Title
Olfactory proteins mediating chemical communication in the navel orangeworm moth, Amyelois transitella.

Permalink
https://escholarship.org/uc/item/8xd8k0g5

Journal
PloS one, 4(9)

ISSN
1932-6203

Authors
Leal, Walter S
Ishida, Yuko
Pelletier, Julien
et al.

Publication Date
2009-09-30

DOI
10.1371/journal.pone.0007235

Peer reviewed
Olfactory Proteins Mediating Chemical Communication in the Navel Orangeworm Moth, Amyelois transitella

Walter S. Leal1*, Yuko Ishida1, Julien Pelletier1, Wei Xu1, Josep Rayo1, Xianzhong Xu2, James B. Ames2

1 Department of Entomology, University of California Davis, Davis, California, United States of America, 2 Department of Chemistry, University of California Davis, Davis, California, United States of America

Abstract

Background: The navel orangeworm, Amyelois transitella Walker (Lepidoptera: Pyralidae), is the most serious insect pest of almonds and pistachios in California for which environmentally friendly alternative methods of control — like pheromone-based approaches — are highly desirable. Some constituents of the sex pheromone are unstable and could be replaced with parapheromones, which may be designed on the basis of molecular interaction of pheromones and pheromone-detecting olfactory proteins.

Methodology: By analyzing extracts from olfactory and non-olfactory tissues, we identified putative olfactory proteins, obtained their N-terminal amino acid sequences by Edman degradation, and used degenerate primers to clone the corresponding cDNAs by SMART RACE. Additionally, we used degenerate primers based on conserved sequences of known proteins to fish out other candidate olfactory genes. We expressed the gene encoding a newly identified pheromone-binding protein, which was analyzed by circular dichroism, fluorescence, and nuclear magnetic resonance, and used in a binding assay to assess affinity to pheromone components.

Conclusion: We have cloned nine cDNAs encoding olfactory proteins from the navel orangeworm, including two pheromone-binding proteins, two general odorant-binding proteins, one chemosensory protein, one glutathione S-transferase, one antennal binding protein X, one sensory neuron membrane protein, and one odorant receptor. Of these, AtraPBP1 is highly enriched in male antennae. Fluorescence, CD and NMR studies suggest a dramatic pH-dependent conformational change, with high affinity to pheromone constituents at neutral pH and no binding at low pH.

Introduction

Insects are biosensors par excellence. They have developed a remarkable ability to detect with extreme sensitivity and selectivity small, hydrophobic molecules that convey essential information for their reproduction and survival. Female moths, for example, advertise their readiness to mate by releasing infinitesimal amounts of a species-specific sex pheromone bouquet, which is remotely detected by males with remarkable precision. Minute amounts of signal deters eavesdropping, but requires such a fine tuning that the male olfactory system may be considered a “gold standard” in olfaction. It has been estimated that males of the silkworm moth, Bombyx mori, can detect one molecule of the pheromone bombykol [1]. Moreover, small modifications in pheromone molecules render them completely inactive, or at least a few order of magnitude less active [2]. There is growing evidence in the literature suggesting that pheromone-binding proteins (PBPs) contribute to the sensitivity and possibly the selectivity of the olfactory system. PBPs are part of a family of olfactory proteins, including odorant-binding proteins (OBPs) and chemosensory proteins (CSPs), postulated to be involved in uptake of odorants, transport through the sensillar lymph, and delivery to membrane-bound odorant receptors.

A detailed mechanism has been proposed for a pheromone-binding protein of the silkworm moth, BmorPBP1, suggesting that a pH-dependent conformational change is involved in pheromone binding and release [3,4,5,6]. Indeed, structural biology studies showed that the C-terminal part of the protein forms an additional α-helix at low pH that competes with pheromone molecules for the binding pocket [7,8,9], thus enabling the delivery of the pheromone in acidic environment similar to that formed by the negatively charged dendrite surfaces of the olfactory receptor neurons [10]. Functional studies also showed that BmorPBP1, when co-expressed with pheromone receptor BmorOR1 in the empty neuron system of Drosophila, enhanced the response to the pheromone, indicating that OBPs contribute to the remarkable sensitivity of the insect’s olfactory system [11].

The navel orangeworm, Amyelois transitella Walker (Lepidoptera: Pyralidae), is the most serious insect pest of almonds and pistachios in California, and a major pest of a number of other crops, including walnuts and figs. The navel orangeworm is primarily controlled during the growing season with pyrethroids and insecticides, which are often applied repeatedly on a large scale, increasing the risk of resistance development.

Citation: Leal WS, Ishida Y, Pelletier J, Xu W, Rayo J, et al. (2009) Olfactory Proteins Mediating Chemical Communication in the Navel Orangeworm Moth, Amyelois transitella. PLoS ONE 4(9): e7235. doi:10.1371/journal.pone.0007235

Editor: Mark A. Frye, UCLA - Physiological Science, United States of America

Received August 11, 2009; Accepted September 8, 2009; Published September 30, 2009

Copyright: © 2009 Leal et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported in part by National Science Foundation (0918177 to W.S.L), the Almond Board of California, California Pistachio Research Board (to W.S.L) and the National Institute of Health (EY012347 to J.B.A), but the funders had no role in study design, data collection, and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: wslleal@ucdavis.edu
growth regulators, but alternative methods of control are sorely needed. Sex pheromones offer an environmentally-friendly alternative to control insect populations by mating disruption or other strategies in integrated pest management. Typically, sex pheromones and other attractants (aka semiochemicals) are identified by a bioassay-guided isolation of natural products. Alternatively, olfactory proteins may be used in a reverse chemical ecology approach [12,13] for screening potential attractants on the basis of their affinity to odorant-binding proteins. These proteins are part of a large family of carrier proteins, for which we coined the term encapsulins [12,14], but those directly involved in semiochemical reception are grouped into pheromone-binding proteins (PBP)s and general odorant-binding proteins (GOBP)s based on their transport of pheromones or other semiochemicals [15], respectively. We have now isolated, cloned and expressed olfactory proteins from the navel orangeworm and set the stage to use them in reverse chemical ecology. Although the sex pheromone system of the navel orangeworm has already been identified [16,17], some of the constituents are unstable. Reverse chemical ecology in this case can be used for the development of alternative compounds (parapheromones).

