Virtual Screening of Plant Volatile Compounds Reveals a High Affinity of *Hylamorpha elegans* (Coleoptera: Scarabaeidae) Odorant-Binding Proteins for Sesquiterpenes From Its Native Host

Angélica González-González,1,* Rubén Palma-Millanao,1,2,* Osvaldo Yáñez,3 Maximiliano Rojas,4 Ana Mutis,5 Herbert Venthur,6 Andrés Quiroz,5 and Claudio C. Ramírez1

1Millennium Nucleus Centre in Molecular Ecology and Evolutionary Applications in Agroecosystems, Instituto de Ciencias Biológicas, Universidad de Talca, 2 Norte 685, Talca, Chile (angelica.gonzalez@utalca.cl; rupalma@utalca.cl; clramirez@utalca.cl), 2Corresponding author, e-mail: rupalma@utalca.cl, 3Doctorado en Fisicoquímica Molecular, Facultad de Ciencias Exactas, Universidad Andrés Bello, República 275, Santiago, Chile (osvyanezoses@gmail.com), 4Instituto de Ciencias Biológicas, Universidad de Talca, Dos Norte 685, 3465548 Talca, Chile (mrojasr@alumnos.utalca.cl), and 5Laboratorio de Ecología Química, Departamento de Ciencias Químicas y Recursos Naturales, Universidad de La Frontera, Av. Francisco Salazar, 01145 Temuco, Chile (ana.mutis@ufrontera.cl; h.venthur01@ufromail.cl; andres.quiroz@ufrontera.cl)

*These authors contributed equally to this work.

Subject Editor: Jurgen Ziesmann

Received 2 November 2015; Accepted 24 January 2016

Abstract

*Hylamorpha elegans* (Burmeister) is a native Chilean scarab beetle considered to be a relevant agricultural pest to pasture and cereal and small fruit crops. Because of their cryptic habits, control with conventional methods is difficult; therefore, alternative and environmentally friendly control strategies are highly desirable. The study of proteins that participate in the recognition of odorants, such as odorant-binding proteins (OBPs), offers interesting opportunities to identify new compounds with the potential to modify pest behavior and computational screening of compounds, which is commonly used in drug discovery, may help to accelerate the discovery of new semiochemicals. Here, we report the discovery of four OBPs in *H. elegans* as well as six new volatiles released by its native host *Nothofagus obliqua* (Mirbel). Molecular docking performed between OBPs and new and previously reported volatiles from *N. obliqua* revealed the best binding energy values for sesquiterpenic compounds. Despite remarkable divergence at the amino acid level, three of the four OBPs evaluated exhibited the best interaction energy for the same ligands. Molecular dynamics investigation reinforced the importance of sesquiterpenes, showing that hydrophobic residues of the OBPs interacted most frequently with the tested ligands, and binding free energy calculations demonstrated van der Waals and hydrophobic interactions to be the most important. Altogether, the results suggest that sesquiterpenes are interesting candidates for in vitro and in vivo assays to assess their potential application in pest management strategies.

Key words: insect olfaction, molecular dynamics, *Nothofagus obliqua*, protein prediction, MM/GBSA

Scarabaeidae is an important family of Coleoptera comprising near 25,000 species distributed worldwide (Gillott 2005), with species that feed on different substrates such as plants, dung, and decomposing plants and animals (Pedigo and Rice 2009). With regard to agriculture, the larval stage is frequently associated with the consumption of organic matter as well as the roots of berries, cereal crops and pasture (Rodríguez et al. 2004). Scarabaeidae pest species are difficult to control due to the cryptic position of the larvae in the soil (Chen et al. 2014) and the typical nocturnal activity of the adults (Jackson and Klein 2006). In addition, exotic plants introduced within the native geographical range of these insects are usually invaded by these beetle species (Lefort et al. 2014).

Environmentally friendly strategies for controlling adults scarab beetles are needed (Wang et al. 2013a,b; Zhuang et al. 2014), and there is much interest in studying the molecular mechanisms of proteins that are highly expressed in sensory organs (Li et al. 2015),
which may lead to the manipulation of behavior driven by olfactory chemoreception (Zhuang et al. 2014, Li et al. 2015).

Among such proteins, odorant-binding proteins (OBPs) are major peripheral olfactory proteins involved in the perception of odorants in insects (Leal 2013). OBPs, small soluble proteins present in the sensillum lymph (Gu et al. 2013), are characterized by six conserved cysteine residues joined by three disulfide bridges (Pelosi 1998) and six alpha-helices that form a cavity for ligand binding (Leite et al. 2009). These proteins serve as carriers of lipophilic odorant molecules to olfactory receptors (Wang et al. 2013a,b) and are linked to odorant recognition in the olfactory process (Biessmann et al. 2010).

Accordingly, the use of their recognition ability has been reported in the identification of candidate compounds for pest control (Leal et al. 2008). This approach, named “reverse chemical ecology”, reduces the number of odorant candidate compounds based on protein-ligand affinity (Leal 2005), thus saving time and cost compared to the conventional trial-and-error screening performed in the field (Leal 1998). This concept was recently updated, and the combination of in silico molecular docking and molecular dynamics (MD) proved to be reliable for predicting behaviorally active compounds for insect pest species (Jayanthi et al. 2014). Indeed, the simultaneous use of both of these computational tools has been widely used for drug design research (Okimoto et al. 2009), whereas only a few modeling studies have been performed on insect OBPs (Venthur et al. 2014).

