PBP synthesis in A. polyphemus *and* M sexta

Based on the *L. dispar* studies, we made a cursory examination of PBP synthesis in *A. polyphemus* and *M. sexta* for comparison. Antennal extracts of male *A. polyphemus* antennae showed little or no detectable PBP on A-3, but a large amount present on A1, detected by Coomassie blue staining (Fig. 9*a*). *In vivo* labeling of the adult proteins showed that PBP synthesis was occurring at a relatively high rate in the adult stage, between A0 and A1 (Fig. 9*b*).

IVTs of adult (A0) antennal mRNAs and subsequent immunoprecipitations of translated proteins showed similar patterns in *A. polyphemus, M. sexta,* and *L. dispar* (Fig. 10). PBP mRNA is apparently very abundant relative to the background of other male antennal mRNAs in all 3 species, as is evident by the abundance of translated PBP in the total translation mixtures. In contrast, there was a total lack of translated PBP in female *A. polyphemus* antennal mRNA translation mixtures, illustrated by the lack of precipitable PBP in the female immunoprecipitation lane. In addition, 2 minor bands, smaller than the major PBPs, were typically observed in immunoprecipitations of translated male antennal mRNAs (Fig. 10). It is not clear what these bands represent, but their mobility pattern was repeatedly observed, and therefore characteristic for each species.

Table 1. Degree of amino acid identity between the 4 *N*-terminal amino acid sequences (see Fig. 4)

	$Ldis_2$	Apol	Msex
$Ldis_1$	0.50	0.50	0.40
$Ldis_2$		0.37	0.37
Apol			0.57

Numbers are fractions of 1.0, which would represent 100% identity. The numbers are not adjusted for property similarities. $Ldis_1$, $Ldis_2$, Apol, and Msex refer to PBP_1 and PBP_2 of *L. dispar,* and the PBPs of *A. polyphemus* and *M. sexta,* respectively.

Discussion

PBPs of L. dispar

We have identified 2 olfactory sensilla proteins in the gypsy moth *L. dispar,* which are uniquely associated with male antennae, the principal olfactory organ in these animals. These 2 sensilla proteins comigrate on SDS-PAGE, showing an apparent molecular mass of ca. 15,000 Da. However, they migrate separately on non-SDS-PAGE, suggesting they have different net charges. Both sensilla proteins bind radiolabeled pheromone, and both react positively with antisera generated against the PBP of *A. polyphemus.* Furthermore, both *L. dispar* proteins share sufficient sequence identity with each other and with the PBPs of *A. polyphemus* and *M. sexta* to consider the entire group structural homologs. We therefore have classified these 2 *L. dispar* proteins as PBPs. Based on their migration differences on non-SDS-PAGE, we have termed the slower-migrating protein PBP_1 and the faster-migrating protein PBP_2. The fact that PBP_1 and PBP_2 share only 50% identity in their *N*-terminal regions argues that these represent 2 distinct gene products.

The amount of PBP per antenna can be estimated by comparing the sensilla and antennal lanes in Figure 2*a*. We determined that the total protein concentration of a sensilla homogenate was 45 μg/mg sensilla. Nearly all of this sensilla protein is PBP (Fig. 2*a*). In Figure 2*a,* the sensilla lane represents 0.19 mg sensilla, or ca. 8.5 μg total protein, equally distributed between PBP_1 and PBP_2. These are approximately the same levels of PBP_1 and PBP_2 observed in the total antennal homogenate lane (Fig. 2*a*), which represented 3.75 antennae. Thus, a single antenna (ca. 0.9 mg wet weight) contains about 2.2 μg of total PBP (8.5 μg protein divided by 3.75 antennae).

