Functional Disparity of Three Pheromone-Binding Proteins to Different Sex Pheromone Components in Hyphantria cunea (Drury)

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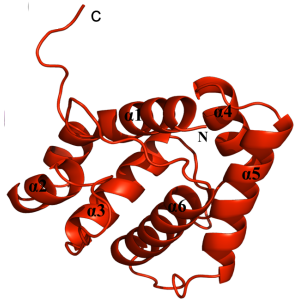
Hyphantria cunea

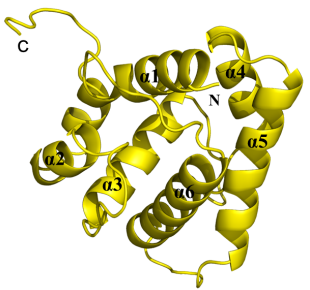
Olfactory System

Sex Communication

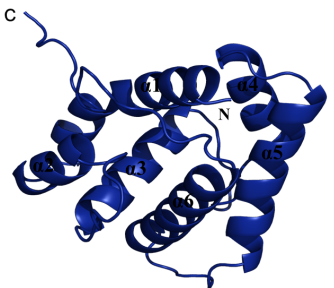
HcunPBP1



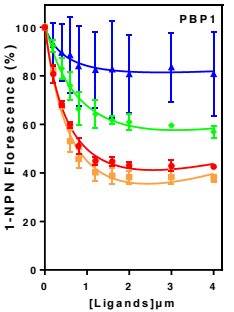
HcunPBP2

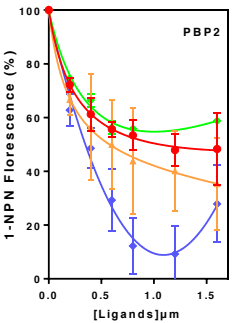


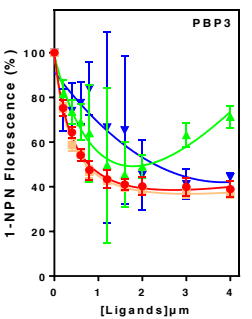
HcunPBP3



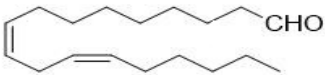
Binding Affinities



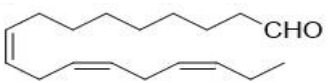




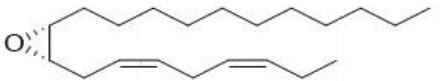
Z9,Z12-18Ald



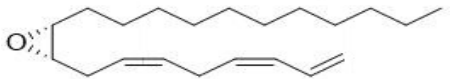
Z9,Z12,Z15-18Ald



Z3,Z6-9S,10R-epoxy-21Hy



1,Z3,Z6-9S,10R-epoxy-21Hy



Type II pheromone components

Functional disparity of three pheromone-binding proteins to different sex pheromone components in *Hyphantria cunea* (Drury)

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ABSTRACT

Hyphantria cunea (Drury) is a destructive invasive pest species in China that uses Type-II sex pheromone components. To date, however, the binding mechanisms of its sex pheromone components to their respective pheromone-binding proteins (HcunPBPs 1/2/3) have not been explored. In the current study, all three *HcunPBPs* were expressed in the antennae of both sexes. The prokaryotic expression and ligand binding assays were employed to study the binding of the moth's four sex pheromone components, including two aldehydes and two epoxides, and 24 plant volatiles to the HcunPBPs. Our results showed that the abilities of these HcunPBPs to bind to the aldehydes were significantly different than binding to the epoxides. These three HcunPBPs also selectively bound some of the plant volatiles tested. Our molecular docking results indicated that some crucial hydrophobic residues might play a role in the binding of HcunPBPs to their sex pheromone components. Three HcunPBPs have different selectivities for pheromone components with both major and minor structural differences. Our study provides fundamental insight into the olfactory mechanism of moths at the molecular level, especially for moth species that use various Type II pheromone components.

KEY WORDS: *Hyphantria cunea* (Drury), pheromone binding protein, ligand binding assay, sex pheromone, plant volatile.

INTRODUCTION

Most moths have developed a highly sophisticated olfactory sensory system to recognize various volatile chemicals and to perceive female-produced sex pheromones. There are several types of peripheral olfactory proteins, including odorant-binding proteins (OBPs), odorant receptors (ORs), odorant-degrading enzymes (ODEs), ionotropic receptors (IRs), and sensory neuron membrane

proteins (SNMPs). These proteins have been demonstrated to be involved in the reception of odorants by insects.[1] Among these, OBPs, as highly concentrated hydrophilic proteins in the sensillum lymph of moth antennae, serve as odorant carriers in the first step of the perception of various odors.[2] Pheromone-binding proteins (PBPs), a sub-class of OBPs, are key components in the detection of insect sex pheromones. Since the first identification of PBPs in male Polyphemus moths, *Antheraea polyphemus*, many PBPs have been identified and reported from various lepidopteran species.[3,4,5,6,7] Traditionally, there are two major groups of moth sex pheromones: Type I and Type II pheromones, classified according to their biosynthesis.[8,9] Type I pheromones typically contain C_{10}-C_{18} unsaturated straight chains and a terminal functional group (e.g. acetate, aldehyde or alcohol) (>75% moth species). In contrast, Type II pheromones are typically C_{17}-C_{23} unsaturated hydrocarbons and the corresponding epoxide derivatives, typically without terminal functional groups.[8,9] However, a small number of moth pheromones are not as easily classified.[8] Recently, Löfstedt et al. (2016) revised and extended the known classifications of lepidopteran pheromones by defining another two pheromone types, Type 0 and Type III pheromones.[16] To date, however, the characterized PBPs reported in moths are mainly responsible for detection of Type I sex pheromones.[3,10,11] In contrast, PBPs for Type-II sex pheromones have only been reported from a few species, including *Ascotis selenaria cretacea,* *Operophtera brumata* and *Ectropis obliqua*.[11,12,13,14,15]

The fall webworm, *Hyphantria cunea* (Drury) (Lepidoptera: Erebidae: Arctiinae), is one of the most destructive invasive pest species in China. It prefers to feed on broad-leaved ornamental trees, and defoliation of these trees during larval outbreaks can cause significant damage to urban landscapes and natural environments. The sex pheromone of *H. cunea* was first partially identified in 1982 in the USA and further identifications from different populations have been reported from

Japan, Europe, New Zealand and China.[17,18,19,20,21,22] These studies reported four female-produced sex pheromone components, including two straight chain aldehydes: (9*Z*,12*Z*)-octadecadienal (*Z*9, *Z*12-18Ald) and (9*Z*,12*Z*,15*Z*)-octadecatrienal (*Z*9, *Z*12, *Z*15-18Ald), and two epoxides: (3*Z*,6*Z*,9*S*,10*R*)-9,10-epoxy-3,6-heneicosadiene (*Z*3, *Z*6-9*S*, 10*R*-epoxy-21Hy) and (3*Z*,6*Z*,9*S*,10*R*)-9,10-epoxy-1,3,6-heneicosatriene (1, *Z*3, *Z*6-9*S*, 10*R*-epoxy-21Hy).[19,21,22,23] Synthetic pheromone lures containing three of the four components were commercialized by Nitto Denko Corp. in Japan and have been widely used for monitoring and mass trapping of this invasive pest species in Japan and China.[24,25,26,27,28]

