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Female *Attacus atlas* Respond to Pheromones of *Antheraea polyphemus*: a Comparative Electrophysiological and Biochemical Study

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Abstract

Female *Attacus atlas* respond electrophysiologically to both of the *Antheraea polyphemus* pheromone components (*E*,*Z*)-6,11-hexadecadienyl acetate and (*E*,*Z*)-6,11-hexadecadienal. Moreover, they possess a pheromone-binding protein (PBP) and general odorant-binding proteins (GOBPs), as well as a pheromone-degrading sensillar esterase and aldehyde oxidase enzymes. They show no electroantennogram responses to their own gland extract. In contrast, female *A. polyphemus* do not respond to their own or to *A. atlas* pheromone. Male *A. atlas* do not detect any of the *A. polyphemus* compounds but only the conspecific female gland extracts. Both male *A. atlas* and female *A. polyphemus* possess PBP and GOBP but lack the pheromone-degrading esterases of male *Antheraea*. The results indicate that the two species use quite distinct classes of chemicals as pheromones. In spite of this, the N-terminal amino acid sequences of the PBPs show homology of 68%.

Introduction

In the communication system of moths the female is usually the sender of the signal, emitting chemical compounds that are sensed and behaviorally answered by the males. In moth species the females appear to be unable to detect their own pheromone and only a few examples of autodetection have been found (Ross *et al.*, 1979; Ljungberg *et al.*, 1993; Den Otter *et al.*, 1996; Schneider *et al.*, 1998; Pearson and Schal, 1999). This has led to the situation that olfaction of male moths has been more thoroughly investigated than that of females.

Priesner (1968) investigated interspecific electrophysiological responses of males of more than 100 saturniid moth species. He defined *reaction groups* (RG) as a group of species that respond electrophysiologically [in electroantennograms (EAGs)] as if their pheromones were identical (though the components may occur in different ratios in the pheromone blend). Moths from one RG may respond to a lesser degree to the pheromones of another RG. The *Saturnia* RG species, which includes *Antheraea polyphemus*, show responses to 14 of the 19 RG defined. In contrast, the *Attacus* RG is very delimited. This reaction group, which includes *Attacus atlas*, has only some overlap with the *Philosamia* RG (the genus *Attacus* is known to have been derived from *Philosamia*). The *Saturnia* RG and the *Attacus* RG thus represent extremes in Priesner's classification, which led us to compare pheromone reception in the two groups.

The pheromone blend of *A. polyphemus* is a mixture of at least two compounds, (*E*,*Z*)-6,11-hexadecadienyl acetate (C16-AC) and (*E*,*Z*)-6,11-hexadecadienal (C16-AL) (Kochanski *et al.*, 1975; Kaissling, 1979). The males also possess olfactory neurons specific for a third compound, (*E*,*Z*)-4,9-tetradecadienyl acetate (C14-AC) (Meng *et al.*, 1989). While this compound has not been found in *A. polyphemus*, it is a component of the pheromone blend of the closely related species *Antheraea pernyi* (Bestmann *et al.*, 1987).

In our comparison of *A. polyphemus* and *A. atlas* we also investigated proteins of the sensillum lymph [odorant-binding proteins (OBPs), sensillar esterase and aldehyde oxidase] that are involved in odorant reception and degradation. OBPs are soluble proteins of low molecular weight distributed in the sensillum lymph that surrounds the dendrites of olfactory receptor cells. They are called pheromone-binding proteins (PBP) if involved in the reception of pheromones or general odorant-binding proteins (GOBPs) and antennal-binding proteins (ABPX) if associated with sensilla specialized for non-pheromonal odorants [reviewed by Steinbrecht (Steinbrecht, 1998)]. Insect OBPs are thought to be involved in solubilization, transport and deactivation of odorants [reviewed by Kaissling and Ziegelberger (Kaissling, 1996; Ziegelberger, 1996)].

Pheromone-degrading enzymes have been characterized in the olfactory system of *A. polyphemus* [reviewed by Vogt *et al.* (Vogt *et al.*, 1990)]. The males of *A. polyphemus* possess esterases and oxidases that metabolize the pheromone components C16-AC and C16-AL into non-excitatory

compounds. The metabolites are more soluble in water and therefore more easily eliminated from the system.