Results and Discussion

Isolation of antennae-specific proteins

To isolate putative olfactory proteins from the navel orangeworm, we extracted proteins from olfactory and non-olfactory tissues dissected from adult males and females, and compared protein profiles of these extracts by native polyacrylamide gel electrophoresis (PAGE). Typically, OBP@s are abundant acidic proteins that migrate faster than non-olfactory proteins thus appearing in the lower part of a native gel. Antennae-specific proteins can be identified by comparing protein extracts from non-olfactory tissues (e.g.: legs) with protein profiles from antennae. In addition, comparison of male and female antennal extracts may identify putative PBPs, which in most cases are specifically expressed or at least enriched in male antennae. This protein-based approach led us to identify several bands (Bands 1–7), which are likely to represent olfactory proteins from the navel orangeworm (Fig. 1). A faint band migrating just above Band 1 (Fig. 1) was also detected in leg extracts when larger control samples were analyzed (data not shown). To obtain the N-terminal amino acid sequences of the target proteins by Edman degradation we re-ran native PAGE analysis, transferred proteins to polyvinyl difluoride membranes and isolated the bands after staining. Band 1 (Fig. 1) was slightly more intense in extracts from female antennae, but samples from male and female antennae gave the same N-terminal sequence: SAEVMSHTAHFGKA. Contrary to the initial assumption that Bands 2 and 3 were different, they gave the same N-terminal sequence: SQEVLMHKMTASF. On the other hand, Band 4 was detected exclusively in male antennae and gave the N-terminal sequence SPEIMKDLISNFGR. Bands 5 and 6 gave nearly equal intensity in extracts from male and female antennae, whereas Band 7 (SDYITGKNIENIQE) was detected with higher intensity in male than female antennae.

Cloning of PBPs, GOBPs and a Chemosensory Protein (CSP)

Our PCR approach to clone the cDNAs encoding the isolated proteins started with the isolation of total RNA from antennal tissues and synthesis of first strand cDNA by the SMART RACE cDNA Amplification. We used 3’-RACE cDNA and 5’-RACE cDNA templates with degenerate primers, designed on the basis of the identified N-terminal amino acid sequences, GCGT15 or universal primer mix (UPM) primers and, subsequently, gene-specific primers (GSPs). With primers designed on the basis of the male-specific Band 4 we obtained a 711 bp-long cDNA encoding 164 amino acid residues, including 22 residues of a signal peptide, which was assigned on the basis of the N-terminal sequence of the mature protein. The mature protein contained the hallmark of insect OBP@s, six cysteine residues. Blast search indicated that this protein had 72, 70, and 69% identities with Synanthedon exitiosa PBP (AAF06142) [18], Antheraea polyphemus PBP1 (CAB35592) [19] and A. pernyi PBP2 (Q17078) [20]. Thus we named this protein AtraPBP1 (Accession Number, GQ433364; calculated molecular weight, 16,072 Da). 

Next, we cloned the genes encoding the other antennae-specific proteins starting with Band 1. Although we observed a rather unusual connection of the SMART II Oligonucleotide (Clontech) to the 5’-end of the cDNA immediately after the 5’-region encoding the N-terminal amino acid sequence, we were able to

![Figure 1. Analysis of proteins extracted from olfactory and non-olfactory tissues.](image-url)
isolate a 511 bp-long cDNA encoding a 141-residue mature protein with a N-terminal sequence identical to that of the isolated protein. The translated protein had six cysteine residues and showed 84, 81 and 79% identities with *Manduca sexta* GOBP2 (AA650015) [21], *A. pernyi* GOBP2 (Q17075) [22], and *Samia cynthia ricini* GOBP2 (BAF91326) (Leal, unpublished), respectively. We, therefore, named the Band 1 protein AtraGOBP2 (Accession Number, GQ433366; calculated molecular weight, 16,166 Da; pI, 4.87).

We had also encountered problems with 5'-RACE when cloning the cDNA encoding the protein in Bands 2 and 3, but in this case the SMART II Oligonucleotide was connected upstream of the 5'-region encoding the N-terminal amino sequence and the signal peptide. The cloned partial cDNA sequence included 614 bp encoding for 21 amino acid residues of the signal peptide and 146 residues of the mature protein. We sequenced 16 independent clones and observed four points of polymorphism at 130<sup>th</sup> C/G, 368<sup>th</sup> T/A, 521<sup>st</sup> A/T and 539<sup>th</sup> T/A, which suggest the occurrence of two forms of the mature protein, one with Phe-102 and the other having Tyr-102, and both having six cysteine residues. Blastp search indicated 67, 66, and 65% identity to pheromone-binding proteins from *Heliothis virescens* PBP2 (CAL48346) [23], *Drosophila melanogaster* PBP2 (ABY28381) (Yang, W. L. et al., unpublished), and *H. armigera* PBP2 (ACD01993) (Zhang, S. et al, unpublished), respectively. Therefore, we named the two forms AtraPBP2F102 (Accession Number, GQ433365; calculated molecular weight, 16,747 Da; pI, 5.15) and AtraPBP2Y102 (Accession Number, GQ433366; calculated molecular weight, 16,747 Da; pI, 5.11).

With degenerate primers for the protein in Bands 5 and 6, we isolated a partial cDNA sequence of 702 bp encoding 144 amino acid residues of the mature protein, which included seven cysteine residues, and four residues of the signal peptide. Blastp search indicated that the protein had 78, 77, and 76% identities with GOBP1s from *Bombyx mori* (CAAA64444) [24], *Plutella xylostella* (ABW05104) (Dong, X.-L. et al., unpublished), and *H. armigera* PBP2 (ACD01993) (Zhang, S. et al, unpublished), respectively. Therefore, we named the two forms AtraPBP2P102 (Accession Number, GQ433365; calculated molecular weight, 16,174 Da; pI, 5.15) and AtraPBP2P102 (Accession Number, GQ433366; calculated molecular weight, 16,174 Da; pI, 5.11).

Lastly, we cloned the cDNA encoding the protein detected in Band 7. A partial 537 bp-long cDNA was isolated, which encoded for 105 amino acid residues, including the N-terminal sequence obtained by Edman degradation. This protein was named AtraCSP (Accession Number, GQ433369; calculated molecular weight, 12,923 Da; pI, 5.63) because the mature protein contains four cysteine residues and showed 80, 77, and 73% amino acid identity to chemosensory proteins from *B. mori* (AAV34688) [26], *Cactoblastis cactoris* (AAC47827) [27], and *H. armigera* (AF363875) (Deyts et. al., unpublished), respectively.