Besides functioning as passive carriers to solubilize lipophilic pheromones in the hydrophilic antennal lymph, PBPs have been postulated to contribute to the exquisite specificity of insects' olfactory systems.[29] The binding specificity between PBPs and pheromones has been reported in several previous works, mostly on Type I sex pheromone-producing lepidopteran species.[5,30,31] *Antherea polyphemus* represents a typical example wherein each of the three PBPs specifically binds one of the three pheromone components.[32] However, information on the specificities of PBPs from moth species that use Type II or III sex pheromones is still limited. Under the newly revised and extended classification by Löfstedt et al. (2016),[16] *H. cunea* uses Type-II sex pheromone components having two different chemical functionalities (aldehydes and internal epoxides) in its pheromone system, and this system provided a unique model that inspired us to unravel the specificity of their PBPs in discriminating these two different types of Type II sex pheromone components. Here, we hypothesized that each of the different *H. cunea* PBPs may bind to different sex pheromone components. Thus, we investigated the binding properties of three recently identified pheromone binding proteins (PBPs: HcunPBP1, 2 and 3) of *H. cunea in vitro* with regard to its four (Type II) sex pheromone components,[15] as well as 24 host or non-host plant volatiles, by using

prokaryotic expressions and ligand binding assays. Subsequent structural modeling of these three HcunPBPs and docking studies with the four sex pheromone components were carried out to estimate their ligand affinities. Our study provided not only a better understanding of sex pheromone perception of *H. cunea* at a molecular level, but also a valuable insight into the different binding mechanisms of PBPs, especially for the moth species that use various Type II pheromone components.

MATERIALS AND METHODS

Insect rearing and tissue collection

Hyphantria cunea pupae were provided by Dr. Yu-Zhu Wang at the Chinese Academy of Forestry, Beijing, and kept in the laboratory under a 14L/10D light cycle at 25 ± 1 °C and 65 ± 5% RH. These pupae were sexed and maintained separately by sex in different cages. After emergence, the adults were provided with 10% honey solution. To eliminate individual variations, different body parts such as antennae, heads (without antennae), abdomens, thoraxes, legs and wings from 50 3-day old virgin males and females were dissected and combined into composite samples. Three biological replicates were performed as our previous report.[15] All collected tissues were immediately flash-frozen in liquid nitrogen and stored separately at -80°C prior to RNA extractions.

RNA extraction and preparation of cDNA library

Total RNA from the dissected body parts of 50 males or 50 females was prepared as previously described. cDNA libraries were prepared with M-MLV reverse transcriptase kits (Takara, Japan). The cDNA product was used directly for PCR amplification or stored at -20 °C.

111

112 **Quantitative real-time PCR (RT-qPCR)**

113 The RT-qPCR experiment was conducted on a CFX96 real-time fluorescence quantitative PCR

114 instrument (BioRad, USA) combined with SYBR® Premix Ex Taq II (TliRNaseH Plus) (TaKaRa,

115 Japan). Elongation factor 1 alpha (*EF1- α*) was used as a reference gene. The RT-qPCR method and

116 primers were the same as those used by Zhang et al. (2016)[15] (Table S1). The HcunPBPs mRNA

117 relative levels were calculated based on the Ct-values of target gene and reference gene *EF1-a* by

118 using the Q-gene method in Microsoft Excel-based software of Visual Basic.[33]

119

120 **Chemicals**

121 Synthetic pheromone compounds, *Z*9, *Z*12-18Ald and *Z*9, *Z*12, *Z*15-18Ald were kindly provided by

122 Dr. Xin Chen (Nimord Inc., Jiangsu, China), *Z*3, *Z*6-9*S*, 10*R*-epoxy-21Hy and 1, *Z*3, *Z*6-9*S*, 10*R*-

123 epoxy-21Hy were kindly provided by Dr. Xiangbo Kong (Chinese Academy of Forestry, Beijing,

124 China) (≥ 95% purity). Plant volatiles (Table S2), and N-phenyl-1-naphthylamine (1-NPN) were all

125 ≥95% pure and purchased from Sigma-Aldrich (Saint Louis, MO, USA).

126

127 **Recombinant protein production, expression, and purification**

128 The SnapGene® 3.2.1 software was used to find out whether there were two restriction endonuclease

129 sites *BamHI* and *XhoI* on the ORF sequences of the three *HcunPBPs,* and then their signal peptides

130 were predicted by using the website SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP-4.1/).

131 *HcunPBP1*, *2* and *3* genes without signal peptide were amplified using specific primers (Table S3),

132 constructed from the 5' and the 3' ends towards the host genome, digested with a restriction enzyme

BamHI and XhoI, respectively. The cloning vector pEASY Blunt3 (TransGen, Beijing, China) was used to subclone the PCR products of *HcunPBP1*, *2* and *3*. The positive clones were selected and sequenced to confirm their identity (GenScript Biology Company, Nanjing, China). The target gene was then bound into the pET-30a (+) expression vector (Novagen, Darmstadt, Germany) which was previously digested with the same enzyme, and then transformed into *Escherichia coli* BL21 (DE3) cells for further protein expression. Because a *BamHI* site was present in the *HcunPBP2* gene, BamHI and XhoI restriction endonuclease for double digestion could not be added. The homologous arms can be added on the 5'and the 3'ends of the designed PBP2 sequence, and homologous recombinant enzymes (Novagen, Darmstadt, Germany) were used for ligation with pET-30a (+).

The protein expression method was similar to the one described previously.[34] In short, all *HcunPBPs* were transformed into BL21 (DE3) *E. coli* cells, formed as insoluble, inactive inclusion bodies. Protein solubilization was done using lysis buffer (8 M urea, 1 mM DTT (Dithiothreitol) in 20 mM Tris-HCl, pH 7.4). Protein purification was conducted using Beaver Beads™ His-tag Protein Purification Kit (Enriching Biotechnology Ltd., China). Recombinant enterokinase (rEK) (GenScript Biology Company, Nanjing, China) was used for His-tag removal. Untagged protein was further purified and desalted by dialysis membrane (MD25, 8000-14000D) and lyophilized. The pure protein was then stored at -80 °C until use.

Fluorescence measurements

The binding assays were conducted using previously reported protocols on a Spectra Max M5 Fluorescence Spectrophotometer (Molecular Devices Co., Sunnyvale, CA, USA) with Greiner Microlon 96-well plates.[5,11] The emission spectra were recorded between 400 and 470 nm with an