In the south of Chile, the most threatening phytophagous species are native insects (Durán 1954, Cisterñas 1992, Aguilera et al. 1996); among these, Hylamorpha elegans (Burmeister) is an abundant species and is considered to be a relevant pest in different crops (Klein and Waterhouse 2000). H. elegans is distributed from Valparaiso to the Los Lagos region of Chile (Ratcliffe and Ocampo 2002, Aguilera et al. 2011). A monovoltine species, adults are present each year from November to January, emerging from pasture fields of the Estación Experimental Maquehue, Araucanía, Chile. The beetles were separated according to sex, transported to the laboratory in a cooler, and preserved at 4°C until processing.

Materials and Methods
Insects
Adult individuals were collected from the foliage of N. obliqua in fields of the Estación Experimental Maquehue, Araucanía, Chile. The beetles were separated according to sex, transported to the laboratory in a cooler, and preserved at 4°C until processing.

cDNA Cloning and RT-PCR
RNA from different tissues was extracted using RNeasy Plant Mini Kit (Qiagen, The Netherlands) following the manufacturer's instructions. A total of 30 males and 50 females were used for extraction of RNA from antennae; 20 males and 20 females for hindleg tibia; and 50 males and 50 females for mouthparts. The RNA quantified using an Epoch spectrophotometer (BioTek, VT). One microgram of total RNA was treated with 1u DNase I (ThermoFisher, PA) according to the manufacturer’s instructions, and cDNA was synthesized by SMART RACE cDNA Amplification Kit (Clontech, CA). Specific primers were designed based on predicted sequences and used to amplify the 5’- and 3’-ends of cDNAs for the following OB sequence: HeleOBP1, HeleOBP3, HeleOBP4, and HeleOBP6 (Table 1). The reactions were performed using 20ng of cDNA and 1 u of Platinum Taq DNA polymerase (Invitrogen, Brazil) under the following conditions: 94°C for 120 s; 30 cycles at 94°C for 30 s, the annealing temperature (Table 1) for 30 s, and 72°C for 30 s; and 94°C for 2 min. The products were visualized on 1.5% agarose gels and sequenced by Macrogen (Seoul, Korea). Actin was used as a control to assess cDNA quality. The sequences were deposited in NCBI under accession numbers KT861417–KT861420.

Homologous sequences were identified by similarity using the Basic Local Alignment Search Tool (BLASTn). Reading frames were obtained using EMBOSS Transeq (http://www.ebi.ac.uk/Tools/st/emboss_transseq/) and then aligned using BLASTp (http://blast.ncbi.nlm.nih.gov/). Signal peptides were predicted using PrediSi (www.predisi.de), and the isoelectric point (pI) and molecular mass were calculated by the Compute pi/MW tool (http://web.expasy.org/compute_pi/).

Ligand Extraction and Identification
Seventeen compounds found in southern beech (N. obliqua), the native host of H. elegans, were used to perform the in silico analyses; the compounds consisted of those obtained from the report of Quirioz et al. (1999) as well as new compounds extracted and identified in this work (Table 2). Moreover, two potential sex pheromone compounds of H. elegans (Quirioz et al. 2007), 1,4-benzoquinone and 1,4-hydroquinone, were among the tested chemicals. For the collection of new compounds, briefly, volatiles from fresh leaves were trapped in the field by two methods that involved enclosing the
Prediction of *H. elegans* Chemoreceptors

Chemosensory sequences of several insect species were used to predict chemoreceptors in *H. elegans* based on the early genome draft (in curation). Sequences from *Acyrthosiphon pisum*, (Harris) *Aedes aegypti*, (L.) *Anopheles gambiae* Giles, *Antheraea polyphemus* (Cramer), *Apis mellifera*, (L.) *Bombus mori*, (L.) *Colex quinquefasciatus*, (Say) *Dendroctonus ponderosae*, Hopkins *Drosophila melanogaster*, Meigen *H. oblitia, Nasonia vitripennis*, (Walker) *Phyllopertha diversa*, Waterhouse *Solenopsis invicta*, Barren and *Trichlorcastaneum*, (Herbst) present in the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank), were translated into amino acid sequences using Bioperl (Stajich et al. 2002) algorithms, including six-frame translations or open reading frames (ORFs). BLASTp local (Altschul et al. 1990) was used to compare all chemoreceptor ORFs from these species with those from the early genome draft of *H. elegans* ORFs. The cut-off parameters used for BLASTp were an E value of 10⁻³ and 25% amino acid identity (Vieira et al. 2007). Finally, all predicted sequences were checked by BLASTx (http://blast.ncbi.nlm.nih.gov/Blast.cgi) searches against the non-redundant GenBank database.

Homology Modeling

Because of the unknown nature of the templates of OBPs from scarab beetle species (Zhang et al. 2014), three-dimensional homology models were constructed for HeleOBP1, HeleOBP3, HeleOBP4, and HeleOBP6. A suitable template for three-dimensional modeling was identified using the position-specific iterated basic local alignment search tool (PSIBLAST) from Protein Data Bank. From four to seven PDB templates were tested for each HeleOBP. The template structures were structurally aligned using Modeller 9.14 (Sali and Blundell 1993) to obtain a multiple-template, structure-based sequence alignment. Each alignment was then used to create a set of 10,000 homology models, and the smallest value of the normalized discrete optimized molecule energy function (Shen and Sali 2006) and the GA341 score, available in NAMD software (Kalé et al. 1999, Phillips et al. 2005). The quality of the models was then analyzed using PROCHECK (Laskowski et al. 1993) to choose the best of them to each HeleOBP.