**Cloning of other olfactory proteins**

While attempting to clone the cDNA encoding AtraCSP, we isolated a cDNA fragment encoding a glutathione S-transferase. Because these enzymes are implicated in odorant reception [28], we have designed gene-specific primers, obtained the entire sequence by 5'-RACE, and named this protein AtraGST (Accession Number, GQ433371).

Using a degenerate primer PCR approach, we have identified a partial cDNA sequence encoding a putative olfactory neuron membrane protein (SNMP). AtraSNMP1 (942 bp, 314aa) (Accession Number, GQ451327) displays 75% amino-acid identity to *Mamestra brassicae* SNMP1 (AF462066) (Jacquin-Joly, E. et al., unpublished) and *H. virescens* SNMP1 (AJ251959) and 74% to *B. mori* SNMP1 (AJ251958) [29]. Likewise, we have identified a partial cDNA sequence encoding a putative putative OR83b-like odorant receptor 2 (OR2). AtraOR2 (813 bp, 271aa) (GQ451326) is highly conserved, sharing 85 to 91% amino-acid identity with orthologs of other lepidopteran species. While attempting to clone cDNAs encoding pheromone receptors using degenerate forward primer and universal primer, we have identified a partial cDNA sequence encoding a putative antennal binding protein X (ABPX). AtraABPX (421 bp, 117aa) (Accession Number, GQ451326) displays 69% amino-acid identity to *H. virescens* ABPX (AJ002518) [30], 64% to *Agrotis ipsilon* ABPX (AY301901) (Picimbon, J.-F. et al., unpublished) and 63% to *B. mori* ABPX (X94990) [31].

**Expression patterns of olfactory proteins and phylogenetic relationships**

To compare transcript patterns with protein profiles (Fig. 1), RT-PCR experiments were performed using gene-specific primers. First, we compared expression of *AtraPBP1*, *AtraPBP2*, *AtraGOBP1*, *AtraGOBP2*, and *AtraCSP* in non-olfactory tissues (male legs) with olfactory tissues (male and female antennae) (Fig. 2). In general, gene expression mirrored protein profiles, except for *AtraCSP*, which was detected not only in male and female antennae, but also in non-olfactory tissues (legs). *AtraPBP2*, *AtraGOBP1* and *AtraGOBP2* genes were detected in both male and female antennae, but not in legs, whereas *AtraPBP1* was apparently expressed exclusively in male antennae. Next, we assessed gene expression during antennal development. Contrary to our previous experience with the wild silkworm moth, *A. polyphemus* [32], sampling antennal pockets from pupae and day 0 adults of the navel orangeworm and extracting RNA were very challenging due to high RNAse activity at this developmental stage as reflected in the irregular amplifications of *actin* control gene (Fig. 3). Indeed, we were unable to extract RNA sample just the day before adult eclosion (day -1). Despite the unavoidable fluctuation in template titers, these experiments suggest that gene expression of most olfactory proteins starts at least two days before adult emergence (Fig. 3). Expression of the male antennae-specific *AtraPBP1* starts at day 0 of adult stage or the day prior to adult emergence.

![ML MAFA](https://example.com/ML_MAFA.png)

**Figure 2. Gene expression analysis by RT-PCR.** Expression of *AtraPBP1*, *AtraPBP2*, *AtraGOBP1*, *AtraGOBP2*, and *AtraCSP* genes in control tissue (ML, male hindlegs) and olfactory tissues (MA, male antennae and FA, female antennae). *Actin* gene was used as endogenous control.

doi:10.1371/journal.pone.0007235.g002
Having observed by non-quantitative RT-PCR that AtraPBP1 gene is expressed only in male antennae, a more thorough examination of gene expression was performed. Indeed, AtraPBP1 was limited to expression in male antennae (Fig. 4), with no trace detected in non-olfactory tissues, including legs, wings, thorax, and abdomen. It is worth mentioning, however, that a faint band was observed when cDNA from female antennae was used as template thus suggesting that AtraPBP1 is highly enriched in male antennae. Consequently, it is reasonable to assume that AtraPBP1 plays male-specific role(s), such as the detection of sex pheromones.

Next, we assessed tissue-specificity of other olfactory proteins we have isolated by cloning, namely, AtraSNMP1, AtraGST, and AtraABPX. RT-PCR data (Fig. 5) suggest that the genes encoding these proteins are highly expressed in male and female antennae. However, AtraGST and AtraABPX have also been detected, albeit with lower intensity, in all non-olfactory tissues tested (Fig. 5). By contrast, the gene encoding the co-receptor AtraOR2 was expressed only in male and female antennae, with no trace being detected in non-olfactory tissues (Fig. 6).

In order to gain insight of the relationships among moth PBPs, we have carried out a phylogenetic analysis in Mega v4.0.2 [33], combining amino acid sequences of the two PBPs from the navel orangeworm (this study) with 57 PBPs previously identified in 33 moth species. A consensus sequence comparison tree was constructed by the neighbor joining method [34] with one thousand bootstrap replicates. The resulting tree suggests that based on their amino acid identity, moth PBPs are clustered into different groups, each comprising related proteins of different moth species (Fig. 7). Indeed, phylogenetic analysis shows the
existence of at least four distinct groups of PBPs in moths, illustrating the diversity of this multigenic family. AtraPBP1 and AtraPBP2 belong to two separate groups, with the protein enriched in male antennae, AtraPBP1, clustering with some of the most well-characterized insect PBPs like BmorPBP1 [3,4,5,36] and ApolPBP1 [37,38,39,40]. Despite little bootstrap support in the tree, these moth PBPs share 65–70% amino acid identity with AtraPBP1, whereas AtraPBP2 is only 48% identical to AtraPBP1. Contrarily to AtraPBP1, AtraPBP2 belongs to a well supported group (95% bootstrap support) comprising 13 PBPs of other moth species.