155 excitation wavelength of 337 nm. The affinity of a fluorescent probe, N-phenyl-1-naphthylamine (1-
156 NPN), for PBP proteins was measured. The 2 μM PBP solution in 50 mM Tris-HCl (pH 7.4) was
157 titrated with a 1 mM 1-NPN aliquot (dissolved in methanol) to a final concentration of 2-20 μM, and
158 fluorescence intensity results were recorded. The binding affinity of the PBPs for each odorant was
159 measured by using 1-NPN as a fluorescent reporter. Mixtures of 250 μL solution (50 mM Tris-HCl)
160 containing PBP (0.2 μM) and 1-NPN (2 μM) were titrated to final concentrations of 0.2-4 μM. For
161 tests with plant volatiles, mixtures of 250 μL solution (50 mM Tris-HCl) containing plant volatiles (2
162 μM) and 1-NPN (2 μM) were titrated to final concentrations of 2-20 μM. Three replications were
163 done for each odorant. The binding data analysis was done using previously described methods.[5,11]
164
165 **Homology modeling and molecular docking**
166 For protein sequence alignment, the NCBI BLAST (http://blast.ncbi.nlm.nih.gov) program was used.
167 The homology of BmorPBP1 (PDB ID: 1dqe) of *Bombyx mori* was used as a template for the analysis
168 of amino acid sequence homology. Homology modeling of the target protein was done by
169 MODELER version 9.19 (http://salilab.org/modeller/) software. The molecular docking and the
170 binding modes of the ligands to HcunPBP1, HcunPBP2 and HcunPBP3 were run by Autodock Vina
171 version 1.1.2.[35] The AutoDock Tools version 1.5.6 was used to produce the docking input files.[36] The
172 detection and classification of binding pockets, and the grid points were identified by BmorPBP1. The
173 Vina docking was performed using the default search parameter with exhaustiveness set to 20 to
174 secure high docking accuracy. Ranking docked confirmation was based on the Vina docking score
175 and visual analysis using PyMOL version 1.9.0 (http:// www.pymol.org/).
176

177 **RESULTS**

178 **Expression of *H. cunea* PBP genes**

179 The expression of *HcunPBPs* gene in different body parts/tissues, including antennae, heads (without
180 antennae), abdomens and legs, was determined by RT-qPCR. Specific primers were designed from
181 conserved cDNA sequences encoding *HcunPBPs* (Table S2). As shown in Figure 1, three *HcunPBPs*
182 gene were highly (and male-biased) expressed in the antennae. *HcunPBP1* expression levels were 6.5
183 times higher in the antennae of males than females, whereas *HcunPBP2* and *HcunPBP3* were
184 expressed 1.7 and 4.2 times higher in the antennae of males than females, respectively. These three
185 *HcunPBPs* gene showed undetectable or low expression levels in the other tissues tested in both
186 sexes.

187

188 **Expression and purification of HcunPBP recombinants**

189 To perform the binding assay experiments, HcunPBPs were expressed in a bacterial system. The
190 recombinant HcunPBP1, 2 and 3 were obtained in high yield in *E. coli* after induction with isopropyl
191 β-D-1-thiogalactopyranoside (IPTG), and all three HcunPBP proteins were found to be present in
192 insoluble inclusion bodies. The protein pellets were solubilized by urea treatment and re-natured by
193 extensive dialysis based on the reported protocol.[5,11] Then the proteins were treated with enterokinase
194 to remove the His-tag. The results of SDS-PAGE indicated that the recombinants of HcunPBP1, 2 and
195 3 were approximately 16.26 kDa, 16.43 kDa and 16.13 kDa, respectively (Figure 2). These results
196 were consistent with the expected molecular weights reported in our previous study.[15]

197

198 **Ligand binding properties of the three HcunPBPs to odorants**

As shown in Figure 3a, the dissociation constants (K_i value) of HcunPBP1, 2 and 3 were calculated as 4.87 ± 0.50 μM, 5.64 ± 0.80 μM and 3.95 ± 0.29 μM, respectively. Next, a competitive binding assay was carried out to determine the binding affinity of HcunPBPs to the four sex pheromone components (Figure 3b) and selected plant volatiles (Figure 3c-g and Figure S1). Based on the criteria of binding affinities for sex pheromones: high (K_i < 2.00 μM), moderate (K_i = 2.01-5.00 μM) and low (K_i = 5.01-10.00 μM),[34,37]all three HcunPBPs had a high binding affinity to the two aldehydes, *Z*9, *Z*12-18Ald and *Z*9, *Z*12, *Z*15-18Ald (K_i = 0.49-1.00 μM) (Figure 3b). HcunPBP1 showed a moderate binding affinity (K_i = 2.15 μM) to *Z*3, *Z*6-9*S*,10*R*-epoxy-21Hy and no detectable binding affinity to 1,*Z*3,*Z*6-9*S*,10*R*-epoxy-21Hy. HcunPBP2 showed no detectable binding affinity to *Z*3,*Z*6-9*S*,10*R*-epoxy-21Hy, but had an extremely high binding affinity to 1,*Z*3,*Z*6-9*S*,10*R*-epoxy-21Hy (K_i = 0.26 μM) (Figure 3b). HcunPBP3 had a moderate binding affinity to both epoxides: *Z*3,*Z*6-9*S*,10*R*-epoxy-21Hy (K_i =1.24 μM) and 1,*Z*3,*Z*6-9*S*,10*R*-epoxy-21Hy (K_i =1.84 μM) (Figure 3b).

For plant volatiles, the binding affinities of HcunPBPs were measured and categorized as high, moderate, and low when their K_i values fell in the following ranges: < 10.00 μM, 10.01 - 20.00 μM and 20.01-30.01 Mm,[38] respectively. Among the 24 plant volatiles tested, all three HcunPBPs had a high binding affinity to palmitic acid and nerolidol (K_i < 10 μM). HcunPBP2 and HcunPBP3 showed high binding affinities (K_i = 5.06 μM and 8.44 μM, respectively) to cedrol, whereas HcunPBP1 had a moderate binding affinity (K_i = 10.75 μM) to it. HcunPBP1 and HcunPBP3 had a high binding affinity to nonanal (K_i < 10 μM), while HcunPBP2 and HcunPBP3 showed a high binding affinity to (*E*)-2-hexenol (K_i = 4.08 μM and 5.89 μM, respectively). HcunPBP1 also expressed a very high binding affinity to β-ocimene, (*Z*)-2-penten-1-ol and 2,4-dimethyl-3-pentanol (K_i < 10 μM). HcunPBP2 had a high binding affinity to 6-methyl-5-hepten-2-one, (*Z*)-3-hexenol and menthol (K_i <

10 μM), and a moderate binding affinity to (*E*)-2-hexenal (K_i = 12.91 μM). HcunPBP3 showed a high binding affinity to (*E*)-2-hexenal (K_i = 9.75 μM) and a moderate binding affinity to β-ocimene (K_i = 10.94 μM) (Figure 3c-g). No binding affinities by these three HcunPBPs were found to the other 12 plant volatiles (Figure S1).

Homology modeling and molecular docking

To determine the residues of HcunPBPs that interact with pheromone components, the 3D protein structure for each of the three HcunPBPs was estimated by using a computational procedure. Sequence alignments showed that the amino acid identities of HcunPBP1, HcunPBP2 and HcunPBP3 were 82%, 67% and 64%, respectively, in common with BmorPBP1. Because the homology values of BmorPBP1 were the highest in the database, it was used as a homology template to analyze the HcunPBPs. As shown in Figure 4, the results of structural comparisons demonstrated that: 1) each of the three HcunPBPs possessed 6 α-helices (α1-α6), the same as BmorPBP1 and other moth PBPs;[38,39] 2) the internal cavity structures of these three HcunPBPs were similar to that of BmorPBP1. These data suggest that all three HcunPBPs may have similar ligand binding mechanisms and that BmorPBP1 could be used as a reference model.