Docking

AutoDock (v 4.2.1) and AutoDock Vina (v 1.0.2) (Trott and Olson 2009) were used for all dockings in this study. The three-dimensional coordinates of ligand structures (Table 3) were obtained from...
Table 3. Interaction energy calculated in molecular docking between *H. elegans* OBPs and the different tested compounds

| Ligand              | Structure | Interaction Energy (kcal/mol) |
|---------------------|-----------|------------------------------|
|                     |           | HeleOBP1 | HeleOBP3 | HeleOBP4 | HeleOBP6 |
| alpha-agarofuran*   | C_{15}H_{24}O | -7.6     | -5.7     | -6.4     | -4.7     |
| alpha-caryophyllene | C_{15}H_{24}  | -7.5     | -5.4     | -6.6     | -3.7     |
| alpha-copaene*      | C_{15}H_{24}  | -7.6     | -7.4     | -6.9     | -7.3     |
| alpha-gurjunene     | C_{15}H_{24}  | -7.8     | -6.7     | -6.7     | -4.7     |
| aromadendrene       | C_{15}H_{24}  | -7.8     | -6.8     | -6.3     | -4.9     |
| benzaldehyde*       | C_{7}H_{6}O   | -5.5     | -5.3     | -4.9     | -4.7     |
| beta-caryophyllene  | C_{15}H_{24}  | -7.5     | -6.2     | -6.7     | -3.1     |
| beta-copaene*       | C_{15}H_{24}  | -7.5     | -7.4     | -6.9     | -7.2     |
| beta-myrcene        | C_{10}H_{16}  | -4.9     | -5.8     | -5.6     | -5.7     |
| cis-3-hexenyl acetate | C_{6}H_{10}O_{2} | -5.0     | -5.5     | -5.0     | -5.1     |
| cis-beta-ocimene    | C_{10}H_{16}  | -5.4     | -5.8     | -5.6     | -5.7     |
| decanal*            | C_{10}H_{20}O | -4.7     | -5.4     | -4.9     | -5.2     |
| dodecane            | C_{12}H_{26}  | -4.3     | -5.6     | -5.1     | -5.8     |
| ethyl-octanoate*    | C_{8}H_{16}O_{2} | -4.9    | -5.7     | -5.1     | -5.3     |
| hexyl-acetate*      | C_{10}H_{20}O_{2} | -4.5    | -5.3     | -4.7     | -4.8     |
| linalool            | C_{10}H_{20}O | -5.5     | -5.9     | -5.3     | -5.5     |
| nonanal*            | C_{8}H_{16}O  | -4.6     | -5.3     | -4.6     | -4.8     |
| phenol*             | C_{6}H_{10}O  | -4.6     | -5.1     | -4.4     | -4.6     |
| trans-beta ocimene  | C_{10}H_{16}  | -5.5     | -5.8     | -5.3     | -5.8     |
| tretradecane        | C_{14}H_{30}  | -4.6     | -5.9     | -5.4     | -5.9     |
| 1,4-benzoquinone    | C_{8}H_{16}O_{2} | -4.9    | -5.4     | -4.4     | -4.9     |
| 1,4-hydroquinone    | C_{8}H_{16}O_{2} | -4.7    | -5.1     | -4.2     | -4.9     |

*Ligands were taken from a previous study (Quiroz et al. 1999).*
the PubChem database (http://pubchem.ncbi.nlm.nih.gov). When ligand structures were not available from PubChem, they were drawn using Discovery Studio 3.1 (Accelrys, CA). The ligand files were prepared using the AutoDockTools package (Sanner 1999) (http://autodock.scripps.edu) provided by AutoDock by accepting all rotatable bonds. The proteins (OBP1, OBP3, OBP4 and OBP6) were treated with the protein preparation wizard by Maestro (Schrodinger NY); polar hydrogen atoms were added, nonpolar hydrogen atoms were merged, and charges were assigned. Docking was treated as rigid and carried out using the empirical free energy function and the Lamarckian Genetic Algorithm provided by AutoDock Vina. The grid map dimensions were 10 by 10 by 10 Å³, with 0.375 Å spacing between the grid points, making the center of the protein the center of the cube, i.e., x, y, and z centers at 0.06, 0.04, and 0.09, respectively. All other parameters were set as the default defined by AutoDock Vina. Dockings were repeated 20 times with 20 conformations. The best interaction energy of binding (kcal/mol) was selected for MD evaluation.

**MD Protocol**

Two complexes were built for each modeled HeleOBP1, HeleOBP3, HeleOBP4, and HeleOBP6, and each model was confined inside a periodic simulation box. Furthermore, an explicit solvent was added to the TIP3P water model (Neria et al. 1996) (≈3.500 water molecules). Na⁺ and Cl⁻ ions were added to neutralize the systems and maintain an ionic concentration of 0.15 mol/liter. MD simulations were carried out using the modeled CHARMM22 and CHARMM36 force fields (MacKerell et al. 1998) within the NAMD software (Kalé et al. 1999, Phillips et al. 2005). First, each system included 15,000 steps of conjugate-gradient energy minimization followed by 1 ns of simulation with the protein backbone atoms fixed and gradually releasing the backbone over 10,000 ps with 10–0.1 kcal/mol Å⁻² restraints. The total duration of simulation was ~5 ns for each system. During the MD simulations, motion equations were integrated with a 1 fs time step in the NPT ensemble at a pressure of 1 atm. The SHAKE algorithm was applied to all hydrogen atoms, and the van der Waals cutoff was set to 12 Å. The temperature was maintained at 300 K, employing the Nosé–Hoover thermostat method with a relaxation time of 1 ps. The Nosé–Hoover Langevin piston was used to control the pressure at 1 atm. Long-range electrostatic forces were taken into account by means of the Particle-Mesh Ewald (PME) approach. Data were collected every 1 ps during the MD runs. Molecular visualization of the systems and MD trajectory analysis were carried out with the VMD software package (Humphrey et al. 1996).