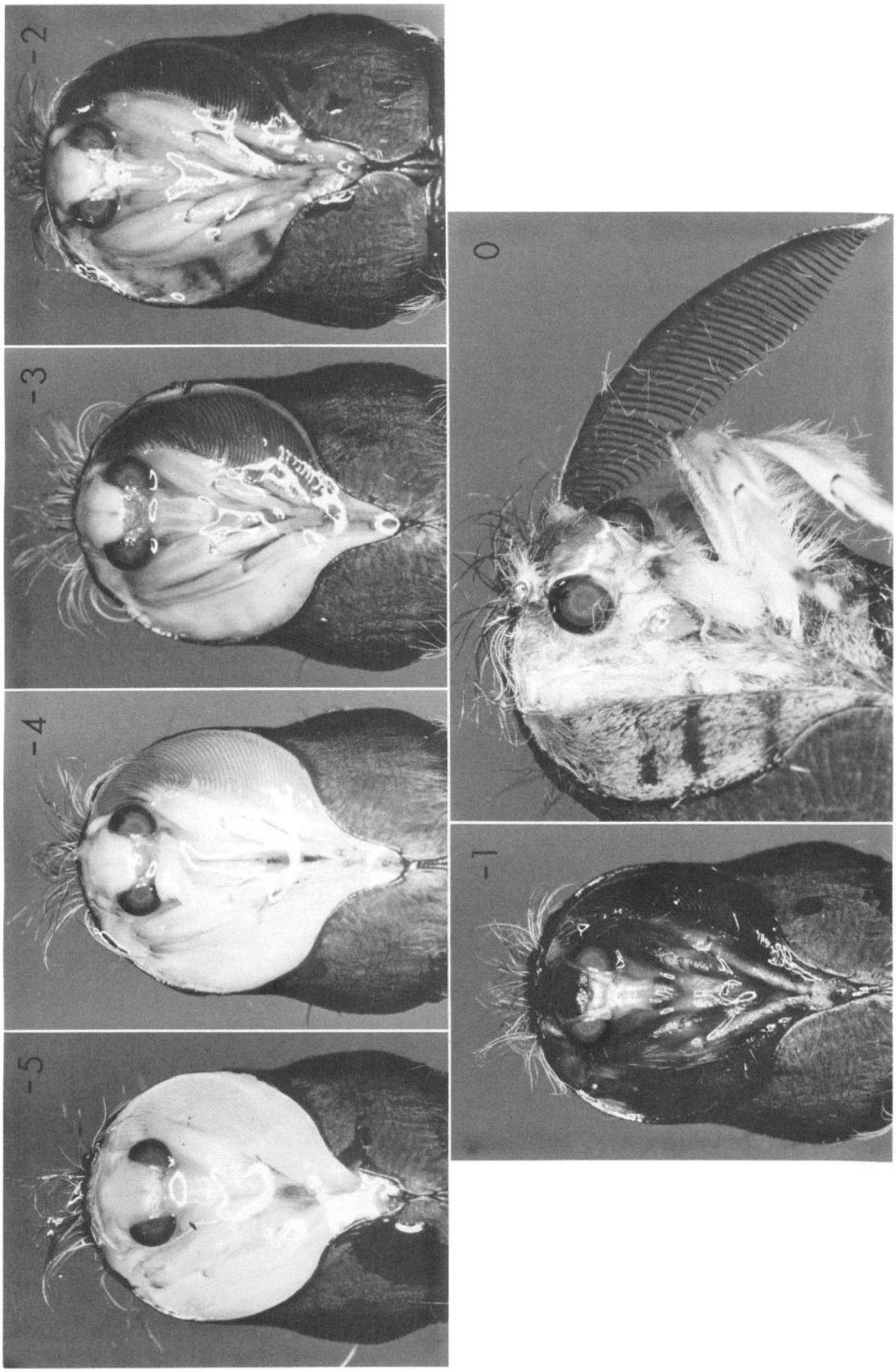

Figure 5. Photographs of developing male *L. dispar* adults. The pupal cuticle was removed over the head/antenna/wing region to reveal underlying tissues, and one antenna has been removed to reveal the wing beneath. Stages were assigned based on the following criteria: *A-5*, wings and antennae white, no pigmentation; *A-4*, wings white, antennae noticeably pigmented against background; *A-3*, wings pigmented but no bars visible, antennae darker; *A-2*, wing bars visible, overall pigmentation ca. ½ of A-1; *A-1*, full pigmentation (very dark), body wet; *A0*, Newly eclosed adult (emerged within 24 h). Animals that were just pre-eclosion, or pharate, were completely dry beneath the pupal cuticle.

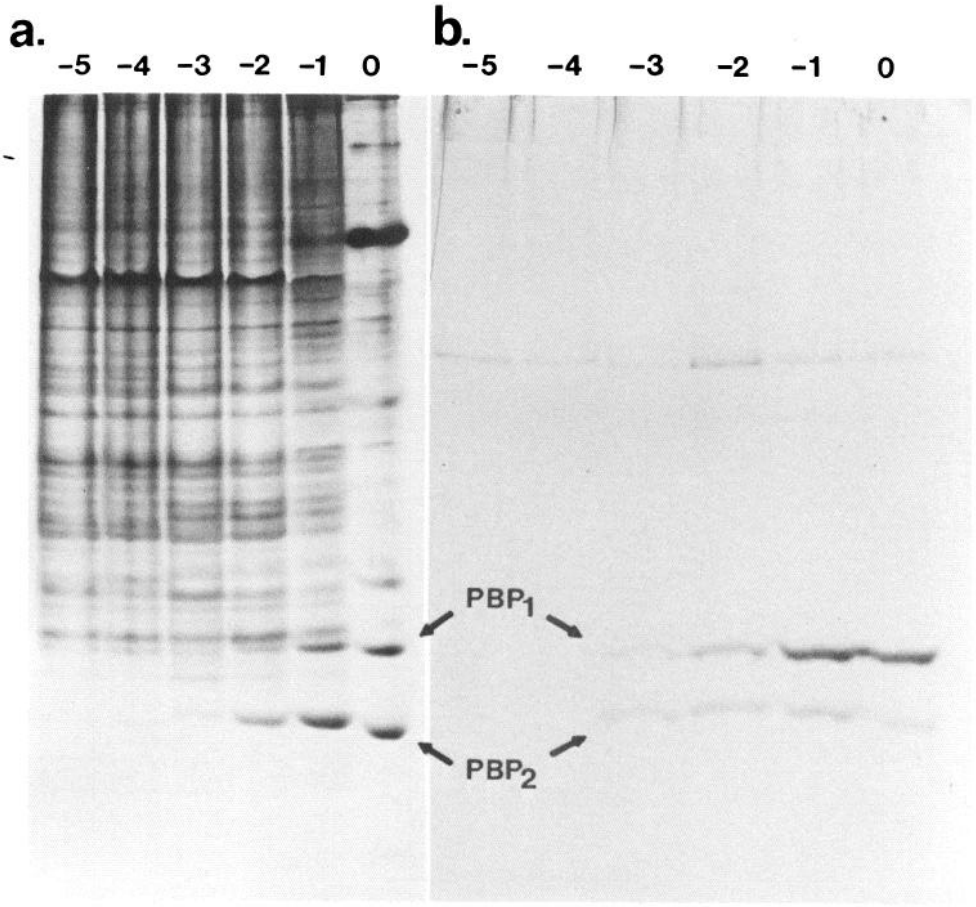

Figure 6. Electrophoretic comparison of male *L. dispar* antennal proteins of different developmental stages, visualized by Coomassie blue (*a*) and immunostaining (*b*). Numbers over the lanes indicate the stage of that individual at the time of antennal removal (e.g., −2 refers to stage A-2). Each lane in *a* represents 4 antennae pooled from 2 individuals; each lane in *b* represents 2 antennae from single individuals. The antiserum used in the immunoblot (*b*) was generated against the *A. polyphemus* PBP and was used at a 1:200 dilution. Protein separation was by non-SDS-PAGE (15%).