Next, the binding energies between the HcunPBPs and the four pheromone components were calculated (Table 1). As shown in Figs. 5-7, all the docking binding energies were shown to have negative values. Moreover, the lengths of all potential interaction residues were less than 4 Å. These results suggested a strong interaction between HcunPBPs and the pheromone components. In addition, the binding models showed that some crucial hydrophobic residues might play a beneficial role in the binding of HcunPBPs to their sex pheromone components, including 12 in HcunPBP1

(Phe-43, Phe-36, Trp-37, Ile-52, Val-136, Phe-119, Val-13, Ala-9, Ile-94, Met-55, Phe-12, Met-8), 9 in HcunPBP2 (Phe-94, Met-18, Phe-118, Ser-115, Met-51, Tyr-36, Phe-12, Leu-135, Thr-9) and 7 in HcunPBP3 (Leu-94, Phe-118, Ala-115, Ile-52, Phe-12, Ile-8, Leu-61).

DISCUSSION

Multiple studies have demonstrated the binding specificity of some PBPs to their pheromone components, for example in *A. polyphemus*, *Antheraea pernyi*, *Lymantria dispar* and *Agrotis ipsilon*.[32,40] However, contradictory results were reported in other species, including *B. mori, Plutella xyllostella*, *Helicoverpa armigera*, *H. assulta*, *Spodoptera exigua* and *S. litura*,[29,41,42,43] which did not show any clear PBP binding discrimination for their own sex pheromone components. In the current study, we first investigated the expression of the three *HcunPBPs* in antennae and other tissues of *H. cunea* adults using RT-qPCR. Consistent with our previous report,[15] these *HcunPBPs* were expressed essentially only in the antennae of both sexes. Interestingly, these three *HcunPBPs* were expressed at much higher levels in the antennae of males than females, corroborating a significant function in detecting the female-produced sex pheromone components in *H. cunea*. Similar antennae-predominant and male-biased PBPs were also reported in other moth species, such as *A. selenaria cretacea*, *E. oblique* and *S. litura*.[5,13,14,44] As earlier reported for *S. exigua*, *S. litura*, *H. armigera*, *H. assulta*, *A. ipsilon*, *S. inferens* and *Carposina sasakii*,[6,41,44] the relatively low (but significant) expression levels of *HcunPBPs* in the antennae of females suggests that females may perceive their own pheromone components as well.

Although the sequences of these HcunPBPs were conserved with a low homology (HcunPBP1 had 43.2% homology with HcunPBP2 and 38.07% with HcunPBP3, while HcunPBP2 showed

42.05% with HcunPBP3), a phylogenetic analysis showed that they were more closely related to PBPs from other species than to each other (Figure S2 and S3). Moreover, these three HcunPBPs were classified into three separate groups. HcunPBP2 and HcunPBP3 showed a high homology with MsexPBP2, HarmPBP3 and HassPBP3 (from Type I pheromone-producing species), respectively (Figure S4). Surprisingly, HcunPBP1 showed a low homology with PBPs from the Type II pheromone-releasing species (e.g. *E. grisescens* and *A. selenaria cretacea*),[13,45] but shared a high level of homology with LdisPBP1 from a recently classified Type III pheromone-producing species, *L. dispar* (Figure S5).[16] These results suggested that the HcunPBPs gene coexisted among lepidopteran species over evolutionary time, and the gene duplication events potentially leading to the three HcunPBPs in *H. cunea* must have occurred before the lepidopteran radiation. On the other hand, PBPs among moth species in general show a limited diversity. Phylogenetic analyses showed that Type II PBPs clustered together but the clade was not separated from other PBPs (Figure S3). In addition, as mentioned above, the three HcunPBPs showed a low homology with each other. These data suggest that the PBPs of Lepidoptera shared a common ancestor; in evolution, however, they formed the different function-specific clades. In agreement with a recent study on pheromone receptors (PRs) of the Type II sex pheromone in *Operophtera brumata*,[46] our results also suggest that moths did not evolve a new type of PBPs, but recruited existing PBPs as carriers of novel Type II components.

Our competitive fluorescence binding assays clearly showed that the binding patterns and abilities of the three HcunPBPs to the two aldehydes were significantly different from those to the two epoxides, indicating a strong binding disparity towards the two chemically different sex pheromone component groups. All three of the HcunPBPs strongly bound to *Z*9, *Z*12-18Ald and *Z*9,*Z*12,*Z*15-18Ald, whereas HcunPBP1 had a moderate binding affinity to *Z*3, *Z*6-9*S*, 10*R*-epoxy-21Hy and no

detectable binding to 1,*Z*3, *Z*6-9*S*, 10*R*-epoxy-21Hy. In contrast, HcunPBP2 showed a highly preferential binding affinity for 1,*Z*3, *Z*6-9*S*,10*R*-epoxy-21Hy, but no detectable binding affinity for *Z*3, *Z*6-9*S*,10*R*-epoxy-21Hy. Interestingly, HcunPBP3 exhibited a moderate binding affinity to both *Z*3, *Z*6-9*S*,10*R*-epoxy-21Hy and 1,*Z*3, *Z*6-9*S*,10*R*-epoxy-21Hy. Consistent with previous studies on *A. polyphemus*, *L. dispar* and *Plutella xylostella*,[31,32,47] our results provide further support for the hypothesis that PBPs act as an additional layer of selectivity and participate in pheromone discrimination.[30] The two aldehydes in the *H. cunea* sex pheromone system are structurally somewhat similar to many Type I pheromone components (*i.e.* C_{10}-C_{18} unsaturated straight chain acetates, aldehydes or alcohols) and markedly different from most other Type II sex pheromones, which lack a terminal functional group. However, they were recently classified as non-typical structures of the extended Type II pheromones from a biosynthetic perspective.[16] That is, these aldehydes share the same biosynthetic origins as the typical Type II epoxides and unsaturated hydrocarbons, *i.e.*, they are derived from linoleic or linolenic acid precursors that must be obtained from the diet, and their unsaturated hydrocarbon skeletons are biosynthesized in the oenocytes and then transported to the pheromone gland for release. In contrast, Type I pheromones are biosynthesized *de novo* from acetate in the pheromone gland.[16] The pheromones of *Ascotis selenaria cretacea, Operophtera brumata* and *Ectropis obliqua* consist of typical Type II sex pheromone components with either long chain unsaturated hydrocarbons alone or unsaturated hydrocarbons plus their corresponding epoxide derivatives. However, the PBPs identified and reported from these species so far did not show any significant disparities in binding affinity to their different Type II pheromone components.[11,12,13,14] In contrast, the three HcunPBPs of *H. cunea* in the current study were not only able to clearly distinguish the differences between the aldehyde and epoxide groups of these four Type II sex pheromone

components, but also showed different binding affinity patterns to the minor structural variations between the two epoxide compounds.

According to the results of *in vitro* ligand binding assays and evidence from *in vivo* studies of PBPs in several other lepidopteran species, multiple PBP genes in one species may have different importance or roles in the perception of sex pheromone components.[4,43,48,49] For example, the HarmPBP1 in *H. armigera*, with the highest male-bias in antennal expression, bound strongly to the two major sex pheromone components, while two other HarmPBPs showed weak affinities for all three pheromone components.[39] In *S. inferens*, SinfPBP1 showed high and similar binding affinities to the 3 sex pheromone components: *Z*-11-hexadecenyl acetate (*Z*11-16:Ac), *Z*-11-hexadecenol (*Z*11-16:OH) and *Z*-11-hexadecenal (*Z*11-16:Ald); SinfPBP3, however, exhibited no apparent binding affinities to sex pheromone components.[50] These results suggested that these two PBPs, HarmPBP1 and SinfPBP1, might play a more important role in the reception of female-produced sex pheromones than other HarmPBPs and SinfPBPs. Moreover, compared to CsupPBP2 and CasuPBP4 in *Chilo suppressalis*, CsupPBP1 and CsupPBP3 showed higher affinities to the tested components in ligand binding assays.[3] *In vivo* functional studies further verified that CsupPBP1 played a more important role than CsupPBP3 in sex pheromone perception.[4] In the current study, *HcunPBP1* had the highest expression (9.67 fold to *HcunPBP2* and 4.02 fold to *HcunPBP3*) in antennae of males, and displayed a high binding affinity to *Z*9,*Z*12,*Z*15-18Ald and *Z*3,*Z*6-9*S*,10*R*-epoxy-21Hy (the two dominant sex pheromone components in *H. cunea*), followed by HcunPBP3 and HcunPBP2. This suggests that among the three HcunPBPs, HcunPBP1 may have a more important function/role in pheromone perception than the other two HcunPBPs.