pH-Dependent conformational change and pheromone binding

Having previously observed that PBPs from the silkworm moth, *B. mori*, and the wild silkworm moth, *A. polyphemus*, undergo pH-dependent conformational changes [3,4,5,36] that lead to lack of binding at low pH [3,5], we assessed the effect of pH on the conformation of AtraPBP1. We prepared samples of recombinant AtraPBP1 by using a recombinant pET vector without His6-Tag that generates PBPs with identical conformation and disulfide bridge formation [3] as the native protein. Samples were highly purified by a combination of ion-exchange chromatography (DEAE), high-resolution ion-exchange chromatography (Mono Q), and gel filtration, with the purity confirmed by SDS-PAGE and LC-ESI/MS (>99.5%). We prepared samples for circular dichroism (CD) and fluorescence analysis by taking aliquots of the same sample and diluting with buffers of the desired pH. Far-UV-CD spectrum of AtraPBP1 (Fig. 8) at pH 7 with a maximum at 193 nm and two minima at 208 and 223 nm demonstrated that this PBP is α-helical rich like BmorPBP1 [3] and ApolPBP1 [38]. At lower pH, the intensity of the second minimum at 223 nm was clearly reduced and thus indicated that there is unwinding of helical secondary structure. Similar changes have been observed with CD spectra of BmorPBP1 [3] and ApolPBP1 [38]. Apparently, the formation of a C-terminal helix does not offset the unwinding of the N-terminal α-helix thus causing a reduction in the overall content of this secondary structure. pH-Titration by intrinsic fluorescence (Fig. 9) showed a dramatic transition between pH values of 5 and 6.5 thus suggesting that AtraPBP1 exists in two distinct conformations, one at the pH of the sensillar lymph and the other at low pH as in the vicinity of dendritic membranes [3,12,14,41,42].

NMR analysis revealed very striking spectral changes upon changing the pH from 4.5 to 7.4. The $^{15}$N-H heteronuclear single quantum coherence spectrum at pH 4.5 (Fig. 10) exhibited the expected number of sharp and well-resolved main-chain amide

Figure 6. Analysis of AtraOR2 gene expression by RT-PCR. This co-receptor gene was detected in male and female antennae (Ant), but not in legs (L), wings (W), thorax (Thx), or abdomen (Abd). Template control is shown in Fig. 4. doi:10.1371/journal.pone.0007235.g006

Figure 7. Phylogenetic relationships of moth PBPs. Four groups were identified (A–D). The dashed line in Group D suggests a possible subdivision into D1 and D2. The following PBPs have been included in phylogenetic analysis: *Agrotsis ipsilon*: AipsPBP1 (AY301985), AipsPBP2 (AY301986); *Antheraea pernyi*: AperPBP1 (X96773), AperPBP2 (X96860), AperPBP3 (AJ277265); *Antheraea polyphemus*: ApolPBP1 (X17559), ApolPBP2 (AJ277266), ApolPBP3 (AJ277267); *Agristona segetum*: AsegPBP (AF134292); *Ascitis selenia*: AselPBP1 (AB265332), AselPBP2 (AB265332); *Argyrotaenia velutinana*: AvelPBP (AF177641); *Bombyx mori*: BmorPBP1 (NM_001044029), BmorPBP2 (AM403100), BmorPBP3 (NM_0010838626); *Choristoneura fumiferana*: CfumPBP (AF177642); *Choristoneura murinana*: CmurPBP (AF177646); *Choristoneura parallelula*: CparPBP (AF177649); *Choristoneura pinus*: CpinPBP (AF177653); *Choristoneura rosaceana*: CrosPBP (AF177652); *Diaphania indica*: DindPBP1 (AB263115); *Ephiphus postvittana*: EposPBP1f (AF416587), EposPBP1s (AF416588), EposPBP2 (AF411459), EposPBP3 (EV811597); *Helicoverpa armigera*: HarmPBP1 (AJ278992), HarmPBP2 (EU647241), HarmPBP3 (AF527054); *Helicoverpa assulta*: HassPBP1 (AY864775), HassPBP2 (EU316186), HassPBP3 (DQ286414); *Heliotoxiscis virens*: HvirPBP1 (X96861), HvirPBP2 (AM403491); *Heliotris zeae*: HzeaPBP (AF090191); *Lymantria dispar*: LdisPBP1 (AF007867), LdisPBP2 (AF007868); *Mamestra brassicae*: MbraPBP1 (AF051143), MbraPBP2 (AF051142); *Mythimna separata*: MsepPBP (AB263112); *Manduca sexta*: MsexPBP1 (AF117593), MsexPBP2 (AF117589), MsexPBP3 (AF117581); *Ostrinia furnacalis*: OfurPBP (AF133630); *Ostrinia nubilalis*: OunbPBP (AF133637); *Pectinophora gossypiella*: PgosPBP (AF177656); *Plutella xylostella*: PxyPBP1 (FJ201994), PxyPBP2 (AB263118); *Samaia cynthis*: ScyPBP1 (AB039793); *Sasnathodon exotica*: SexiPBP1 (AF177657); *Spodoptera littoralis*: SlitPBP1 (EF396284); *Spodoptera litura*: SlitPBP1 (DQ004497), SlitPBP2 (DQ114219); *Sesamia nonagrioides*: SnonPBP1 (AY485219), SnonPBP2 (AY485220); *SexiPBP*: SexiPBP1 (AY743351), SexiPBP2 (AY545363); *Yponomeuta cagnagellus*: YcagPBP (AF177661). doi:10.1371/journal.pone.0007235.g007
have already determined NMR backbone assignments for AtraPBP1 at low pH [43] and a full structure determination is currently underway. On the other hand, we were able to co-crystallize AtraPBP1 with pheromone constituents and obtain crystals that diffract to atomic resolution thus allowing determination of structures of AtraPBP1-pheromone complexes.

To assess affinity of AtraPBP1 for pheromone constituents, we used a previously developed binding assay [5], which is based on the separation of bound and unbound ligand by a centrifugal device. After the free ligand is removed by filtration, the PBP-bound ligand is released from the protein by lowering the pH, extracted with organic solvent and analyzed by gas chromatography (GC) for quantification and gas chromatography-mass spectrometry (GC-MS) for identification of the bound ligand. The major constituent of the sex pheromone system, (Z,Z, -11,13-hexadecadienal, hereafter referred to as Z11Z13-16Ald [16,17], bound to AtraPBP1 with apparent high affinity at neutral pH (Fig. 11) and low or no binding affinity at low pH. This pH-dependent binding affinity may be explained by the formation of a C-terminal α-helix, which competes with the ligand for the binding cavity at low pH [7,8,37]. Although only one of the four isomers of 11,13-hexadecadienal is known to be behaviorally active [17], pheromone-detecting sensilla in male antennae are sensitive to the four isomers of this compound, namely, Z11Z13-16Ald, Z11E13-16Ald, E11E13-16Ald, and E11Z13-16Ald [17]. We compared binding of Z11Z13-16Ald and E11E13-16Ald and found no difference (data not shown) thus suggesting that AtraPBP1 alone cannot discriminate stereoisomers of the major constituent of the sex pheromone. It is not known how many odorant receptors are expressed in the pheromone-detecting sensilla of the navel orangeworm male antennae and if they can discriminate isomers of the major constituent alone or in combination with AtraPBP1.