The *in situ* concentration of total PBP can be estimated based on the amount of PBP per antenna and the assumption that all of the PBP is located within the sensilla lumen (Fig. 1). A single *A. polyphemus* pheromone-sensitive sensillum is ca. 300 μm long with a volume of ca. 10^{-12} liter (Gnatzy et al., 1984; Vogt et al., 1985). *L. dispar* sensilla are ca. 200 μm long (Scheffler, 1975), and thus might have half the volume, or 5×10^{-13} liter. In addition, there are ca. 22,000 sensilla per *L. dispar* antenna (Scheffler, 1975). The volume of sensilla lumen per *L. dispar* antenna is thus ca. 1.1×10^{-8} liter (5×10^{-13} liter per sensillum times 22,000 sensilla per antenna). If we assume that all of the *L. dispar* PBP is associated with the sensillum lumen, then the *in situ* concentration of total *L. dispar* PBP is 2.2 μg/1.1×10^{-8} liter. This converts to a molar concentration of 13.4 mM PBP (MW = 15,000 Da; 2.2×10^{-6} gm PBP per antenna divided by 15,000 gm/mol divided by 1.1×10^{-8} liter). The concentrations of PBP_1 and PBP_2 would be half this value, or 6.7 mM, respectively. This value is very close to the 10 mM concentration estimated for the PBP of *A. polyphemus* (Klein, 1987).

The PBPs of both *L. dispar* and *A. polyphemus* appeared to be sex specific in their expression, being absent from female antennae (Fig. 1 and 10; Vogt and Riddiford, 1981b). However, Gyorgyi et al. (1988) have observed that PBP is expressed in female antennae of *M. sexta,* though at a considerably lower level than in male antennae. These authors suggested that the presence of PBP in female *M. sexta* antennae is due to the large number of female olfactory sensilla in this species and that PBP might bind other odorants besides pheromone (Gyorgyi et al., 1988). It is certainly the case that female antennae of *L. dispar* and *A. polyphemus* are very reduced compared with male antennae, possessing comparatively few and very small olfactory sensilla (Boeckh et al., 1960; Scheffler, 1975; Koontz and Schneider, 1987), and that female *M. sexta* antennae possess a comparatively large number of olfactory sensilla relative to males (Sanes and Hildebrand, 1976a; Oland et al., 1988). It is therefore possible that we did not observe PBP in female antennae of *L. dispar* and *A. polyphemus* because there are very few female sensilla and PBP expression was merely too low to detect in these studies.

Expression of PBPs in moth antennae

Adult antennal development occurs during the pupal stage in Lepidoptera and has been described in depth for *M. sexta* by Sanes and Hildebrand (1976a, b). At the larval–pupal transition (P0), the antennal imaginal disks evert and grow into a mass of tissue approximately the size of the adult antennae. At the start of adult antennal development this tissue mass is 2 cell layers thick. The tissue mass then passes through a sequence of morphogenetic events that rapidly result in the adult form (Sanes and Hildebrand, 1976a, b). In *L. dispar,* adult development takes ca. 11 d at 27°C (Fig. 11). Antennal branches and sensilla are visibly forming during days P2 and P3 (R. G. Vogt, unpublished observations). We observed that both PBP_1 and PBP_2 were first expressed about 3 d prior to adult eclosion (A-3), corresponding to pupal stage P8. Translatable PBP mRNA was detectable at very low levels a day earlier, assayed by IVT. PBP mRNA levels rose to a plateau on P9 (A-2) where they remained until at least a day into the adult stage. The amount of PBP