**MM/GBSA Calculations**

The molecular mechanics/generalized born surface area (MM/GBSA) method was employed to estimate the binding free energy of the OBP–ligand complexes. For calculations from a total of 5 ns of MD, 2 ns were extracted for analysis, and the explicit water molecules and ions were removed. The MM/GBSA analysis was performed on three subsets of each system: the protein alone, the ligand alone, and the complex (protein–ligand). For each of these subsets, the total free energy (G_{tot}) was calculated as follows:

$$G_{tot} = H_{MM} + G_{solv} - T\Delta S_{conf}$$

where $H_{MM}$ is the bonded and Lennard–Jones energy terms; $G_{solv}$ is the polar contribution of solvation energy and nonpolar
Fig. 2. Alignment generated by the ESPript 3.0 webtool of *H. elegans* OBPs with the PDB accessions used to build the models indicating identity and similarity percentage. Asterisks indicate conserved cysteine residues.
Both   Alphворотis  2011,  Vergara-Jaque et al.  2013 ), both to their amino acidic identity ( Fig. 2 ).

To build the OBP model, different templates were chosen from the PDB database (http://www.rcsb.org/pdb/home/home.do) according to their amino acidic identity (Fig. 2). C.  quinquefasciatus  OBP1 (PDB code 2L2C), with 24% identity, was used for HeleOBP1. The HeleOBP3 model was generated using the  A. gambiae  OBP4 (PDB code 3Q8I)  (Davrazou et al.  2011 ) crystal structure, with 25% identity. For HeleOBP4,  A. gambiae  OBP1 (PDB code 2ERB)  (Wogulis et al.  2006 ), with 19% identity, was used.  Rhyparobia maderae  (F.) pheromone-binding protein (PDB code 1OW4)  (Lartigue et al.  2003 ), reaching 23% identity, was used for the HeleOBP6 model (Fig. 2).

The quality of the models was analyzed with PROCHECK, which showed that 85.6% of the HeleOBP1 residues are in the most favored region and the rest in the additionally allowed region of Ramachandran plots  (Laskowski et al.  1993 ). The values obtained for HeleOBP3, HeleOBP4 and HeleOBP6 were 93.1, 89.4, and 89.2%, respectively.

The overall structure of HeleOBP1 comprises six helices (α1–α6) stabilized by three disulfide bridges formed between α1 and α3 (Cys19–Cys50), α3 and α6 (Cys46–Cys98), and α5 and α6 (Cys89–Cys107). The structure of HeleOBP3 is also composed of six helices stabilized by three disulfide bridges formed between α1 and α3 (Cys14–Cys45), α3 and α6 (Cys41–Cys93), and α5 and α6 (Cys84–Cys102). Similarly, the structure of HeleOBP4 consists of three disulfide bridges formed between α2 and α4 (Cys22–Cys53), α4 and α7 (Cys49–Cys108), and α6 and α7 (Cys96–Cys117) (Figs. 2 and 4).

Docking

Docking studies of the protein–ligand complexes resulted in calculation of the affinity energy (Table 3). For HeleOBP1, the best results were obtained for alpha-gurjunene and aromadendrene, at −7.8 kcal/mol each. For HeleOBP3 and HeleOBP4, the best results were obtained for alpha- and beta-copaene, at −7.4 and −6.9 kcal/mol, respectively. The best results for HeleOBP6 were −7.3 kcal/mol for alpha-copaene and −7.2 kcal/mol for beta-copaene. Finally, the best affinity scores for all the proteins were obtained with sesquiterpenic compounds, whereas pheromones exhibited the lowest interaction energy values.

| Identity | HeleOBP1 | 100% | HeleOBP3 | 45% | HeleOBP4 | 22% | HeleOBP6 | 50% |
|----------|----------|------|----------|------|----------|------|----------|------|
| HeleOBP1 | 100%     |      | 100%     |      | 100%     |      |
| HeleOBP3 | 45%      | 100% | 23%      | 100% |
| HeleOBP4 | 22%      | 100% | 100%     | 100% |
| HeleOBP6 | 50%      | 23%  | 23%      | 100% |

| Similarity | HeleOBP1 | 100% | HeleOBP3 | 71% | HeleOBP4 | 42% | HeleOBP6 | 71% |
|------------|----------|------|----------|------|----------|------|----------|------|
| HeleOBP1   | 100%     |      | 100%     |      | 100%     |      |
| HeleOBP3   | 71%      | 100% | 40%      | 100% |
| HeleOBP4   | 42%      | 100% | 39%      | 100% |
| HeleOBP6   | 71%      | 45%  | 39%      | 100% |

Fig. 3. (A) Alignment of different HeleOBPs made with T-Coffee. Conserved Cys residues in red color. Amino acids participating in the binding pocket in green color. (B) Identity and similarity percentages of HeleOBPs obtained with BLASTp.
Fig. 4. Folded OBP models. Images in the left side show models with ligand inside the pockets and highlighted S-S bridges (red sticks). Cysteine residues and \( \alpha \)-helices formed are labeled. Images in the right show superimposed template (gray) on their respective model.
Fig. 5. RMSD calculated for OBP backbones (A) and for the ligands (B) used in the MD procedures.