A previous study on *P. xylostella* has demonstrated that five mutants of PxylGOBP2 with only one or two amino acid substitutions can completely abolish binding to the pheromone, and shift the affinity to plant-derived compounds.[54] Liu et al. (2019) reported that *Apolygus lucorum* OBP (AlucOBP22) mutants of five hydrophobic residues Leu5, Ile40, Met41, Val44 and Met45 significantly decreased or completely abolished binding affinities to the ligands.[55] In addition, site-directed mutagenesis *Ectropis oblique* PBP2 (EoblPBP2) indicated that different components of Type II sex pheromone play different binding characters under specific conditions in the physicochemical behavior such as pH, temperature and amino acid mutations under specific conditions.[56] In our current study, the binding energies and the lengths of all potential interaction residues suggested a strong interaction between HcunPBPs and the pheromone components (Figure 5, 6 and 7). These findings allowed us to speculate that these hydrophobic residues and their hydrophobic interactions were crucial for the binding of HcunPBPs to their sex pheromone components. These HcunPBPs may serve similar functions as those of PxylGOBP2, AlucOBP22 and EoblPBP2. Further *in vivo* functional studies, such as RNAi or CRISPR/Cas9 technique, combined with electrophysiological and behavioral assays, will be needed to validate these assumptions.

Previous studies demonstrated that *Z*9,*Z*12,*Z*15-18Ald, *Z*3,*Z*6-9*S*,10*R*-epoxy-21Hy and 1,*Z*3, *Z*6-9*S*,10*R*-epoxy-21Hy were the three essential components for significant attraction of *H. cunea* males in the field.[24,25,27,28] Further field tests with the four synthetic sex pheromone components showed that all individual components were inactive alone, but their quaternary blend was highly attractive to *H. cunea* males.[21] The minor epoxide component 1,*Z*3, *Z*6-9*S*,10*R*-epoxy-21Hy, which was specifically bound by HcunPBP2, seems to be a critical part of the *H. cunea* sex pheromone system. These findings allowed us to speculate that although there was a strong disparity in the

affinities and expression of these three HcunPBPs, all HcunPBPs may act cooperatively to ensure the effective perception of sex pheromones in *H. cunea*. Indeed, in nature, the full activation of males of many moth species occurs only when the full pheromone blend of their own species is presented.[30] In tests of a panel of plant volatiles, all three HcunPBPs showed a relatively higher binding ability to fatty acid derivatives and 6-carbon alcohols and aldehydes in the competitive fluorescence binding assays. In addition, HcunPBP1 and HcunPBP2 exhibited an obvious binding affinity to two other plant volatiles (HIPVs), β-ocimene and (*Z*)-2-penten-1-ol. (*Z*)-2-Penten-1-ol is released by intact and mechanically-damaged leaves, whereas β-ocimene is released only by herbivore-damaged leaves.[51] Moreover, the attraction of *H. cunea* males to a sex pheromone lure was increased by β-ocimene but reduced by (*Z*)-2-penten-1-ol.[51,52] Based on our binding data, Tang et al.'s results and previous reports on *E. oblique*,[11] and *C. sasakii*,[6] we suspect β-ocimene and (*Z*)-2-penten-1-ol are potential HIPVs for *H. cunea*. In addition, LdisPBPs in *L. dispar* females were found to have a primary function in recognizing plant volatiles and its own sex pheromone.[53] Thus, based on our results and those of others, we believe that HcunPBPs also participate in the perception and discrimination of host-plant kairomones in *H. cunea*.

The molecular docking results appear to provide further evidence that these HcunPBPs might contribute to the perception and discrimination of not only the sex pheromones but also host-plant kairomones in *H. cunea*, although with much stronger binding to the four sex pheromone components than to the plant volatiles (Figure 3). Furthermore, we also found that some crucial hydrophobic residues may play a role in the binding of HcunPBPs to their sex pheromone components (Figure 4-7). Therefore, these residues could serve as potential targets for future mechanistic studies of

HcunPBP ligand binding, by integrating various omics techniques, such as the CRISPR/Cas9 editing system or site-directed mutagenesis methods.

In summary, we studied the functionality of three HcunPBPs in *H. cunea*, a moth species that uses Type II sex pheromone components from two different chemical classes (aldehyde and epoxide) in its pheromone system. Different HcunPBPs might have different selectivities for pheromone components with both major and minor structural differences. The results of the molecular docking studies demonstrated that some key amino acids may play an important role in the ligand binding of HcunPBPs. These residues, therefore, could serve as potential targets for future mechanistic studies of HcunPBPs' ligand binding. Our study provided not only a better understanding of sex pheromone perception of *H. cunea* at a molecular level, but also a valuable insight into the different binding mechanisms of PBPs, especially for moth species that use various Type II pheromone components.

SUPPORTING INFORMATION

Binding affinities of 12 plant volatiles to HcunPBP1, HcunPBP2 and HcunPBP3; comparison of the amino acid sequences of HcunPBP1, HcunPBP2 and HcunPBP3; molecular phylogeny comparing HcunPBPs with PBPs from twenty-seven insect species; comparison of the amino acid sequences of HcunPBP2 and HcunPBP3 with pheromone-binding proteins from Type I pheromone releasing species; comparison of the amino acid sequences of HcunPBP1 with pheromone-binding proteins from different species of Lepidoptera; pheromone-binding proteins from Type II pheromone releasing species and Type III pheromone releasing species; primers of *H. cunea* PBP genes used for RT-qPCR; plant volatiles used in the binding assays of HcunPBPs; and primers of *H. cunea* PBP genes used for prokaryotic expression (PE).

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

LZ conceived and designed experiments. XZ, LH, JY and JQ performed experiments. XZ, LZ, DM, YZ and HP analyzed data. SD, YZ and PH contributed, materials. XZ, LZ, DM, QHZ and EP wrote the paper. All authors read and approved the manuscript.