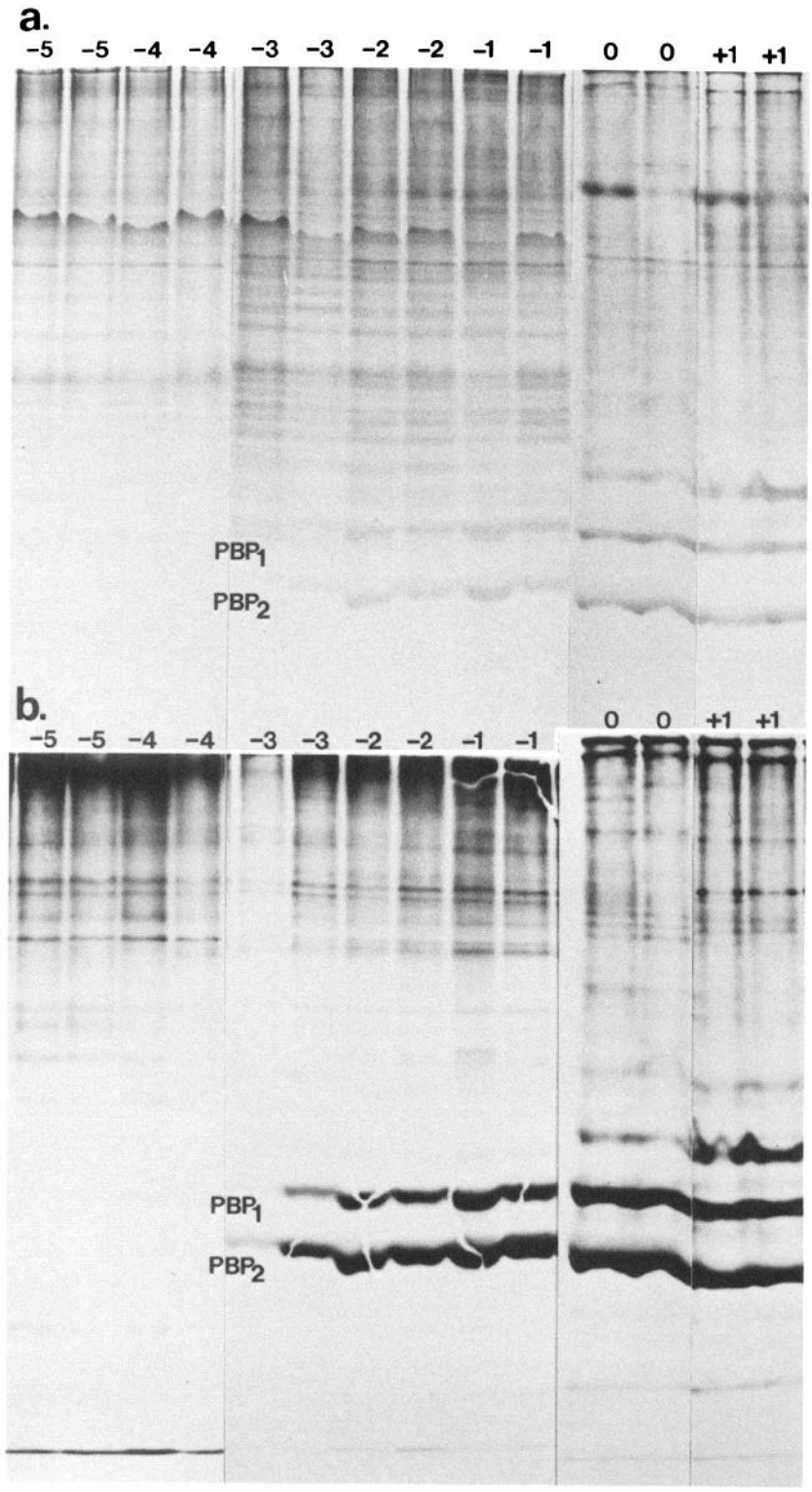

Figure 7. Electrophoretic comparison of total (*a*) and newly synthesized (*b*) male *L. dispar* antennal proteins of different developmental stages. Male pupae were injected with ^{35}S-methionine (33 μCi), and 24 hr later antennae were removed, staged, and processed for non-SDS-PAGE (15%). Proteins were visualized by Coomassie blue staining (*a*) followed by fluorography (*b*). Each lane represents protein of one individual, with the stage at the time of antennal removal indicated (e.g., −2 refers to stage A-2). One antennal equivalent was loaded per lane.

increased to a constant level by P10 (A-1), where it remained at least a day into the adult stage (A1). It is notable that male and female *L. dispar* will readily mate on the day of adult eclosion (A0). These results are summarized in Figure 11. Our observations of PBP expression in *A. polyphemus* and *M. sexta* were consistent with the *L. dispar* findings, suggesting that this scheme is general.

The observation that the concentration of PBP is held at a constant level while under a constant rate of synthesis suggests that it is under a steady-state rate of turnover. An estimate can be made of this turnover rate. Earlier in this discussion it was estimated that a single antenna contains about 2.2 μg of total PBP. The studies illustrated in Figures 6 and 7 suggest that it takes 2 d to reach this full antennal compliment of 1.1 μg of