Table 4. Decomposition of energies calculated by MM/GBSA to the interactions between different H. elegans OBPs and the tested ligands

| Protein  | Ligand    | $\Delta H_{\text{MM,vdW}}$ | $\Delta H_{\text{MM,elec}}$ | $\Delta G_{\text{Solv}}$ | $\Delta G_{\text{bind}}$ |
|----------|-----------|-----------------------------|-----------------------------|--------------------------|--------------------------|
| HeleOBP1 | alpha-gurjunene | −28.04                      | 9.30                        | −4.37                    | −23.11 (±0.04)           |
|          | aromadendrene  | −25.95                      | 8.79                        | −3.97                    | −21.13 (±0.04)           |
| HeleOBP3 | alpha-copaene  | −32.68                      | 10.96                       | −1.63                    | −26.03 (±0.06)           |
|          | beta-copaene   | −33.63                      | 10.50                       | −4.29                    | −27.43 (±0.05)           |
| HeleOBP4 | alpha-copaene  | −27.25                      | 7.81                        | −4.28                    | −23.72 (±0.06)           |
|          | beta-copaene   | −25.85                      | 8.08                        | −4.24                    | −22.01 (±0.09)           |
| HeleOBP6 | alpha-copaene  | −29.86                      | 7.53                        | −4.41                    | −26.75 (±0.04)           |
|          | beta-copaene   | −29.77                      | 6.76                        | −4.40                    | −27.41 (±0.03)           |

$\Delta H_{\text{MM,vdW}}, \Delta H_{\text{MM,elec}}, \Delta G_{\text{Solv}}, \text{and } \Delta G_{\text{bind}}$ represent energy attributable to van der Waals interactions, electrostatic interactions, solvation, and free binding energy respectively.
Fig. 6. Frequency of the appearance of residues at a distance of 3 Å or closer from a ligand for *H. elegans* OBPs calculated using MD procedures.
Fig. 7. Ligand interaction diagrams generated by Maestro showing residues interacting in the OBP pocket. Green color represents hydrophobic residues, blue is positively charged residues, red is negatively charged, and cyan denotes polar. The gray atom background represents the solvent-accessible surface area (SASA) of that atom. (A) HeleOBP1/alpha-gurjunene; (B) HeleOBP1/aromadendrene; (C) HeleOBP3/alpha-copaene; (D) HeleOBP3/beta-copaene; (E) HeleOBP4/ alpha-copaene; (F) HeleOBP4/ beta-copaene; (G) HeleOBP6/ alpha-copaene; and (H) HeleOBP6/ beta-copaene.
MD and MM/GBSA
Temporal root mean square deviation (RMSD) calculations were performed on all the atoms of each complex (protein and ligand) during the 5 ns of simulation. For the same OBP, the average RMSD values calculated for the protein backbones (Fig. 5A) did not show important differences. Comparing the ligand RMSD values (Fig. 5B), HeleOBP1 showed the largest difference, with 0.486 Å for aromadendrene and 0.214 Å for alpha-gurjunene; compared to alpha- and beta-copaene to HeleOBP3, HeleOBP4, and HeleOBP6 which showed lower differences. The temporal RMSD results suggest that the compounds were well accommodated inside the binding sites during the MD simulations, demonstrating the stabilization of the systems and confirming the results obtained by docking (Table 3) and free energy of binding via MM/GBSA (Table 4).

During the MD simulations, the frequency of the closer residues was calculated using a 3 Å cutoff (Fig. 6). The three most frequent residues in HeleOBP1 were TYR51, HIS117, and LEU116 for alpha-gurjunene and ILE72, PHE56, and ARG7 for aromadendrene. With regard to HeleOBP3, the most frequent amino acids were LEU102, ILE63, and LYS70 for alpha-copaene and ILE63, LYS108, and LEU 102 for beta-copaene. In the case of HeleOBP4, ARG13, LEU55, and LEU51 were most frequently close to alpha-copaene and ARG13, LEU55, and MET9 to beta-copaene. Lastly, HeleOBP6 LEU78, LYS38, and PHE117 were the amino acids most frequently close to alpha-copaene and LEU10, PHE54, and LYS38 to beta-copaene.

MM/GBSA analyses for calculating the binding free energy to the selected ligands (Table 4) showed that every couple of ligands tested to each OBP had similar energy. The largest difference was obtained with HeleOBP1, where $\Delta_{GBSA}$ for alpha-gurjunene reached $-23.11$ kcal/mol compared to $-21.13$ kcal/mol for aromadendrene. Moreover, it is important to note the significant contribution of $\Delta H_{MM^{\mathrm{elec}}}$ to the binding free energy when compared to the null influence of $\Delta H_{MM^{\mathrm{ew}}}$ on all of the complexes studied.

Discussion
The method used in this study allowed the prediction of four novel OBP sequence in H. elegans. This is an important results when considering the lack of genomic information for scarab beetles species (Chen et al. 2014). Moreover, RT-PCR analyses showed that the proteins are more expressed in organs related to the olfactory and gustatory senses, in both males and females (Fig. 1), supporting their chemosensitive role. HeleOBP1 and HeleOBP3 appear to be much more expressed in antenna than other tissues and HeleOBP3 appear to be remarkably expressed in female antenna respect to male antenna. In contrast, HeleOBP4 and HeleOBP6 appear to be more expressed in mouth parts than antenna or hindleg tibia, but HeleOBP4 show higher expression in male antenna also.