Next, we tested binding affinity of other constituents of the navel orangeworm sex pheromone. Female-produced sex pheromones in moths are normally complex mixtures of straight chain acetates, alcohols and aldehydes, with 10–18 carbon atoms and up to three unsaturations, the so-called Type I pheromones. Type II sex pheromone is comprised of polyunsaturated hydrocarbons and epoxy derivatives with long straight chains. The navel orangeworm is unusual in that its sex pheromone system in composed of a complex mixture that includes constituents of both types: Z11Z13-16Ald, Z11Z13-16OH, Z11Z13-16OAc (behavioral antagonist), (Z,Z,Z,Z,Z)-3,6,9,12,15-tricosapentaene and (Z,Z,Z,Z,Z)-3,6,9,12,15-pentacosapentaene, and other minor constituents [17]. As opposed to Type I pheromones that gave very low background indicating negligible non-specific binding (see buffer in Fig. 12), it was difficult to assess binding of the pentaene compounds because their hydrophobicity led to high background levels. On the other hand, the secondary constituent, Z11Z13-16OH bound to AtraPBP1 with affinity comparable to that of the major constituent, but showed no affinity at low pH (data not shown). Interestingly, the behavioral antagonist, Z11Z13-16OAc showed the highest affinity to AtraPBP1 of all tested ligands (data not shown). Next, we performed competitive binding studies with AtraPBP1 incubated with the three ligands at the same concentration. These competitive binding assays mirrored what was observed with non-competitive binding assays, AtraPBP1 was bound with the highest affinity to Z11Z13-16OAc, whereas the aldehyde and alcohol showed similar affinity (Fig. 12). These results suggest that a single PBP may be involved in the reception of multiple constituents of sex pheromones.

To further explore the potential use of AtraPBP1 for the development of parapheromones, we tested binding of a
A uniform, stable, and monomeric tertiary structure is inferred by the sharp, well-resolved peaks of main-chain amide resonances. The broader peaks at pH 7.4 suggest that AtraPBP1 exists as a heterogeneous mixture of monomer, dimer, and multimer, as previously suggested for BmorPBP1 [36]. The NMR spectrum at intermediate pH is in agreement with a transition state with at least two conformations in equilibrium.

doi:10.1371/journal.pone.0007235.g010

Z11Z13-16Ald showed high affinity for the pheromone-binding protein at pH 7, but low or no binding activity at low pH. Minimal non-specific binding is indicated by the low amounts of ligand detected after incubation with buffer only.

doi:10.1371/journal.pone.0007235.g011

Two major constituents of the sex pheromone of the navel orangeworm, Z11Z13-16Ald and Z11Z13-16OH, and a behavioral antagonist, Z11Z13-16OAc, were incubated with AtraPBP1 at the same concentration. The two pheromone constituents bound to AtraPBP1 with nearly, equally high affinity, whereas the behavioral antagonist showed even higher apparent affinity.

doi:10.1371/journal.pone.0007235.g012
pheromone analog, (\(\text{Z}_{11}1,1,1\text{-trifluoro-13-octadecen-2-one}\) (hereafter referred to as Z11C16OCOF3). Trifluoromethyl ketones (TFMK) [44] are compounds which inhibit a variety of hydrolytic enzymes, such as acetylcholinesterase, chymotrypsin, trypsin, juvenile hormone esterase, human liver microsomal CEs, and pheromone degrading esterases in male olfactory tissues. They have been demonstrated to interrupt insect chemical communication [45,46] and to bind to pheromone-binding proteins [47], but their mode of action is still a matter of debate. We compared by competitive binding the affinity of Z11C16OCOF3 and Z11Z13-16OAc to AtraPBP1. Surprisingly, Z11C16OCOF3 binds to AtraPBP1 with much higher affinity than the behavioural antagonist Z11Z13-16OAc (Fig. 13). Although binding activity decreased dramatically at low pH, this TFMK showed binding affinity at low pH almost half of that of the best natural ligand (Z11Z13-16OAc) at neutral pH (Fig. 13). We, therefore, concluded that AtraPBP1 may be employed for the development of a affinity-based approach for the development of parapheromones.

Conclusion

We have isolated and cloned olfactory proteins from the navel orangeworm, including pheromone-binding proteins, general odorant-binding proteins, chemosensory protein, antennal binding protein X, glutathione S-transferase, sensory neuron membrane protein and an odor receptor. Our goal was to identify olfactory proteins involved in the reception of pheromones for future applications in a reverse chemical ecology approach to explore the development of alternative attractants (parapheromones) as substitutes for unstable constituents of the navel orangeworm sex pheromone system. One of the identified olfactory proteins, AtraPBP1, was expressed almost exclusively in male antennae. The major constituent of the sex pheromone, Z11Z13-16Ald bound AtraPBP1 with high affinity at the sensillar lymph pH, but no affinity at the postulated pH at the close vicinity of the pheromone receptor. Because unsaturated aldehydes in general have limited lifetime under UV light and other field conditions, more chemically stable attractants (parapheromones) are needed. AtraPBP1 seems an ideal molecular target for screening parapheromones. Indeed, binding of a pheromone analog, Z11C16OCOF3, to AtraPBP1 highlights the potential use of this protein for screening non-natural ligands. The current project paved the way for future structural biology studies aimed at unveiling molecular interactions between AtraPBP1 and Z11Z13-16Ald, and mechanisms of binding and release to set the stage for design of parapheromones.