In order to understand intra- and interspecific pheromone reception in these two saturniid species belonging to different reaction groups, we investigated EAG responses of both sexes of *A. atlas* and *A. polyphemus* to female sex pheromone gland extracts as well as to the known *A. polyphemus* pheromone components. By using three antisera raised against moth OBPs and colorimetric enzymatic assays we have also compared the sensillar proteins in antennal homogenates of both species, to determine if species using different pheromones employ a different repertoire of soluble proteins.

Materials and methods

Animals and reagents

D. Oehlke (New Jersey, USA) and W. Oehlke (Montague, Canada) supplied pupae of *A. polyphemus*. The cocoons were kept at 4°C with a light/dark cycle of 11/13 h. For emergence, the cocoons were warmed to room temperature at 65% relative humidity in a light/dark cycle of 13/11 h for 3 weeks.

M. de Cleen (Belgium) and R. Burgess (London Pupae Supply, UK) supplied pupae of *A. atlas*. For emergence they were kept under the same conditions as *A. polyphemus*.

C16-AC, C16-AL and C14-AC were synthesized by H.J. Bestmann (Erlangen, Germany) and F. Griepink (Wageningen, The Netherlands).

The anti-PBP and anti-GOBP2 antisera were raised by immunizing rabbits with purified, native PBP or GOBP2 of *A. polyphemus* as previously described (Steinbrecht *et al.*, 1992, 1995). The anti-GOBP1 antiserum was generated against the respective *Bombyx mori* recombinant protein, following the protocol of Steinbrecht and co-workers (Steinbrecht *et al.*, 1992).

All reagents were purchased from Sigma, unless stated otherwise.

Preparation of sex pheromone gland extracts

One- or two-day-old calling females were killed by freezing in liquid nitrogen. After 30 min the glands were excised and immersed in 200 µl of distilled hexane. For cleaning and filtration the raw extract was put on a 0.5 ml silica gel column (230–400 mesh; Merck) and washed through the column with 500 µl of hexane. All extracts were stored at –20°C until required.

Electrophysiology

EAGs were recorded using glass electrodes filled with hemolymph ringer (Kaissling, 1995), which were inserted into the tip and base of isolated antennae. We evaluated maximum amplitude of the EAG during a 1 s stimulation. The preparation was placed in a filtered air stream issuing from a glass tube. The tube had an aperture through which odor stimuli could be presented. Filter papers (7.2 × 14 mm) were impregnated with the chemical substance to be tested and placed in Pasteur pipettes. Stimulation of the antennal preparation was carried out by means of a controlled airflow (100 ml/s) that passed through the pipette with the filter paper. To measure dose–response characteristics the concentrations of the test stimuli were successively increased, with control stimulations interspersed between successive concentration steps. Between stimulus presentations, purified air was passed over the antennal preparation for at least 30 s.

Gas chromatography (GC) and coupled GC–EAG

GC analyses of the pheromone extracts were conducted on a Shimadzu GC-17A instrument fitted with a split/splitless injector (220°C) and a flame ionization detector (260°C). The compounds were separated on a fused silica capillary column (25 m × 0.25 mm i.d., Permabond SE-54; Macherey-Nagel, Germany). The carrier gas was helium (1.3 ml/min). At the end of the column the flow was split, half going to the flame ionization detector, the other half to the EAG recording system. Injections onto the analytical column were made with a split valve closed for 30 s. The column was held at 130°C for 3 min, then the temperature was increased by 15°C/min to 195°C, then by 4°C/min to 240°C and finally by 15°C/min to 285°C and held at that temperature for 10 min.

For coupled GC–EAG the outlet of the column (~14 cm from the preparation) delivered the separated compounds into a continuous air stream blown over the preparation. If EAG recording was not needed, the flow was discarded into the room air.

Preparation of antennal extracts

Antennal side branches of male and female antennae of *A. polyphemus* and *A. atlas* were homogenized on ice for 20 min with a home made, motor driven homogenizer in 20 mM Tris–HCl buffer, pH 7.2, in the presence of 1 mM phenylmethylsulphonyl fluoride (PMSF), as protease inhibitor. Homogenates were centrifuged at 10 000 r.p.m. for 15 min and the supernatants used in further experiments.