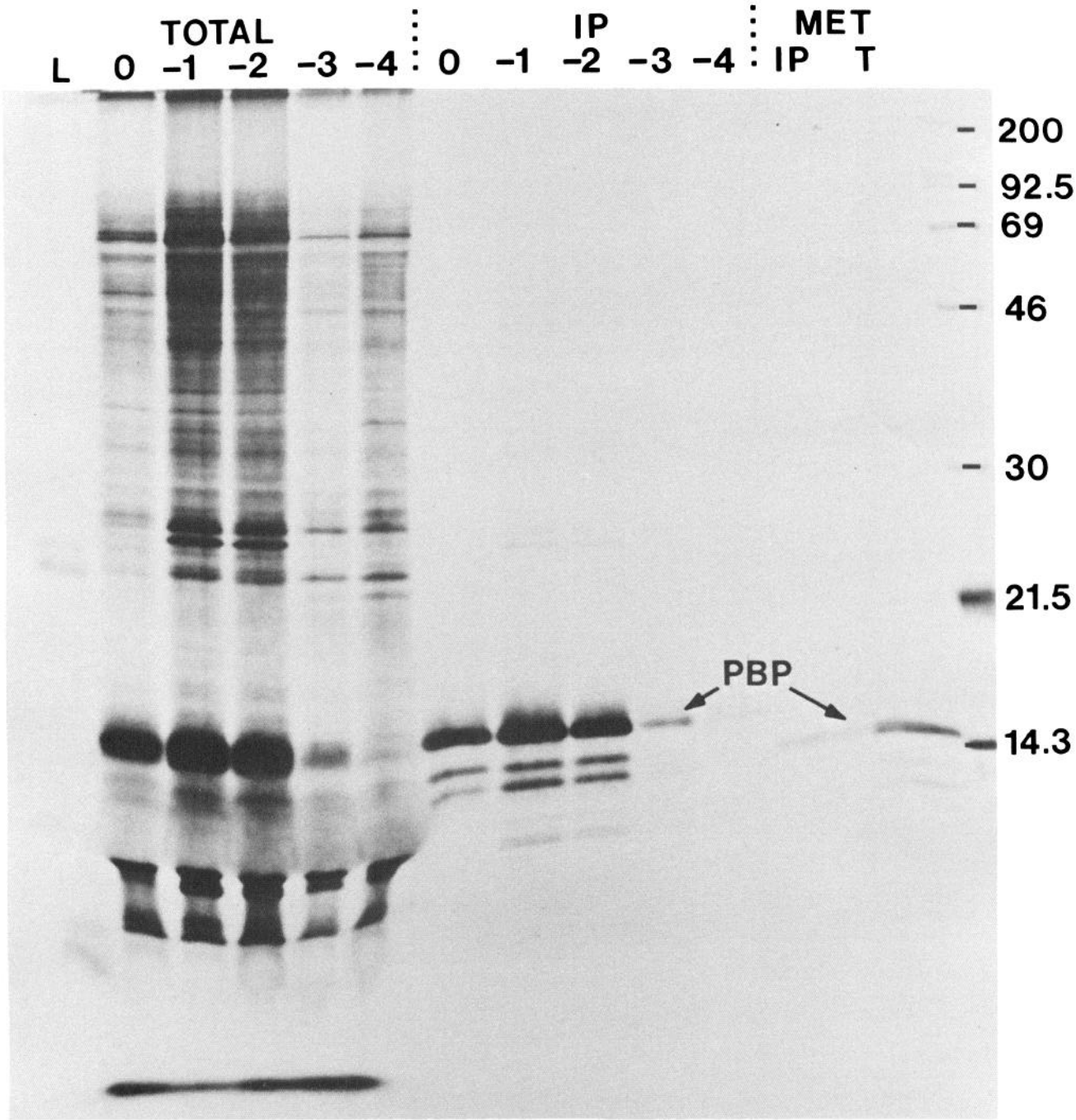

Figure 8. Electrophoretic comparison of *in vitro* translated antennal mRNAs isolated from different developmental stages. *Total,* Total *in vitro* translated proteins from mRNA of male antennal stages A-1 (−1) to A0 (0) and from lysate control (*L*). *IP,* Immunoprecipitated proteins from total *in vitro* translated mixtures, utilizing combined antisera generated against *L. dispar* PBP_1 and PBP_2 at 1:100 dilution, respectively. For each stage, RNA was isolated from 44 antennae, and tissue equivalent fractions of total and immunoprecipitated proteins were examined. *MET,* Total (*T*) and immunoprecipitated (*IP*) proteins from a homogenate of male antennae, metabolically labeled *in vivo* with ^{35}S-methionine. Label was injected into A0 males, and antennae were processed 24 hr later. Immunoprecipitation conditions were the same as for the *in vitro* translated proteins. The blurred region and distortion towards the bottom of the *Total* lanes was due to the large amount of endogenous hemoglobin present in the lysates. Molecular-weight markers are in kilodalton units.

each PBP. The *in vivo* labeling study (Fig. 7) suggests that the rate of PBP synthesis reached a maximum after a day and remained at that maximum. If (1) the full complement of PBP is synthesized in 2 d and (2) the rate of synthesis remains at this level, but (3) the level of PBP does not continue to increase, then (4) after day A-1 the rate of turnover must match the rate of synthesis. Thus, a steady-state turnover can be estimated to be 1.1 μg PBP_1 and PBP_2, respectively, every 2 d per antenna, at least from A-1 to A1.

While the site is not yet confirmed, PBP synthesis most likely occurs in the support cells that surround the neuronal cell bodies at the base of each sensillum (Fig. 1). The PBPs are then presumably exported across the apical membranes of these cells into the sensillum lumen. Coated vesicles and pits, which might