Materials and Methods

Protein identification and characterization

A laboratory colony of the navel orangeworm was initiated from larvae collected in Bakersfield, CA, according to a previously published protocol [48]. Tissues were collected with clean forceps under a microscope, immediately homogenized in 10 mM Tris-HCl, pH 8, with an ice cold Dounce tissue grinder (Wheaton, Millville, NJ) and centrifuged twice at 12,000 xg for 10 min. Samples per batch were typically 50–100 antennae and 50–100 legs. Prior to tissue extraction, adults were sexed [49]. An aliquot of supernatant was concentrated to the appropriate volume with vacuum concentrator and analyzed by 15% native-PAGE. After separations, gels were either stained with Coomassie Brilliant Blue R-250 (CBB, Bio-Rad, Hercules, CA) or proteins were transferred by electroblotting to polyvinyl difluoride (PVDF) membranes, visualized with CBB, bands were cut off, and N-terminal amino acid sequences were obtained on a Precise Protein Sequencing System (Applied Biosystems, Foster City, CA).

cDNA cloning

Tissues were collected with clean forceps and immediately extracted with TRIzol (Invitrogen, Carlsbad, CA) on an ice-cold Dounce tissue grinder. First strand DNA was synthesized from total RNA using reverse transcriptase and a SMART™ RACE cDNA Amplification Kit (Clontech, Mountain View, CA). 3'-RACE PCR was carried out with appropriate template and degenerate primers based on N-terminal amino acid sequence of the target cDNA, UPM primer, or GCGT15 primer. Taq DNA polymerase (ID Labs, London, ON, Canada), PfuUltra HotStart DNA polymerase (Stratagene, Cedar Creek, TX) and Advantage GG-2 Polymerase Mix (Clontech) were used as polymerases for PCR. The PCR products were subcloned into pBluescript SK(+) (Stratagene) and sequenced. 5'-RACE PCR was performed according to instruction manual using gene specific primers designed on the basis of the sequences obtained by 3'-RACE. Multiple (10–16) independent clones were sequenced to eliminate possible PCR-derived mutations. For cloning AtraPBP1, one degenerate primer and two gene-specific primers were designed: 5'-GA(A/G)AT(A/C/T)ATGAA(A/G)GA(C/T)TT(A/G)TC(A/G) –3' (based on EIMKDSLINF); AtraPBP1-1, 5'-CTCATGCACTTGGTGCCGATCAGCTGTCTC-3'; AtraPBP1-2, 5'-CAACTCTCAGTGTAGGAGCCAGGACTTGAG-3'. For AtraPBP2, the following primers were used:

5'-CA(A/G)AT(A/C/T)ATGAA(A/G)GA(C/T)TT(A/G)TC(A/G) –3' (based on QEVVLHKMTA); AtraPBP2-1, 5'-ATCATGTGGCATGGCAGCCAGCTGAC-3'; AtraPBP2-2, 5'-CCACGGTCAGGGTGCCGAGGGCAGTTGGTCGC-3'; AtraPBP2-3, 5'-TCTGATGTTACAAA-TATCAGCATACAAATCC-3'; AtraPBP2-4, 5'-GCCGTTAAGATGGCCACTTGCGGGCAGTTGGTCGC-3'.
For AtraGOBP1: degenerate primer, 5'-AA(A/G)GA(C/TGT[A/C/G]/T[AC/AG]CA/G/T/CT[AC/G/CT/GA/G/C/T]C/GA/G/T[AG/TA/CA/G/CG/GA/G/GC-G3' (based on KDVTLFGEEA). AtraGOBP1-1, 5'-AA(A/G)GACCAGTTGACATAGAAGCTTATGCG-3'; AtraGOBP2-1, 5'-GAAGTTGCTGACCCGGGAGGTTGCTGCAG/CGC3'; AtraGOBP2-2, 5'-CATTGGACATCTGCTGCGGAGAGACC-3'.

For AtraGOBP2, 5'-CA(C/T)GCTGCA/TCA/G/A/G/T[AG/TA/CA/G/CG/GA/G/GC-G3' (based on KDVNL-NIQE) generated a 1-klb-long PCR fragment by misannealing that led to cloning AtraGST. A second degenerate primer was then designed: 5'-GA(A/G)/CT/TA/A/G/AA(A/G)/CT/TA/GC/G/A/C/G/TA/AA/(C/T)A/T/CT/TA/GA/G/A/G/C/AA(C/T)A/T/CT/TA/GA/G/A/G/G3' (based on DYTKGKIE-NIQE). The following GSPs were used for AtraABPX cloning.

For AtraSNMP1, 5'-GACGCTCCTGGAAGGAAAGGAAC-3'; AtraSNMP1Ido: 5'-TTCAGAAGTTGCCTGATCGTGCTC-3'; AtraGSTup: 5'-ATGCACCGCGAAGCTTAATGTGTT-3'; AtraGSTdo: 5'-CGTCGCGCCCAATACTGGTT-3'; AtraABPXup: 5'-CAGATGTGTCAGCAAATCTGGCG-3'; AtraABPxido: 5'-TCC-TTCTGGTGGCACTCTGACCA-3'.

Phylogenetic analysis of moth PBPs

Amino-acid sequences of PBPs identified in different moth species (37 proteins) have been retrieved from GenBank database have been combined to AtraPBP1 and AtraPBP2 to create an entry file for phylogenetic analysis in MEGA 4.0.2 [33]. An unrooted consensus neighbor joining tree [34] was calculated at default settings with pairwise gaps deletions. Branch support was based on bootstrap analysis based on 1000 replicates.

Protein expression and binding assays

pBluescript clones including 3’- and 5’-regions of AtraPBP1 cDNA were used as template for PCR. Following GSPs including recognition sites of Kpn I and Xho I were used: AtraPBP1-5, 5'-CCGGGGTTACCCCTCGCCGGAGATCTGAAAG-3'; AtraPBP1-4, 5'-CCGCGCTCGAGTATGACTTGCTCAAGGACCT-3' (PfuUltra High-Fidelity DNA Polymerase (Stratagene) was selected as DNA polymerase. PCR: 95°C for 2 min; 30 cycles of 95°C for 30 sec, 40°C for 30 sec and 72°C for 1 min; 72°C for 10 min. After gel-purification 500 bp of PCR product was inserted into Eco RV recognition site of pBluescript SK (+) (Stratagene). DNA sequences of two clones were confirmed. Sixteen micrograms of DNA mixed with both clones was treated with 40 U of Kpn I (New England Biolabs, Ipswich, MA) at 37°C for 3 h and subsequently re-purified by QIAquick PCR Purification Kit (Qiagen, Valencia, CA). Purified DNA was treated with T4 DNA polymerase (New England Biolabs) at 12°C for 20 min to remove 5’-protruding single strand DNA fragment. Reaction of T4 DNA polymerase was stopped at 75°C for 10 min. After re-purification of DNA by QIAquick PCR Purification Kit, DNA was digested with 40 U of Xho I at 37°C for 3 h. 500 bp of DNA fragment was gel-purified and ligated into previously digested pET-22b(+). Digestion of 1.2 μg of pET-22b (+) plasmid DNA (Novagen, Gibbstown, NJ) was done with 6 U of Msc I and 5 U of Xho I (New England Biolabs) at 37°C for 3 h. After heat denaturing enzymes at 65°C for 20 min, digested plasmid DNA was gel-purified. Connection between pET vector and AtraPBP1 DNA insert was confirmed by sequencing using T4 terminator primer (Novagen).