SDS–PAGE and native PAGE

Electrophoresis under denaturating conditions was performed in polyacrylamide gels with 5% SDS (SDS–PAGE) using a Bio-Rad Mini-Protean II apparatus and a discontinuous buffer system according to Laemmli (Laemmli, 1970). Native electrophoresis was performed under the same conditions but without SDS (native PAGE). Proteins were stained with Coomassie Brilliant Blue R250 or silver stain.

Immunoblotting

After electrophoretic separation proteins were electrotransferred onto nitrocellulose membranes, according to the semi-dry blotting procedure of Kyhse-Anderson (1984).

First, the nitrocellulose membranes were blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) and 0.05% Tween 20 for 1 h at room temperature and then incubated overnight with the anti-PBP, anti-GOBP1 or anti-GOBP2 antisera at a dilution of 1:1000 using a Mini-Protean II Multiscreen apparatus (Bio-Rad). Bound antibodies were detected by goat anti-rabbit IgG coupled to horseradish peroxidase (dilution 1:3000; Bio-Rad) and 4-chloro-1-naphtol as substrate.

N-terminal sequence analysis

Proteins from antennal side branches of male *A. atlas* were separated by 14% SDS–PAGE, electroblotted onto PVDF membrane (Schleicher & Schuell, Germany) and Coomassie stained. About 3 μg of each protein (200 pM) were used for N-terminal sequence analysis, performed by automated Edman degradation in a Protein Sequencer 476A (Applied Biosystems GmbH, Weiterstadt, Germany). All reagents and solvents used for N-terminal sequence analysis were obtained from Applied Biosystems. Prior to analysis, Coomassie stained protein bands were excised and placed directly onto the reaction cartridge. As cysteine was not modified and, therefore, not detectable, gaps in the resulting sequence were tentatively assessed as cysteine residues. Data were analyzed using Model 610A Data Analysis Program v.1.2.1.

Esterase staining

Gels were stained for esterase activity as previously described (Shaw and Prasad, 1970) after separation of proteins by 15% native PAGE and using as substrate a mixture of α- and β-naphthyl acetate, in the presence of Fast Blue RR. After esterase staining, protein patterns were visualized with Coomassie Brilliant Blue R-250.

Aldehyde oxidase staining

Following separation of proteins from antennal homogenates by 15% native PAGE, aldehyde oxidation was visualized according to the described protocol (Rybczynski *et al.*, 1989). Gels were washed five times (for 10 min) with 100 mM Na_3PO_4, pH 8.0, and stained with 50 mM propanal, 0.3 mM NBT and 0.12 mM PES in Na_3PO_4, pH 8.0. Staining was halted with several washes of 10% ethanol/5% acetic acid.

Results

Electrophysiological responses to pheromones

The males of each species gave a large EAG response to the pheromones of their own females but did not respond to the pheromone of the other species (Figure 1). *Attacus atlas* males do not show an electrophysiological response to *A. polyphemus* pheromone even when stimulated with a concentration of 1 mg/filter paper, which is ~1000 times higher than the threshold for *A. polyphemus* (Figure 2). For females, detection of their own pheromone is rarely found in Lepidoptera and females of both *Attacus* and *Antheraea* do not respond to their own pheromone. The females of *A. polyphemus* also do not respond to *Attacus* gland extracts,