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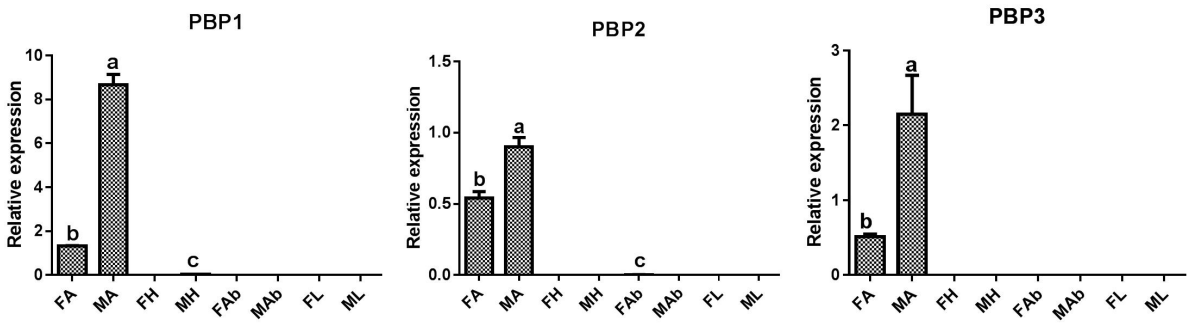
590 **Figures and figure legends**



591

592 **Figure 1.** Relative mRNA expression of *HcunPBPs* in *H. cunea* tissues. F, female; M, male; A,

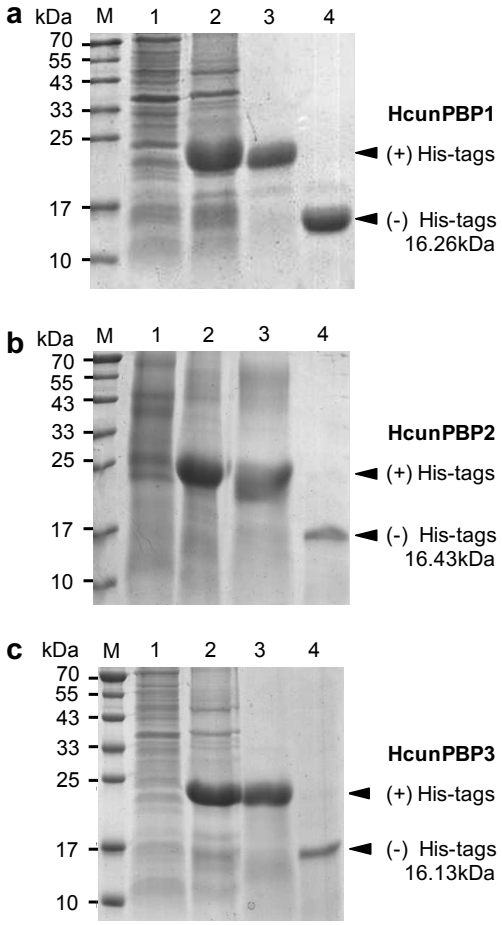
593 antennae; H, heads (without antennae); Ab, abdomens; L, legs. The relative mRNA levels were

594 normalized to those of the EF1-a gene and analyzed using the Q-gene method. All values are shown

595 as the mean ± SEM normalized. The data were analyzed by the least significant difference (LSD) test

596 after one-way analysis of variance (ANOVA). Different letters (a-e) indicate significant differences

597 between means ($P< 0.05$).



598

599 **Figure 2.** Expression and purification of recombinant HcunPBP1 (a), HcunPBP2 (b) and HcunPBP3

600 (c) by SDS-PAGE analysis. Lane 1 and 2, crude bacterial extracts after induction with IPTG,

601 following by supernatant (lane 1) and bacterial pellet (lane 2). Lane 3 and 4, purified pET/HcunPBPs

602 protein with and without His-tags, respectively.

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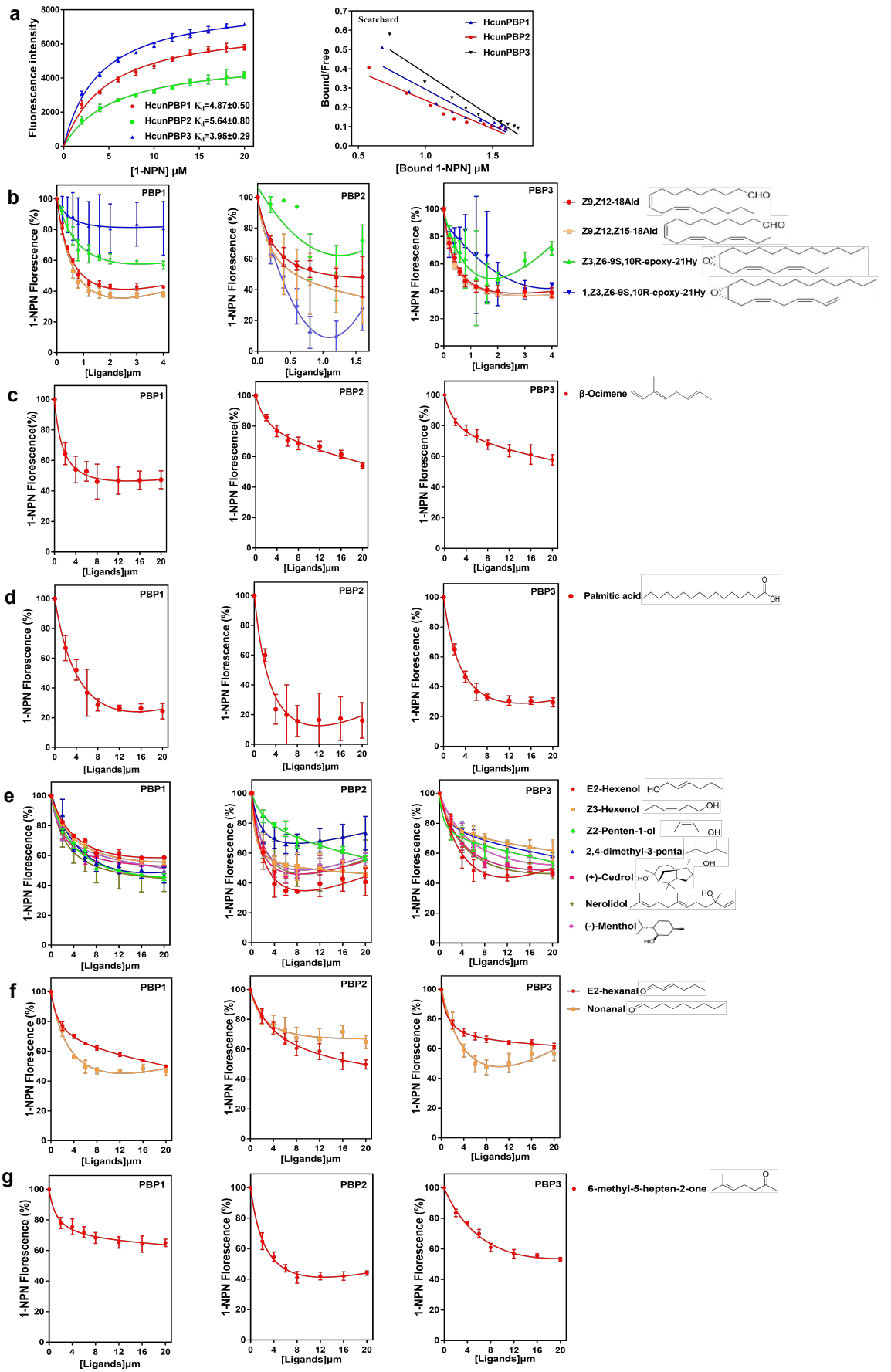
a
Fluorescence intensity
[1-NPN] μM
HcunPBP1 K_d=4.87±0.50
HcunPBP2 K_d=5.64±0.80
HcunPBP3 K_d=3.95±0.29
Scatchard
Bound/Free
[Bound 1-NPN] μM
HcunPBP1
HcunPBP2
HcunPBP3
b
c
d
e
f
g
PBP1
PBP2
PBP3
1-NPN Florescence (%)
[Ligands]μm
Z9,Z12-18Ald
Z9,Z12,Z15-18Ald
Z3,Z6-9S,10R-epoxy-21Hy
1,Z3,Z6-9S,10R-epoxy-21Hy
β-Ocimene
Palmitic acid
E2-Hexenol
Z3-Hexenol
Z2-Penten-1-ol
2,4-dimethyl-3-penta
(+)-Cedrol
Nerolidol
(-)-Menthol
E2-hexanal
Nonanal
6-methyl-5-hepten-2-one