These differences may represent a significative clue about the importance of those proteins, due to the similar habits of adults congregating and feeding on leaves of their hosts, HeleOBP3 could be related to the perception of semiochemicals informative of suitable oviposition sites. Host-seeking and egg-laying task have been suggested for similar female-biased pattern of expression of chemosensitive proteins in A. gambiae (Diptera: Culicidae) (Latrou and Biessmann 2008) and Apolobus lucorum (Meyer-Dur) (Hemiptera: Miridae) (Yuan et al. 2015). Male-biased expression of HeleOBP4 is interesting. Some studies show that certain OBPs have the ability to bind pheromones as well as pheromone-binding proteins do (Liu et al. 2010, Jin et al. 2015). The tissue-biased expression of OBPs has been reported (Pelletier and Leal 2011), and it may be related to the multitasking role played by tissues different than antenna in olfaction (Choo et al. 2015). Although further studies are required, these evidences provide a starting point for using OBPs as targets to regulate the insect behavior of mating, feeding, and oviposition and further to develop novel crop protection strategies (Yuan et al. 2015).

It is important to note that modeling was challenging in this case, in part due to the highly divergent nature of OBPs as well as the scarcity of beetle protein template structures available for model building. However, the analyses performed using PROCHECK showed similar values to those previously reported for other binding proteins in insects (Tomaselli et al. 2006, Leite et al. 2009, Pesenti et al. 2009). Of note, multiple sequence alignment between OBPs and templates (Fig. 2) showed several conserved residues, highlighting six highly conserved cysteines due to their roles in the structural stability of insect OBPs as globular proteins (Leal et al. 1999).

Assessing a group of volatiles previously reported as well as novel compounds first reported herein, docking analyses revealed interaction energies between the compounds and the four OBPs. It is important to note that the highest interaction energy values were obtained for sesquiterpenic compounds compared to the remaining molecules for all the OBPs analyzed.

Interestingly, in the molecular docking, three of the four OBPs studied exhibited the best interaction energy values with the same ligands, and all of them showed the highest interaction energy with compounds that share similar physicochemical properties (sesquiterpenes), despite their marked divergence at the amino acid level. The identity among the different H. elegans OBPs’ ranged from 22 to 50% (Fig. 3), lower than the identity of 57–87% when comparing the amino acidic sequences to the OBPs of other scarab beetle species (data not shown). The affinity for sesquiterpenes may rely in the fact that products from genes that are members of the same family may be able to bind compounds with similar physicochemical properties (Hopkins and Groom 2002). In the other hand, it has been suggested that the binding site is more important to the process of ligand recognition than the entire polypeptide sequence (Zhuang et al. 2014).

Considering that at the moment of performing the analyses, 1,4 benzoquinone and 1,4 hydroquinone were the only compounds reported as responsible to eliciting behavioral response in H. elegans (Quiroz et al. 2007), they were included in the docking analyses. These compounds did not show high interaction energy, in the same manner that pheromones of H. oblitata did not have good binding affinities when they were tested with HoblOBPs (Deng et al. 2012, Wang et al. 2013a,b). However, is feasible that further studies could reveal the existence of new OBPs in H. elegans, especially considering the number of OBPs reported in transcriptome of different scarab beetle species is higher than the number of HeleOBPs reported here (Li et al. 2015).

Molecular docking techniques are used to explore translations, orientations, and conformations until an ideal site is found (Morris et al. 1998) and may help delineate the amino acid residues that form cavities (Venturin et al. 2014). By utilizing more detailed molecular mechanics, it is then possible to calculate the energy of the ligand within the context of the putative cavity (Morris et al. 1998).

Some studies have used docking to predict the residues of OBPs that form the binding site (Zhuang et al. 2014), to predict their interactions with putative ligands (Jiang et al. 2009), and to relate them to their biological activity (He et al. 2010) in the design of new insect repellents (Affonso et al. 2013) and olfactory biosensors (Lu et al. 2014). However, molecular docking has some limitations and
is not completely reliable for establishing ligand affinity (Okimoto et al. 2009, Hayes and Archontis 2011), and complementary analyses are required for improved accuracy (Jayanthi et al. 2014). Thus, MD simulation-based free energy calculations have been used extensively to predict the strength of protein–ligand interactions (Wang et al. 2013a,b). Indeed, these methods provide more accurate binding affinity, though the cost and time required for computational calculations are greater compared to molecular docking (Okimoto et al. 2009). Furthermore, methods for calculating binding free energy offer additional accuracy. One such method is MM/GBSA, which is considered a computationally efficient method (Hou et al. 2011). It provides moderately accurate results at a comparatively low computational cost (Vergara-Jaque et al. 2013) and offers an alternative to rigorous free-energy methods (Wang et al. 2013a,b).

Based on the frequency with which amino acids were 3 Å or closer during the molecular simulation (Fig. 6), despite differences in OBP sequences, residues with hydrophobic properties appear more frequently (Fig. 7). Because of their nature, it is expected that van der Waals and hydrophobic interactions will occur in the same manner as that reported for the OBP-binding site of the scarab beetle H. oblitata, whereby amino acids such as methionine, tyrosine and isoleucine were predicted as important in the binding site (Zhuang et al. 2014). Altogether, MD simulations are useful for establishing compounds are stabilized in binding pockets (Affonso et al. 2013). Thus, physicochemical properties of amino acids in the binding pockets of HeleOBPs determined in MD simulations appear to be complementary to the physicochemical properties of the ligands that showed the highest interaction energies among the HeleOBPs in molecular docking.