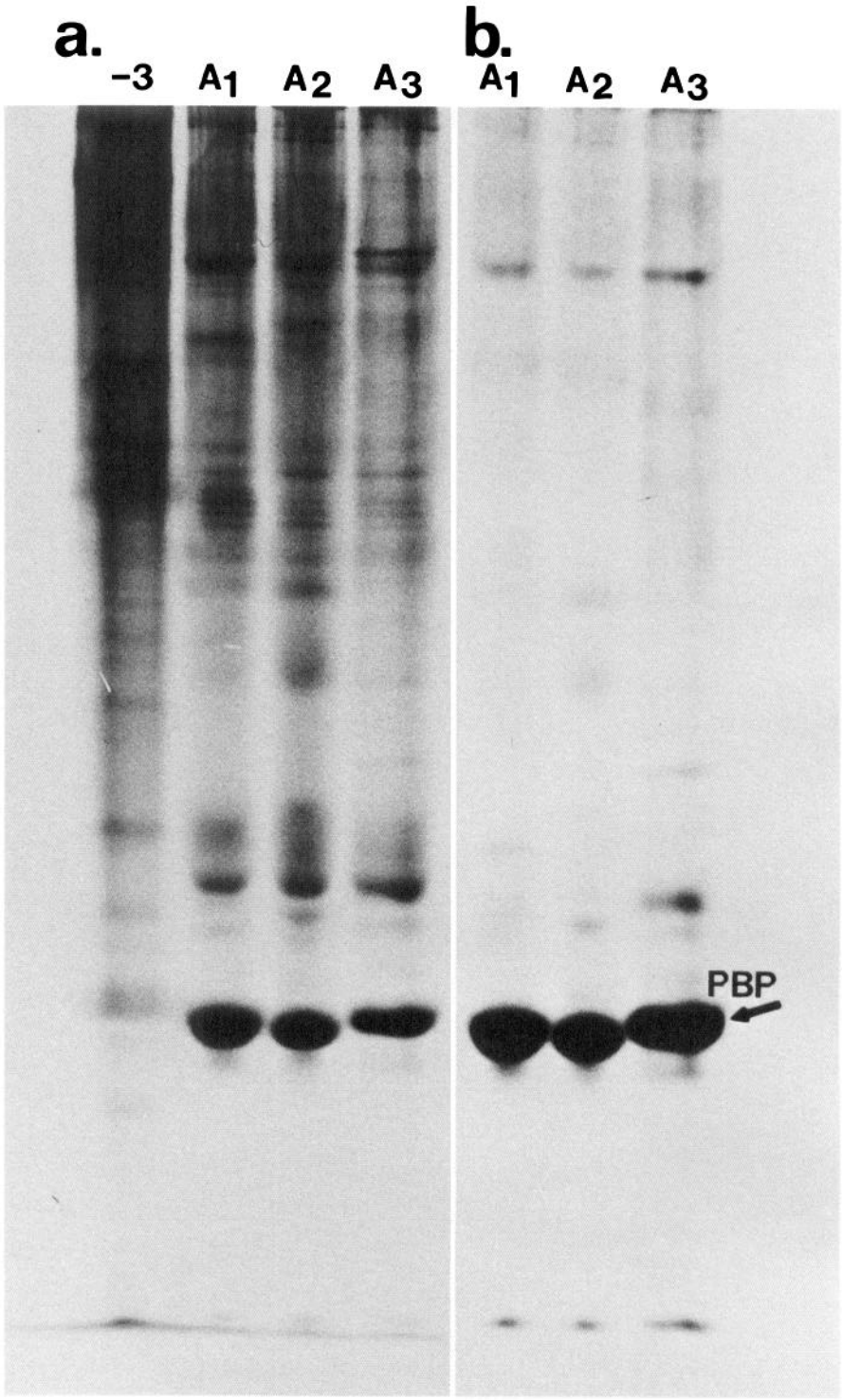

Figure 9. Electrophoretic comparison of total and newly synthesized male *A. polyphemus* antennal proteins. *a,* Comparison of the total protein of an A-3 (*−3*) individual with 3 A1 individuals (*A1, A2, A3*), visualized by Coomassie blue staining. One antennal equivalent was loaded per lane. The A-3 antennae was staged by allowing its donor to continue its development and eclose, which it did 3 d later. *b,* Fluorograph of the gel in *a,* representing protein synthesized during the previous 24 hr. The A1 individuals were injected with ^{35}S-methionine on their respective days of eclosion (A0). The A-3 individual was not injected. The proteins were separated by non-SDS-PAGE (15%).

serve as the route of PBP secretion, have been observed associated with these apical membranes (Steinbrecht and Gnatzy, 1984). Assuming that all of the PBP synthesis is restricted to these support cells, the steady-state turnover per sensillum would be 10^{-10} gm PBP per sensillum/2 d (2.2×10^{-6} gm PBP per antenna divided by 22,000 sensilla per antenna), or 2×10^{-12} gm PBP/hr (10^{-10} gm PBP per 2 d divided by 48 hr per 2 d). This finally converts to a turnover rate of 8×10^{7} molecules PBP/hr/sensillum (MW = 15,000 Da; 2×10^{-12} gm PBP/sensillum divided by 15,000 gm/mol multiplied by 6×10^{23} molecules/mol). The turnover rates of PBP_1 and PBP_2 would each be half this value, or 4×10^{7} molecules/hr/sensillum, respectively.

This high rate of turnover is curious because it is of an extracellular protein that exists in a somewhat closed space, the sensillum lumen. Turnover implies that the protein is exported and imported across the same cell membranes (Fig. 1). It is normal for insect epithelium to export and import large amounts