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Figure 3. Binding of selected ligands to HcunPBPs. (a) Binding curves and Scatchard plots (insert) of the fluorescence probe 1-NPN to HcunPBP1, HcunPBP2 and HcunPBP3. The binding curves and the relative Scatchard plots indicate the binding constants of HcunPBPs/1-NPN complex: 4.87 ± 0.50 μM, 5.64 ± 0.80 μM and 3.95 ± 0.29 μM for HcunPBP1, HcunPBP2 and HcunPBP3, respectively. (b-g) Comparison of binding properties of the three HcunPBPs to four sex pheromone components (b) and 12 plant volatiles: alkenes (c), acids (d), alcohols (e), aldehydes (f) and ketones (g).

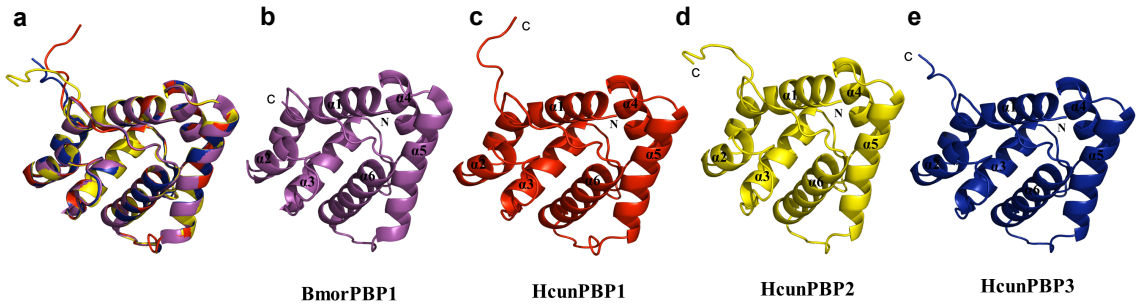
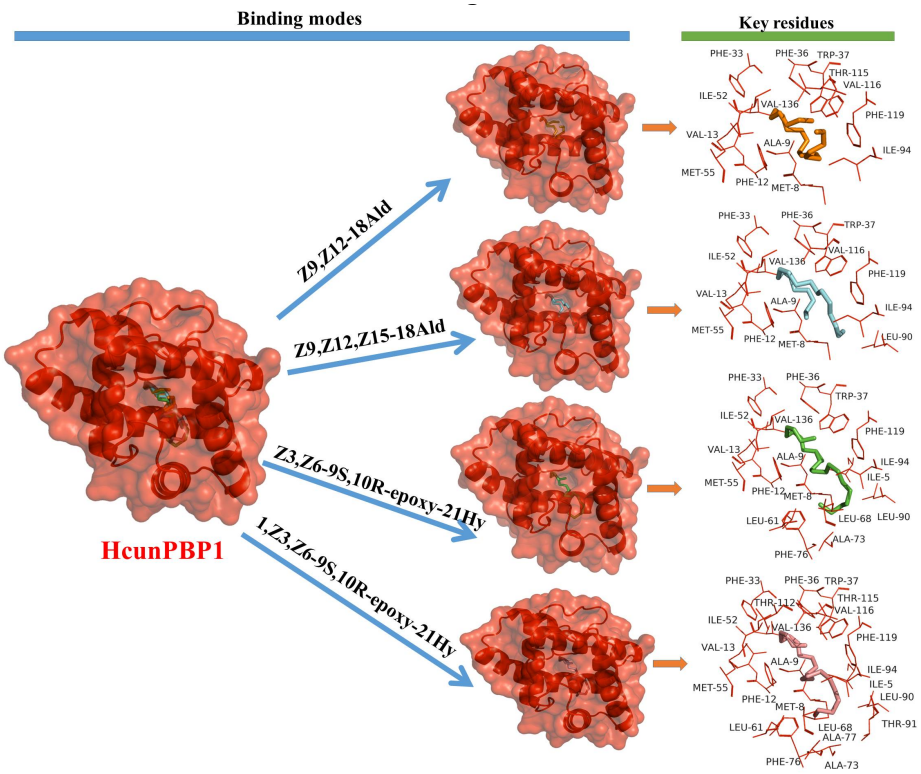


Figure 4. Structural modeling of HcunPBPs. (a) Superposition of the four PBPs from the matching panels B-E in the same orientation. (b) BmorPBP1 (PDB ID: 1dqe). (c) HcunPBP1. (d) HcunPBP2. (e) HcunPBP3.



623

624 **Figure 5.** Binding modes and key residues of HcunPBP1 to the four sex pheromone components.

625 *Z*9,*Z*12-18Ald (orange), *Z*9,*Z*12,*Z*15-18Ald (blue), *Z*3,*Z*6-9*S*,10*R*-epoxy-21Hy (green) and 1,*Z*3,*Z*6-

626 9*S*,10*R*-epoxy-21Hy (rose) in the putative binding pocket of chain A of HcunPBP1. The key residues

627 of the different ligands that interact with HcunPBP1 are shown. The residues within 4 Å of the ligands

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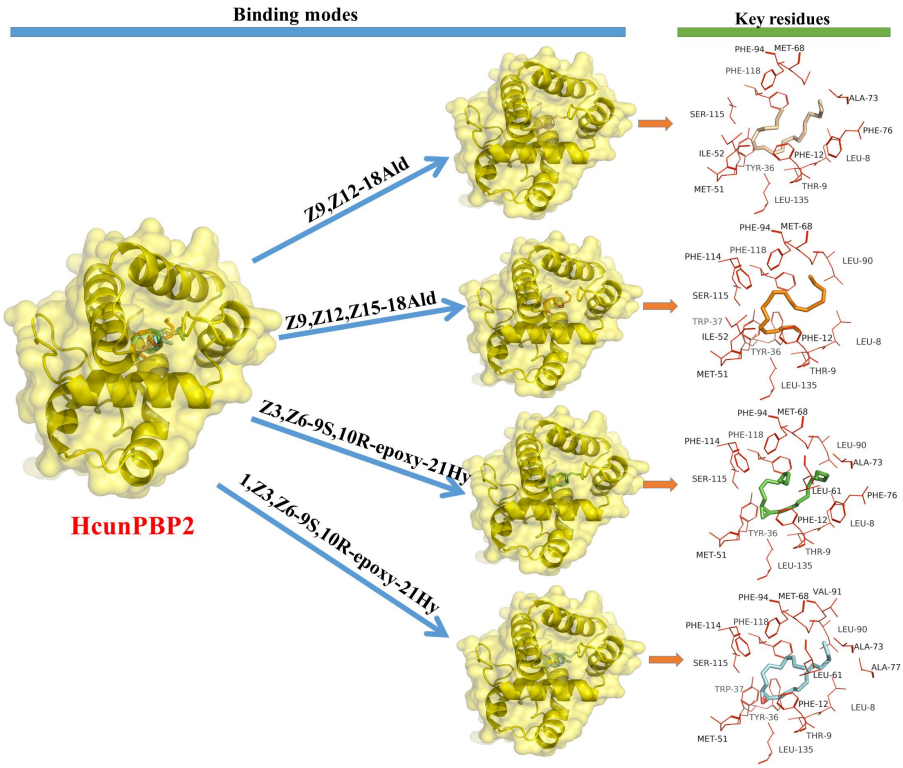

635

636 **Figure 6.** Binding modes and key residues of HcunPBP2 to the four sex pheromone components.