Our study did not show a contribution of $\Delta H_{\text{MM,elec}}$, indicating that no electrostatic interactions can be formed, presumably because the ligands tested (i.e., sesquiterpenes) do not have high-electro negativity atoms in their structures or there is an absence of functional groups, such as aldehydes, alcohols, and ketones. Accordingly, it was expected that van der Waals forces would have the highest contribution to the interaction between OBPs and sesquiterpenes. The partition of energy obtained from MM/GBSA analyses supports this claim. The binding free energies calculated for the compounds in this report are higher than those used previously to predict behavioral activity (Jayanthi et al. 2014).

Conclusions

This work reported four novel OBPs in the scarab beetle H. elegans. All of them appear to be much more expressed in chemosensitive tissues and some of them show a sex-biased expression which provides a starting point for future researches looking for ways to disturb the behavior of this pest species. In parallel from the identification of volatiles in the foliage of the preferred host of adult stage, H. oblitata was reported six new volatiles. Different in silico analyses were performed to study the interaction of HeleOBPs with the volatiles. Molecular docking showed better interaction energy values with sesquiterpenes found in foliage volatiles, and MD reported that hydrophobic amino acids would take part of these relationships. Free-binding energy estimations made by MM/GBSA reinforce the idea that physicochemical properties of binding pocket in HeleOBPs are prepared to interact with compounds like sesquiterpenes despite of the shape and size of binding cavities may be different among these proteins, conferring a certain specificity for this type of compounds. Therefore, these compounds and others that are chemically related could represent interesting candidates for further studies, and in vitro testing (i.e., fluorescence binding assays) should elucidate the properties of these novel OBPs.

Acknowledgments

This work was supported by Fondecyt Postdoctoral Project 3130464 (to R.P.-M.), Fondecyt Regular Project 11311008 (to C.C.R.), R.P.-M. thanks to Facultad de Ciencias Agropecuarias y Forestales from Universidad de La Frontera for permission to collect samples at Maquehue Experimental Station and Rucamanque Field. We thank to the anonymous reviewers, whose comments and critics were very useful to improve this manuscript.

References Cited

Abroshan, H., H. Akharzadeh, and G. A. Parsafar. 2010. Molecular dynamics simulation and MM-PBSA calculations of sickle cell hemoglobin in dimer form with Val, Trp, or Phe at the lateral contact. J. Phys. Organ. Chem. 23: 866–877.

Affonso, R. da S., A. P. Guimarães, A. A. Oliveira, G. B. Slana, and T.C.C. França. 2013. Applications of molecular modeling in the design of new insect repellents targeting the odorant binding protein of Anopheles gambiae. J. Braz. Chem. Soc. 24: 473–482.

Aguilera, A., E. Cisternas, M. Gerdung, and H. Norambuena. 1996. Plagas de las praderas, pp. 309–339. In L. Ruiz (ed.), Praderas para Chile. Instituto de Investigaciones Agropecuarias, Ministerio de Agricultura. Santiago de Chile.

Aguilera, A., J. Guerrero, and R. Rebolloso. 2011. Plagas y enfermedades del avellano europeo en la Araucanía. Ediciones Universidad de La Frontera, Temuco.

Achtschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. 215: 403–410.

Biessmann, H., E. Andronopoulou, M. R. Biessmann, V. Douris, S. D. Dimitratos, E. Eliopoulos, P. M. Guerin, K. Iatrou, R. W. Justice, T. Kröber, et al. 2010. The Anopheles gambiae odorant binding protein 1 (AgamOBP1) mediates odor preference in the antennae of female mosquitoes. PLoS One 5: e9471.

Chen, H., L. Lin, M. Xie, G. Zhang, and W. Su. 2014. De novo sequencing, assembly and characterization of antennal transcriptome of Anomala corpulenta Motschulsky (Coleoptera: Rutelidae). PLoS One 9: e114238.

Choo, Y.-M., G. K. Buss, K. Tan, and W. S. Leal. 2015. Multitasking roles of mosquito labrum in oviposition and blood feeding. Frontiers in Physiology 6: 306.

Cisternas, E. 1992. Biología y control de insectos plagas en praderas, pp. 87–117. In Seminario Manejo de Praderas Permanentes. Serie Remehue N° 31, Instituto de Investigaciones Agropecuarias, Estacion Experimental Remehue, Osorno de Chile.

Davrazou, F., E. Dong, E. J. Murphy, H. T. Johnson, and D.N.M. Jones. 2011. New insights into the mechanism of odorant detection by the malaria-transmitting mosquito Anopheles gambiae. J. Biol. Chem. 286: 34175–34183.

Deng, S., J. Yin, T. Zhong, Y. Cao, and K. Li. 2012. Function and immuno-chemochemical localization of two novel odorant-binding proteins in olfactory sensilla of the scarab beetle Holotricha oblitata Faldernmann (Coleoptera: Scarabaeidae). Chem. Senses 37: 141–150.

Durán, L. 1990. Aspeossológicas de la biología del sanjuan verde, Hyalomorpha elegans (Burm.) y mención de las demás especies de escarabeídos perjudiciales en Cauitin. Agric. Téc. 12:24–36.

Durán, L. 1954. La biología del Phytoloma biermanni Germ. y mención de otros escarabeídos perjudiciales a la agricultura en las provincias australes de Chile. Rev. Chil. Hist. Nat. 54: 5–20.

Gillott, C. 2005. Entomology, 3rd ed. Springer, Dordrecht, The Netherlands.