Expression of non-labeled AtraPBP1 was performed in LB medium with transformed BL21(DE3) cells [3]. Proteins in the periplasmic fraction were extracted with 10 mM Tris-HCl, pH 8 by
using three cycles of freeze-and-thaw and centrifuging at 16,000 × g to remove debris [30]. The supernatant was loaded on a HiPrep™ 16/10 DEAE 16/10 column (GE Healthcare, Piscataway, NJ). Separations by ion-exchange chromatography were done with a linear gradient of 0–100 mM NaCl in 10 mM Tris-HCl, pH 8. Fractions containing the target protein were further purified on a Q-Sepharose HiPrep™ 16/10 column (GE Healthcare), and, subsequently, on a Mono-Q HR 10/10 column (GE Healthcare). PB fractions were concentrated by using Centricon-10, desalted on four 5-ml Hitrap desalting columns (GE Healthcare) in tandem and by using water as mobile phase, analyzed by LC-ESI/MS, lyophilized, and stored at −80°C until use. The concentrations of the recombined proteins were measured by UV absorbance at 280 nm in 20 mM sodium phosphate, pH 6.5 and 6 M guanidine HCl by using the theoretical extinction coefficients calculated with EXPASY software (http://us.expasy.org/tools/protparam.html). LC-ESI-MS was performed with a LC/MS-2010 (Shimadzu, Kyoto, Japan). HPLC separations were done on a Zorbax CB C8 column (150×2.1 mm; 5 μm; Agilent Technologies, Palo Alto, CA) with a gradient of water and acetonitrile plus 2% acetic acid as a modifier. The desolvation line and heat block at 250°C. Fluorescence spectra were recorded on a Shimadzu RF-5301 PC spectrofluorometer equipped with a four-channel interface and triple-resonance cryogenic probe. The 15N–1H HSQC spectra were obtained with 15N-labeled 0.5 mM AtraPBP1 in 95% H2O and 5% 2H2O, with pH adjusted to 4.3 (20 mM sodium acetate), 5.5 (20 mM sodium acetate), and 7.4 (20 mM sodium phosphate).

Binding was measured by incubating AtraPBP1 with test ligands, separating unbound and bound protein, extracting pheromone from the latter sample, and analyzing by gas chromatography, according to a previously reported protocol [5]. After lowering pH to release ligand, bound protein fractions were extracted and analyzed by gas chromatography (GC) for quantification and by GC-mass spectrometry (GC-MS) for confirmation of ligand identity. GC and GC-MS were done on a 6890 series GC and a 5973 Network Mass Selective Detector (Agilent Technologies, Palo Alto, CA), respectively. Both instruments were equipped with the same type of capillary column (HP-5MS, 25 m ×0.25 mm; 0.25 μm; Agilent Technologies) operated under the same temperature program (150°C for 1 min, increased to 250°C at a rate of 10°C/min, and held at the final temperature for 10 min.) Pure pheromone samples, including isolomers of 11,13-hexadecadienal, were supplied by Bedoukian Research Inc. Each compound was tested at least five times. Test compounds were incubated with AtraPBP1 in the ratio of 10:1, ligand:protein. For competitive binding assays, all ligands were added to a protein solution at the same concentration.

Acknowledgments

We are grateful to Angela M. Chen and Stephanie Dickey for assistance in protein expression and binding assays. We are indebted to Bedoukian Research Inc. for providing samples of pheromone constituents of the navel orangeworm, Dr. Angel Guerrero (Chemical Ecology Unit, IQAC-CSIC, Barcelona) for providing a sample of Z11C16COCF3, and Dr. Brad Higbee (Paramount Farming) for providing moths to start and pistachio to maintain a laboratory colony of the navel orangeworm. We are also thankful to lab members for insightful discussions, particularly Zainulabedin Syed, Ana Claudia A. Melo, Zhao Lin, and Rabieñ Palma, and to Dr. David Wilson (UC Davis) for insightful comments on a draft version of the manuscript.

Author Contributions

Conceived and designed the experiments: WSL. Performed the experiments: WSL, YJ, JW, JR, XX, JBA. Analyzed the data: WSL, YJ, JP WX JBA. Wrote the paper: WSL, YJ, JP.

References

1. Kaissling K-E, Priesner E (1970) Die Reichschwelle des Seidenspinners. Naturwissenschaften 57: 23–28.
2. Kaissling K-E (1987) R. H. Wright lectures on insect olfaction; Colburn K, ed. Burnaby, British Columbia: Simon Fraser University. 75 p.
3. Wojtaszek H, Leal WS (1999) Conformational change in the pheromone-binding protein of Bombyx mori revealed by cationic markers. Tissue Cell 16: 705–717.
4. Damberger F, Nikonova L, Horst R, Peng G, Leal WS, et al. (2000) NMR detection of pheromones and plant volatiles. London: Elsevier Academic Press. pp 447–476.
5. Vogt RG, Prestwich GD, Lerner MR (1991) Odoartor-binding-protein subfamilies associate with distinct classes of olfactory receptor neurons in insects. J Neurobiol 22: 74–84.
6. Coffelt JA, Vick KW, Sonnet PE, Doolittle RE (1979) Isolation, identification, and synthesis of a female sex pheromone of the navel orangeworm, Amyelois transella (Lepidoptera: Pyralidae). J Chem Ecol 5: 955–966.
7. Leal WS, Barbossa RM, Xu W, Ishida Y, Syed Z, et al. (2008) Reverse and conventional chemical ecology approaches for the development of oviposition attractants for Culex mosquitos. PLoS ONE 3: e3045.
8. Willett CS (2000) Do pheromone binding proteins converge in amino acid sequence when pheromones converge? J Mol Evol 50: 175–183.
9. Raming K, Krieger J, Breer H (1989) Molecular cloning of an insect pheromone-binding protein. FEBS Lett 236: 215–218.
10. Keil TA (1984) Surface coats of pore tubules and olfactory sensory dendrites of a silkworm moth revealed by cationic markers. Tissue Cell 16: 705–717.
21. Vogt RG, Rybczynski R, Lerner MR (1991) Molecular cloning and sequencing of general odorant-binding proteins GOBP1 and GOBP2 from the tobacco hawk moth Manduca sexta: comparisons with other insect OBPs and their signal peptides. J Neurosci 11: 2972–2984.