637 *Z*9,*Z*12-18Ald (rose), *Z*9,*Z*12,*Z*15-18Ald (orange), *Z*3,*Z*6-9*S*,10*R*-epoxy-21Hy (green) and 1,*Z*3,*Z*6-

638 9*S*,10*R*-epoxy-21Hy (blue) in the putative binding pocket of chain A of HcunPBP2. The key residues

639 of the different ligands that interact with HcunPBP2 are shown. The residues within 4 Å of the ligands

640 are highlighted in red.

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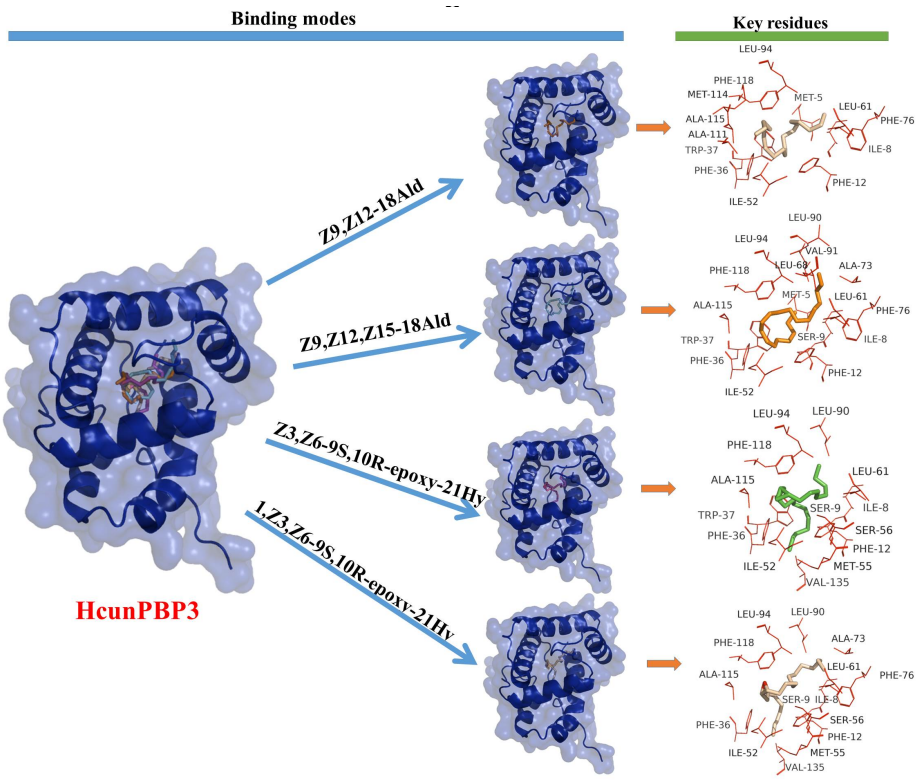

Figure 7. Binding modes and key residues of HcunPBP3 to four sex pheromone components. *Z*9,*Z*12-18Ald (rose), *Z*9,*Z*12,*Z*15-18Ald (orange), *Z*3,*Z*6-9*S*,10*R*-epoxy-21Hy (green) and 1,*Z*3,*Z*6-9*S*,10*R*-epoxy-21Hy (pink) in the putative binding pocket of chain A of HcunPBP3. The key residues of the different ligands that interact with HcunPBP3 are shown. The residues within 4 Å of the ligands are highlighted in red.

Table 1 Binding data of different ligands to HcunPBPs and molecular docking

Ligand name	PBP1			PBP2			PBP3		
	IC50(μM)	Ki(μM)	Free binding energy (kcal mol−1)	IC50(μM)	Ki(μM)	Free binding energy (kcal mol−1)	IC50(μM)	Ki(μM)	Free binding energy (kcal mol−1)
Type II Sex pheromones									
Srain chain aldehydes									
Z3,Z6-9S,10R-epoxy-21Hy	>4	-	-8.2	>4	-	-8	1.1±0.1	0.76±0.07	-7.5
1,Z3,Z6-9S,10R-epoxy-21Hy	>4	-	-8	0.36±0.04	0.27±0.03	-7.9	2.48±0.73	1.73±0.51	-7.5
Epoxides									
Z9,Z12-18Ald	1.2±0.03	0.89±0.03	-7.1	0.72±0.01	0.55±0.01	-7.2	1.06±0.07	0.74±0.05	-6.8
Z9,Z12,Z15-18Ald	1.02±0.05	0.76±0.04	-7.8	0.45±0.04	0.35±0.03	-7.5	1.0±0.03	0.70±0.02	-7
Green leaf volatiles								-	
β-Ocimene	7.59±1.4	5.74±1.01	-6.9	>20	-	-6.2	>20	-	-6.3
Palmitic acid	4.82±0.59	3.6±0.43	-6.7	3.36±0.54	2.59±0.41	-6.9	4.78±0.19	3.43±0.13	-6.5
E2-Hexenol	>20	-	-4.9	4.55±0.32	3.51±0.25	-4.5	7.26±0.80	4.82±0.53	-4.3
Z3-Hexenol	>20	-	-4.8	8.07±1.47	6.21±1.13	-4.6	>20	-	-4.3
Z2-Penten-1-ol	9.7±0.37	7.3±0.28	-4.4	>20	-	-4.1	>20	-	-4
2,4-dimethyl-3-pentanol	9.57±0.66	7.24±0.51	-5.3	>20	-	-5.2	>20	-	-5
(+)-Cedrol	>20	-	-9.7	6.36±0.20	4.90±0.16	-9.8	10.56±1.0	7.53±0.70	-9.3
Nerolidol	9.75±1.98	7.26±1.47	-8.1	6.80±0.62	5.23±0.48	-7.7	9.43±0.32	6.73±0.25	-7.9
(-)-Menthol	>20	-	-7.1	7.30±0.36	5.63±0.28	-7.2	>20	-	-6.6
E2-hexanal	>20	-	-4.9	>20	-	-4.2	>20	-	-4.3
Nonanal	7.26±0.34	5.396±0.25	-5.6	>20	-	-5	6.85±0.10	4.76±0.07	-4.9
6-methyl-5-hepten-2-one	>20	-	-5.8	6.04±0.30	4.64±0.23	-5.5	>20	-	-5.5

In fluorescence competitive binding assays, dissociation constants (K_i) were calculated from the corresponding IC_{50} values (the concentrations of ligands halving the fluorescence of 1-NPN, the IC_{50} values of ligands without binding ability or weak binding ability cannot be obtained in the experiment and were presented as ">4 (sex pheromone) or >20 (non sex pheromone) ", so the dissociation constant K_i of these ligands cannot be calculated and was presented as "-".