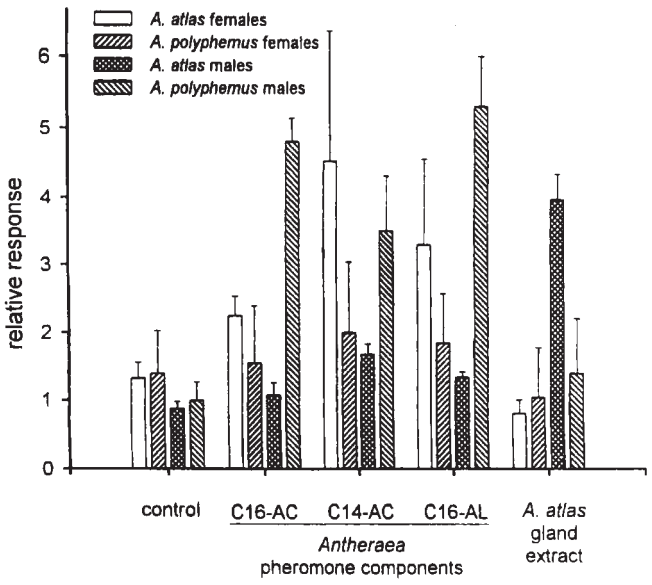


Figure 1 Electrophysiological responses (EAG) of *A. atlas* and *A. polyphemus*. Male *A. polyphemus* and female *A. atlas* respond to each of the *A. polyphemus* compounds (for C16-AL P 0.01, two-sided t-test). Male *A. atlas* respond to the gland extract of their females only; the responses of female *A. polyphemus* were not significantly different from the controls (for C16-AL P 0.2, two-sided t-test). Shown are mean responses (±SD) of nine animals for each compound (10 μg/filter paper). EAG response to an empty pipette was set as unity. Hexane on filter paper was used as the control.

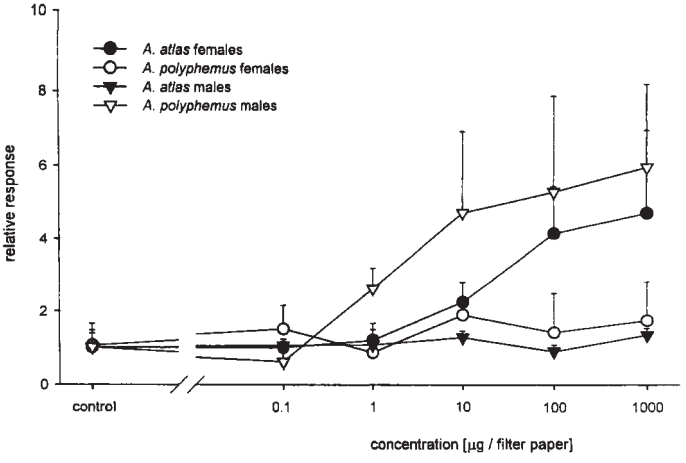


Figure 2 Electroantennogram responses of *A. atlas* and *A. polyphemus* to (*E*,*Z*)-6,11-hexadecadienyl acetate. Male *A. polyphemus* and female *A. atlas* show dose-dependent responses to C16-AC, but the latter need ~10-fold higher concentrations for the same degree of response. Female *A. polyphemus* and male *A. atlas* EAG responses to this compound were not different from the solvent control. Mean response (±SD) of seven animals, recorded at increasing stimulus concentrations. The EAG response to hexane on filter paper was set as 1; the range of responses was 0.3–8 mV.

but instead to some flower and green leaf volatiles (not shown). Surprisingly, *Attacus* females do respond to the *Antheraea* pheromone components (C16 AC, C16 AL and C14 AC) (Figure 1) and the response is clearly dose-dependent (Figure 2, not shown for C16-AL and C14-AC). The sensitivity of *A. atlas* females to C16-AC is only one order of magnitude lower than that of *Antheraea* males (Figure 2). *Attacus atlas* and *A. polyphemus* were additionally tested with *A. polyphemus* female gland extracts at a concentration of 5 gland equivalents/filter paper. Male *A. polyphemus* and female *A. atlas* showed qualitatively the same EAG responses as to the single compounds, while male *A. atlas* and female *A. polyphemus* did not respond (data not shown).