22. Breer H, Krieger J, Raming K (1990) A novel class of binding proteins in the antennae of the silkmoth Antheraea polyphemus. Insect Biochem 20: 735–740.

23. Grosser-Wilde E, Gohl T, Bouche E, Breer H, Krieger J (2007) Candidate pheromone receptors provide the basis for the response of distinct antennal neurons to pheromonal compounds. Eur J Neurosci 22: 2364–2373.

24. Krieger J, von Nickisch-Rosenegk E, Mameli M, Pelosi P, Breer H (1996) Binding proteins from the antennae of Bombyx mori. Insect Biochem Mol Biol. pp 26.

25. Krieger J, Gansle H, Raming K, Breer H (1993) Olfactory binding proteins of Helix aspersa maxima. Insect Biochem Mol Biol 23: 449–456.

26. Jansen S, Chmelik J, Zidek L, Padtra P, Novak P, et al. (2007) Structure of Bombyx mori chemosensory protein 1 in solution. Arch Insect Biochem Physiol 66: 135–145.

27. Malecka Z, Stange G (1997) Molecular cloning, by a novel approach, of a cDNA encoding a putative olfactory protein in the labial palps of the moth Callithea calcarea. Gene 202: 39–43.

28. Rogers ME, Jani MK, Vogt RG (1999) An olfactory-specific glutathione-S-transferase in the sphinx moth Manduca sexta. J Exp Biol 202: 1625–1637.

29. Rogers ME, Krieger J, Vogt RG (2001) Antennal SNMPs (sensory neuron membrane proteins) of Lepidoptera define a unique family of invertebrate CD36-like proteins. J Neurobiol 40: 47–64.

30. Krieger J, Mameli M, Breer H (1997) Elements of the olfactory signaling pathways in insect antennae. Invert Neurosci 3: 137–144.

31. Krieger J, von Nickisch-Rosenegk E, Mameli M, Pelosi P, Breer H (1996) Binding proteins from the antennae of Bombyx mori. Insect Biochem Mol Biol 26: 297–307.

32. Ishida Y, Leal WS (2000) Rapid inactivation of a moth pheromone. Proc Natl Acad Sci U S A 102: 14075–14079.

33. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24: 1596–1599.

34. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. J Mol Biol 208: 21–38.

35. Lautenschlager C, Leal WS, Clardy J (2007) Candidate pheromone receptors provide the basis for the response of distinct antennal neurons to pheromonal compounds. Eur J Neurosci 22: 2364–2373.

36. Leal WS (2000) Duality monomer-dimer of the pheromone-binding protein PBP1 of the moth Antheraea polyphemus. J Mol Biol 301: 1135–1150.

37. Damberger FF, Ishida Y, Leal WS, Wuthrich K (2007) Structural basis of ligand binding and release in insect pheromone-binding proteins: NMR structure of Antheraea polyphemus PBP1 at pH 4.5. J Mol Biol 373: 811–819.

38. Leal WS, Chen AM, Erickson ML (2005) Selective and pH-dependent binding of a moth pheromone to a pheromone-binding protein. J Chem Ecol 31: 2493–2499.

39. Mohrny S, Zabkow S, Gronenborn AM (2004) The solution NMR structure of Antheraea polyphemus PBP provides new insight into pheromone recognition by pheromone-binding proteins. J Mol Biol 339: 443–451.

40. Mohrny S, Zabkow S, Gronenborn AM (2004) Erratum to “The solution NMR structure of Antheraea polyphemus PBP provides new insight into pheromone recognition by pheromone-binding proteins” [J Mol Biol (2004) 337, 443-451]. J Mol Biol 338: 1037.

41. Keil TA (1987) Lectin-binding sites in olfactory sensilla of the silkmoth, Antheraea polyphemus. Annu New York Acad Sci 310: 403–405.

42. Xu X, Li Y, Rayo J, Ishida Y, Leal WS, et al. (2008) 1H, 15N, and 13C Chemical shift assignments of the navel orange worm pheromone-binding protein-1 (Atra-PBP1). Biomol NMR Assign 2: 105–106.

43. Hammock BD, Wing KD, McLaughlin J, Lovell VM, Sparks TC (1982) Trifluoromethylketones as possible transition-state analog inhibitors of juvenile hormone esterase. Pesticide Biochem Physiol 17: 1158–1163.

44. Hammock BD, Wing KD, McLaughlin J, Lovell VM, Sparks TC (1982) Trifluoromethylketones as possible transition-state analog inhibitors of juvenile hormone esterase. Pesticide Biochem Physiol 17: 1158–1163.

45. Riba M, Sans A, Sole J, Munoz L, Bosch MP, et al. (2005) Antagonism of pheromone response of Ostrinia nubilalis males and implications on behavior in the laboratory and in the field. J Agric Food Chem 53: 1158–1165.

46. Sole J, Sans A, Riba M, Riera E, Bosch MP, et al. (2005) Reduction of damage by the Mediterranean corn borer, Serratia nonagrioides, and the European corn borer, Ostrinia nubilalis, in maize fields by a trifluoromethyl ketone pheromone analog. Environ Entomol Exp Appl 109: 26–29.

47. Phophol B, Gebauer T, Ziegenhanger G, McLaughlin J, Lovell VM, Sparks TC (1982) Decyl-trifluoromethylketone, a competitive inhibitor of moth pheromone receptors. J Comp Physiol A 106: 315–323.

48. Parra-Pedrazzoli AL, Leal WS (2006) Sexual behavior of the navel orange worm, Antheraea polyphemus (Lepidoptera: Pyralidae). Neotrop Entomol 35: 769–774.

49. Hussey MM, Madern HF (1964) Sterilization of navel orangeworm, Parasa ruminula (Walker), by gamma radiation (Lepidoptera: Pyralidae). Exp Parasitol 12: 113–117.