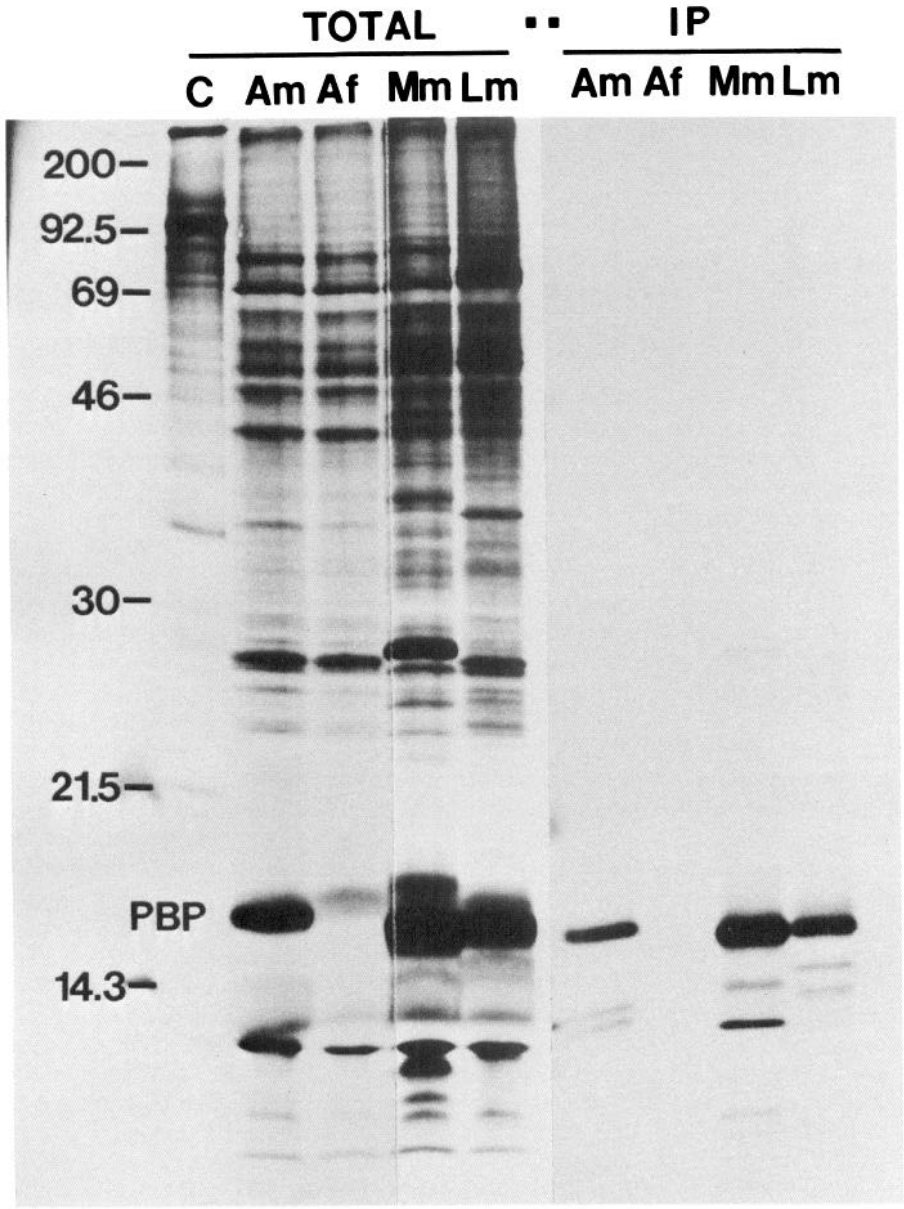

Figure 10. Electrophoretic comparison of *in vitro* translated proteins from antennal mRNAs of adult (A0) male and female *A. polyphemus* (*Am* and *Af*), male *M. sexta* (*Mm*) and male *L. dispar* (*Lm*). *Total* and immunoprecipitated (*IP*) proteins are shown. Antisera used in the immunoprecipitations were generated against the PBPs of the respective species. Proteins were separated by SDS-PAGE (15%). Molecular weight-markers are in kilodalton units.

of cuticle protein during the molt cycle, secreting protein to build cuticle, and then resorbing partially degraded cuticle proteins to be recycled into the next stage cuticle (Hepburn, 1985). However, these 2 processes are separated in time in the case of cuticle production. In the sensilla, secretion and apparent resorption of PBP seem to be occurring simultaneously. Do these processes occur in discretely different regions of cell membrane, or do they even occur within the same cell? Do the cells resorb intact or degraded PBP? Is the site of PBP degradation in the sensillum lumen or within the support cells? Does recycling of PBP contribute in any way to the dynamic properties of these sensilla?

Our observations indicate that *L. dispar* PBP expression is initiated ca. 3 d before adult eclosion. Similarly, *M. sexta* PBP (Gyorgyi et al., 1988), pheromone-degrading enzyme (Rybczynski et al., 1989), and male olfactory specific antigen (MOSA) (Hishinuma et al., 1988a, b) were also first observed about 3 d before adult eclosion, even though *M. sexta* adult development requires 20 d (Sanes and Hildebrand, 1976a) compared with the 11 d of *L. dispar*. This suggests that the final molecular maturation of the olfactory sensilla is coordinated relative to adult eclosion. Schwartz and Truman (1983) have suggested that many of the events occurring late in adult development are coordinated by falling levels of the steroid hormone 20-hydroxy ecdysone, and this may prove to be the case for molecular maturation of the olfactory system as well.

The function of PBPs

We have previously suggested a model for pheromone reception in which (1) pheromone enters a sensillum via a pore through the cuticle wall, (2) pheromone becomes solubilized in the lumen by interacting with PBP, (3) PBP-solubilized pheromone migrates through the lumen until it encounters either a membrane-associated receptor protein or an inactivating enzyme (Vogt et al., 1985; Vogt, 1987). Two key points in this scheme are (1) rapid pheromone degradation by specific enzymes and (2) the

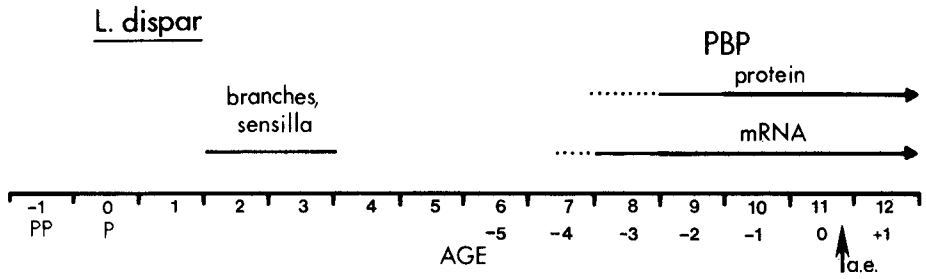

Figure 11. Summary of PBP expression during antennal development. The axis represents age, both relative to pupal eclosion (*upper numbers, −1 to 12*) and adult eclosion (*lower numbers, −5 to +1*). *PP* and *P*, days of prepupal and pupal selection, respectively; *a.e.* (at *arrow*), time of adult eclosion.

need for pheromone molecules to be solubilized (Kaissling, 1986; Vogt, 1987). Pheromone-degrading enzymes have been described in *A. polyphemus* (Vogt and Riddiford, 1981b; Vogt et al., 1985), *M. sexta* (Rybczynski et al., 1989), and *L. dispar* (Prestwich et al., 1989). The *A. polyphemus* and *M. sexta* enzymes are an esterase and an aldehyde oxidase, respectively, which are uniquely associated with the antennae and present in the lumen of the pheromone-sensitive sensilla. These enzymes degrade sex pheromone sufficiently fast *in vitro* to suggest they are responsible for rapid stimulus inactivation. A similarly rapid odorant degradation process has been described in the olfactory organ of a lobster (Trapido-Rosenthal et al., 1987). Thus, rapid enzymatic inactivation of olfactory signal appears to be general, at least in arthropods.