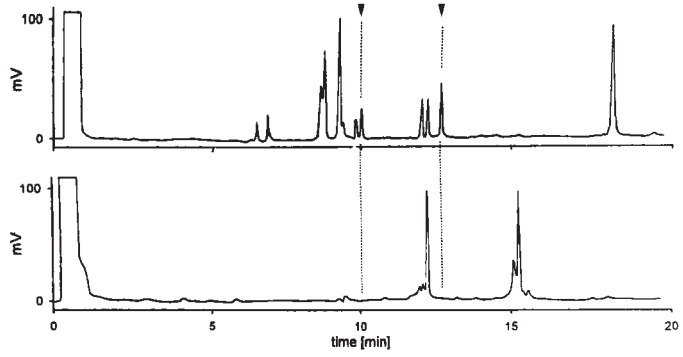


Figure 3 GC traces of gland extracts. (Upper) Hexane extract of 1 gland equivalent of female *A. polyphemus*; (lower) hexane extract of 1 gland equivalent of female *A. atlas*. In the *A. polyphemus* extract there are two peaks eluting at the same retention times as C16-AC (12.749) and C16-AL (10.029) (arrowheads). In this GC system C14-AC has a retention time of 9.998. In the *A. polyphemus* extract there is no peak eluting at this time. There are no corresponding peaks in the *A. atlas* extract to any of the compounds.

Comparison of female gland extracts by GC

In order to identify the sex pheromone components of *A. atlas*, 30 runs of coupled GC–EAG of the female gland extracts were performed. While *A. atlas* males responded electrophysiologically with clear depolarization in the normal EAG set-up when tested with 0.1–1.0 gland equivalents on the filter paper, no clear and reproducible responses to GC peaks in the GC–EAG mode were obtained even after concentration of the injected gland extract to 5 gland equivalents. GC–EAG recordings from *A. polyphemus* revealed responses to peaks of their conspecific gland extract.

When gland extracts from female *A. polyphemus* and *A. atlas* were compared, that of *A. polyphemus* contained both of the known pheromone compounds (Figure 3, arrowheads), while there were no corresponding peaks in the *A. atlas* extract.

Pheromone-degrading enzymes

The presence and activity of two pheromone-degrading enzymes, sensillar esterase, which metabolizes C16-AC, and aldehyde oxidase, which metabolizes C16-AL, in homogenates of male and female antennae of *A. polyphemus* and *A. atlas* were investigated. After separation of proteins in the homogenates by 15% native PAGE and Coomassie staining (Figure 4A), sensillar esterase staining demonstrated the presence and activity of this enzyme in *A. polyphemus* males, but not in females (Figure 4B). Sensillar esterase staining could be detected in the homogenate of *A. atlas* females, but not of males (Figure 4B). The sensillar esterase of female *A. atlas* showed a slightly different electrophoretic mobility than that detected in *A. polyphemus* males, likely representing different isoforms of the enzyme.

The aldehyde oxidase stain revealed positive reactions with both *A. polyphemus* male and female homogenates.

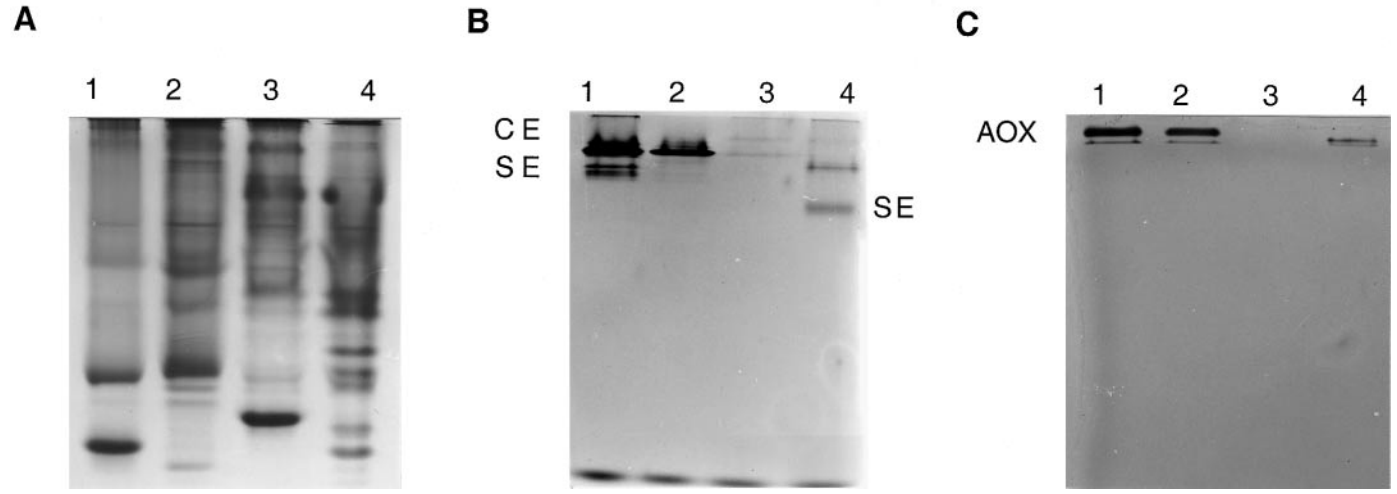


Figure 4 **(A)** Coomassie staining of proteins from homogenates of antennae of males and females of *A. polyphemus* (lanes 1 and 2) and of *A. atlas* (lanes 3 and 4) after separation by 15% non-SDS denaturing PAGE. **(B)** Esterase staining. **(C)** Aldehyde oxidase staining. CE, cuticular esterase; SE, sensillar esterase; AOX, aldehyde oxidase.

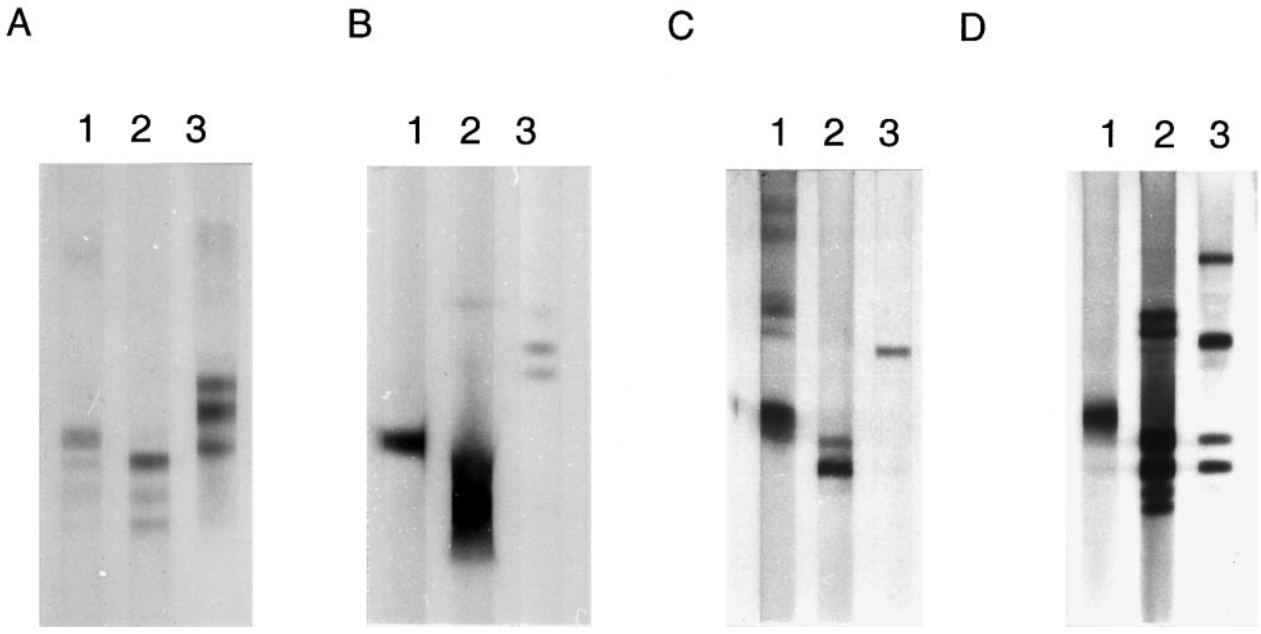


Figure 5 Western blot after separation of proteins by 15% native PAGE of antennal homogenates of male **(A)** and female **(B)** *A. polyphemus*, male **(C)** and female **(D)** *A. atlas*. Polyclonal antisera were raised against PBP of *A. polyphemus* (lane 1), GOBP2 of *A. polyphemus* (lane 2) and recombinant GOBP1 of *B.mori* (lane 3). All the antisera were used at 1:1000 dilution. Immunoreactive bands were detected in males and females of both species with all three antisera tested.