The need for odorant molecules to be water soluble also appears to be general. While fully aquatic animals smell water-soluble odorants such as amino acids and purines, terrestrial animals smell volatile, hydrophobic odorants. Olfactory specific proteins that can enhance the solubility of hydrophobic odorants have been described in vertebrates (Pelosi et al., 1982; Pevsner et al., 1985, 1986; Lee et al., 1987) and invertebrates (Vogt and Riddiford, 1981a; Chase and Tolloczko, 1985; Chase, 1986; Gyorgyi et al., 1988). Both the rat (Pevsner et al., 1988a) and insect (Vogt, 1987; Gyorgyi et al., 1988) odorant binding proteins have been suggested to trap or solubilize odorants, aiding in their transport to membrane-associated receptor proteins. In the land snail, the olfactory epithelium is located on the tip-surface of each tentacle, surrounding the eye. Epithelial glands secrete large quantities of proteins that apparently act to wet the surface of the epithelium hygroscopically, and may otherwise aid in the capture of volatile odorants (Chase and Tolloczko, 1985; Chase, 1986). It may be that odorant binding proteins represent a terrestrial adaptation, evolved to deal with volatile odorants. Gyorgyi et al. (1988) have found that the vertebrate and insect odorant binding proteins are not related, based on comparison of their cDNA-derived full-length sequences (Gyorgyi et al., 1988; Pevsner et al., 1988b). This suggests that if these odorant binding proteins were terrestrial adaptations, they evolved independently and represent a convergence in evolution.

Two genetically distinct PBPs are expressed in *L. dispar* antennae, in contrast to other species we have examined where only a single PBP is expressed. These species include *A. polyphemus* (Vogt and Riddiford, 1981b), *Antheraea pernyi* (Vogt and Riddiford, 1981a), *M. sexta* (Gyorgyi et al., 1988), and *Heliothis virescens, Orgyia pseudotsuga,* and *Hyalophora cecropia* (R. G. Vogt, unpublished observations). Furthermore, the 2 *L. dispar* PBPs apparently bind the sex pheromone disparlure with different affinities. Both (+) and (−)disparlure remained associated with PBP_2 to an equivalent and substantially greater extent than to PBP_1 through electrophoretic separation (Fig. 3).

There are several hypotheses to account both for the existence of 2 *L. dispar* PBPs and for the difference in apparent disparlure binding affinities for PBP_1 and PBP_2. First, there may be 2 sensilla types, equally represented and containing PBP_1 and PBP_2, respectively. Second, PBP_1 and PBP_2 might coexist in the same sensilla and through different pheromone affinities affect the dynamic response of the hair. The lower-affinity PBP_1 might either increase the capacity of pheromone within the sensillum or influence the partitioning and mobility of pheromone within the sensillum. Third, the presence of PBP_1 and its apparent low affinity for disparlure may predict an additional pheromone in *L. dispar,* one with considerably different structure than disparlure. The identity table (Table 1) indicates that PBP_1 shares more identity with the *A. polyphemus* and *M. sexta* PBPs than does PBP_2. The pheromones of *A. polyphemus* and *M. sexta* are very similar long-chain hydrocarbons with polar functional end groups (Kochansky et al., 1975; Starratt et al., 1979; Doolittle et al., 1988), in contrast to the very different structure of the epoxide disparlure (Bierl et al., 1970). Perhaps the presence of PBP_1 suggests an additional *L. dispar* pheromone component structurally more similar to the *A. polyphemus* or *M. sexta* pheromones than to disparlure. A fourth, but unlikely, hypothesis is that PBP_1 binds the degraded product of disparlure. Homogenates of whole *L. dispar* antennae readily convert the epoxide disparlure to a diol (Prestwich et al., 1989), while sensilla isolated under our conditions do not (R. G. Vogt, unpublished observations). When we attempted the binding study illustrated in Figure 3 utilizing whole antennal homogenates under conditions where epoxide degradation was rapid, we observed no radiolabeling of either PBP, suggesting that the diol was not binding. A similar lack of PBP–pheromone product association was previously observed in the *A. polyphemus* system (de Kramer and Hemberger, 1987).

Many of the issues raised by these studies can be addressed at the histological level utilizing appropriate nucleic acid and antibody probes. These issues include the cellular localization, sex specificity, and sensillar distribution of PBP expression, as well as the subcellular sites of PBP turnover and recycling. It is becoming increasingly clear that the insect odorant binding proteins represent a family of proteins that have a specific role in the sexual behavior of these animals. Considering the central role olfaction plays in reproduction in these animals, it is difficult to imagine that the molecular components of this system have been selected to play other than dynamic roles in this process.

There are clear cross-phyla parallels in the olfactory system at both the anatomical and molecular levels. An anatomy of primary olfactory neurons terminating in glomerular synapses within an olfactory bulblike structure exists in vertebrates (Shepherd, 1972), insects (Schneiderman et al., 1982; Ernst and Boeckh 1983; Koontz and Schneider, 1987), and molluscs (Chase and Tolloczko, 1986). A biochemistry of documented and candidate

odorant binding proteins exists within the same groups (Chase, 1986; Vogt, 1987; Pevsner et al., 1988a). Insects provide an outstanding model system for understanding the principals underlying olfactory function. The moth olfactory organ is particularly, well suited to molecular level studies because of its considerable size and accessibility and its highly focused function of detecting and monitoring sex pheromone. Testing the roles the molecular components play requires an ability to manipulate these components, and the development of such an ability seems paramount to the comprehension of this system. Nevertheless, in light of the role that odor detection plays in the sexual behavior of these animals, insight into the molecular basis of insect olfaction contributes directly to our understanding of the role the PNS has in coordinating complex behavior.

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