However in *A. atlas* only the homogenate of females was reactive to oxidase staining (Figure 4C). In male *A. atlas* we could not detect either of the two pheromone-degrading enzymes.

Insect OBPs

Three polyclonal antisera, generated against native PBP and native GOBP2 of *A. polyphemus* and against recombinant GOBP1 of *B.mori*, were used in Western blots to compare the presence of insect OBPs in the two species. Both species and sexes revealed immunoreactivity with the three antisera with bands of different electrophoretic mobility (Figure 5) but similar molecular weight (data not shown).

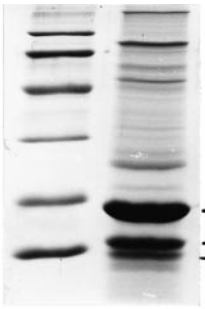
N-terminal amino acid sequence of proteins of male *A. atlas*

The three most abundant bands present in the homogenate of *A. atlas* males with molecular weights between 15 and 20 kDa were analyzed for their N-terminal amino acid sequences. After separation of the proteins by SDS–PAGE, proteins were transferred to PVDF, Coomassie stained (Figure 6) and the three major bands with apparent molecular weights between 15 and 20 kDa excised and subjected to microsequencing (Figure 6).

One of the bands (band 2) showed good amino acid sequence homology with PBPs of other lepidopteran species. In particular, the PBPs of *A. atlas* and *A. polyphemus* males showed ~68% amino acid sequence homology (Figure 6). Band 1 proved to be related to proteins belonging to the apolipoprotein class, sharing ~70% homology with apolipophorin of other moths (Figure 6). This class of proteins is involved in the increase in lipid-carrying capacity of lipophorin in the hemolymph. In insects they have been described for *Manduca sexta* (Cole *et al.*, 1987), *Galleria mellonella* (Weise, 1997; GenBank accession no. P80703) and *Diatrea grandiosella* (Burks *et al.*, 1992). Finally, the amino acid sequence of band 3 appears to be a new protein, with no homology to any of the proteins reported in the SwissProt database.

Discussion

Surprisingly, female *A. atlas* respond electrophysiologically to the *A. polyphemus* pheromone components as well as to C14-AC, a pheromone compound of *A. pernyi* (Bestmann *et al.*, 1987). To our knowledge, female sensitivity to sex pheromones of other species has not been reported for any other lepidopteran species. Moreover, *A. atlas* females possess the enzymes necessary for degradation of *A. polyphemus* pheromone compounds. Therefore, *A. atlas* females seem to be equally well adapted as the males of *A. polyphemus* to detect such compounds. There is no geographical overlap reported for the two species. However, several *Antheraea* species (e.g. *Antheraea gschwandneri*, *Antheraea pratti*, *Antheraea helferi*, *Antheraea roylei* and *Antheraea lampei*) are sympatric with *A. atlas* (Peigler, 1989). Although their pheromones are not known, it is likely that one of these species may use similar pheromones (Naessig *et al.*, 1996). The biological function of the ability of *A. atlas* females to detect such pheromone compounds remains unknown.



1 10 20 30

Attacus atlas Band 1 DAPNIFQDIEKHAQEFQKTF

Attacus atlas Band 2 SSEIMKTLSQNFXKAMDQXKKDQENNLPXA

Attacus atlas Band 3 DAAPASTGNALDEIQKQFEEIRRKELENNQ

	G. mellonella APL 3	*M. sexta* APL 3	*D. grandiosella* APL 3
Attacus atlas band 1	70%	65%	63%

	A. polyphemus PBP	*A. pernyi* PBP1	*A. pernyi* PBP2	*B. mori* PBP
Attacus atlas band 2	68%	67%	60%	59%

Figure 6 **(A)** N-terminal amino acid sequences of the three major proteins (inset) from homogenates of antennae of male *A. atlas* after separation by 14% SDS–PAGE. **(B)** Amino acid sequence homology of two *A. atlas* bands to the related proteins. Band 1 is related to proteins of the apolipoprotein family; band 2 revealed homology to PBPs from other moths; band 3 shows no homology with known proteins. APL, apolipophorin. Molecular weight markers, from bottom to top: 14, 20, 36, 45 and 66 kDa.

Comparison of gas chromatograms of the extract of glands of *A. atlas* females with those of *A. polyphemus* females indicates that the two species use different sex pheromone components for attracting receptive conspecific males.

The GC–EAG technique is routinely used in a number of laboratories as a tool in the identification of insect pheromones (Arn *et al.*, 1975; Wadhams, 1990). GC–EAG with *A. polyphemus* tested on conspecific gland extracts gave clear and reproducible responses. However, we could not obtain any reproducible responses with *A. atlas* using the coupled GC–EAG technique. Lack of material in the extracts is no explanation, because we injected up to 50 times more gland equivalents into the GC column than necessary on the filter paper to repeatedly elicit responses. The difference between EAG and GC–EAG also cannot be explained by heat leaking from the GC, as all antennae were tested before and after, and a few even while running, the GC in normal GC mode, which always led to responses. Other possible reasons for our failure to measure GC–EAGs could be as follows. (i) The pheromone compounds of *A. atlas* might be unstable under the temperature conditions in the GC. (ii) The compounds elute from the GC over a time span of several seconds and, therefore, the concentration necessary to elicit an EAG might not be reached. (iii) Olfactory receptors are adapted to detect fast transient rather than long lasting stimuli, thus the slow rise in concentration during odor stimulation as delivered by the GC is not optimal for stimulation of the olfactory system (Marion-Poll and Thiéry, 1996). The receptors might adapt very quickly and any subsequent EAG response may be inhibited. Recently, a new method generating a pulsed GC effluent to overcome this problem was introduced (Gouinguené *et al.*, 1998). (iv) The pheromone compounds might be permanently adsorbed to the GC column or deactivated fused silica transfer lines. Partial adsorption has been observed in several studies (Campbell *et al.*, 1990; Gouinguené *et al.*, 1998).

For *A. polyphemus* males our study is in agreement with previous results (Meng *et al.*, 1989) that show that they have receptor neurons tuned to each of the three compounds C16-AC, C16-AL and C14-AC. The antennal homogenates contained PBP as well as the pheromone-degrading enzymes sensillar esterase and aldehyde oxidase.

Attacus atlas males responded to conspecific female gland extracts and contained PBP, but surprisingly no sensillar esterase or aldehyde oxidase. As in a previous study (Priesner, 1968), the males of the two species showed no interspecific reactivity to the pheromones even with the highest concentrations tested. This suggests that the pheromone of *A. atlas* probably does not contain C16-AC, C14-AC or C16-AL.

Males of *A. polyphemus* and *A. atlas* contain PBPs with a high degree of amino acid sequence homology. Steinbrecht compared the immunoreactivity of PBPs of species from different moth families with an antiserum raised against the *A. polyphemus* PBP (Steinbrecht, 1996). He concluded that cross-reactivity was correlated more with the chemical structure, mainly chain length, of the pheromones used than with the taxonomical relatedness of the species tested. If homology between PBPs implies similarity of the pheromone ligands, our findings would not support such a conclusion. Unfortunately, the chemical structure of the *Attacus* pheromone is still unknown.

The content of pheromone-degrading enzymes in antennal homogenates of the two species correlates well with the electrophysiological responses. Females of *A. atlas* and males of *A. polyphemus* both respond to *A. polyphemus* pheromones and both contain the same repertoire of pheromone-degrading enzymes. Males of *A. atlas* do not detect

these pheromones and do not have esterases or aldehyde oxidases. The presence of aldehyde oxidase in the homogenate of female *A. polyphemus* antennae could be related to the detection of non-pheromonal aldehydes, i.e. common leaf components, such as has been reported for the antennal-specific aldehyde oxidase of female *M.sexta* (Rybczynski *et al.*, 1989).

In summary, our electrophysiological, GC and biochemical findings presented here, besides providing evidence of *A. atlas* females responding to the three pheromone components of female *A. polyphemus*, also suggest that *A. atlas* pheromone components are molecules with different chemical functional groups to acetate esters or aldehydes.

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