Gu, S.-H., K.-M. Wu, Y.-Y. Guo, L. M. Field, J. A. Pickett, Y.-J. Zhang, and J.-J. Zhou. 2013. Identification and expression profiling of odorant binding proteins and chemosensory proteins between two wingless morphs and a winged morph of the cotton aphid Aphis gossypii Glover. PLoS One 8: e73524.
Hayes, J. M., and G. Archontis. 2011. MM-GB/GB/SA calculations of protein-ligand binding free energies, pp. 171–190. In Wang, L. (ed.), Journal of the Royal Society Interface. InTech. Vienna, Austria.

He, X., G. Tzotzos, C. Woodcock, J. A. Pickett, T. Hooper, L. M. Field, and J. J. Zhou. 2010. Binding of the general odorant binding protein of Bombyx mori BmORGFP2 to the moth sex pheromone components. J. Chem. Ecol. 36: 1293–1305.

Hopkins, A. L., and C. R. Groom. 2002. The druggable genome. Nat. Rev. Drug Discov. 1: 727–30.

Hou, T., J. Wang, Y. Li, and W. Wang. 2011. Assessing the performance of the MM/PBSA and MM/GBSA methods. 1. The accuracy of binding free energy calculations based on molecular dynamics simulations. J. Chem. Inf. Mod. 51: 69–82.

Humphrey, W., A. Dalke, and K. Schulten. 1996. Visual molecular dynamics. J. Mol. Graph. 14: 33–38.

Jackson, T. A., and M. G. Klein. 2006. Scarabs as pests: a continuing problem. Coleops. Bull. 60: 102–119.

Jayanthi, K., V. Kempraj, R. M. Aurade, T. Kumar-Roy, K.S. Shivashankara, T. A. Jackson, T. A., and M. G. Klein. 2006. Odorant reception in insects: roles of receptors, binding proteins, and development. J. Mol. Graph. Model 17: 57–61.

Leal, W. S., L. Nikonova, and G. Peng. 2001. Transcriptome and tissue-specific expression analysis of Obp and Csp genes in adult antennae of Anomala corpulenta Motschulsky (Coleoptera: Scarabaeidae: Rutelinae). PLoS One 10: e0121504.

Leal, W. S., and G. Peng. 2001. Odorant-binding proteins: structural aspects. Ann. N. Y. Acad. Sci. 855: 281–293.

Lee, S. W., J.-Y. Han, S. J. Kean, and G. Peng. 2011. Odorant-binding proteins: structural aspects. Ann. N. Y. Acad. Sci. 855: 281–293.

Leone, N., R. Krogh, W. Xu, Y. Ishida, J. Iulek, W. S., Leal, and G. Oliva. 2009. Structure of an odorant-binding protein from the mosquito Aedes aegypti suggests a binding pocket covered by a ph-sensitive “lid.” PLoS One 4: e8006.

Li, X., Q. Ju, W. Jie, F. Li, X. Jiang, J. Hu, and M. Qu. 2015. Chemosensory gene families in adult antennae of Anomala corpulenta Motschulsky (Coleoptera: Scarabaeidae). PLoS One 10: e0121504.

Liu, Z., Z. Vidal, Z. Syed, Y. Ishida, and W., Leal. 2010. Pheromone binding to general odorant-binding proteins from the navel orangeworm. J. Chem. Ecol. 36: 787–794.

Lu, Y., H. Li, S. Zhuang, D. Zhang, Q. Zhang, J. Zhou, S. Dong, Q. Liu, and P. Wang. 2014. Olfactory biosensor using odorant-binding proteins from honeybee: Ligands of floral odors and pheromones detection by electro-chemical impedance. Sens. Actuators B Chem. 193: 420–427.

Mackrell, A. D., Jr., D. Bashford, M. Bellott, R. L. Dunbrack, J. D. Evanseck, M. Field, S. Fischer, J. Gao, H. Guo, S. Ha, et al. 1998. All-atom empirical potential for molecular modeling and dynamics studies of proteins. J. Phys. Chem. B 102: 358–3616.

Morriss, G. M., D. S. Goodsell, R. S. Halliday, R. Huey, W. E. Hart, R. K. Belew, and A. J. Olson. 1998. Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function. J. Comput. Chem. 19: 1639–1662.

Neria, E., S. Fischer, and M. Karplus. 1996. Simulation of activation free energies in molecular systems. J. Chem. Phys. 105: 1902.

Okimoto, N., N. Futatsugi, H. Fuji, A. Suwaga, G. Morimoto, R. Yanai, Y. Ohno, T. Narumi, and M. Taji. 2009. High-performance drug discovery: computational screening by combining docking and molecular dynamics simulations. PLoS Comput. Biol. 5: e1000528.

Palma, R., A. Mutis, L. Manosalva, R. Ceballos, and A. Quiroz. 2012. Behavioral and electrophysiological responses of Hyla tamincola olivacea to volatiles released from the roots of Trifolium pratense L. J. Soil Sci. Plant Nutr. 12: 183–193.

Pedro, L. P., and M. E. Rice. 2009. Entomology and pest management. PHI Learning Private Limited, New Delhi.

Pelosi, P. 1998. Odorant-binding proteins: structural aspects. Ann. N. Y. Acad. Sci. 855: 281–293.

Peng, G., and W. S. Leal. 2001. Identification and cloning of a pheromone-binding protein from the Oriental beetle, Exomala orientalis. J. Chem. Ecol. 27: 2183–2192.

Pelletier, J., and W. S. Leal. 2011. Characterization of olfactory genes in the antennae of the Shouthern house mosquito, Culex quinquefasciatus. J. Insect Physiol. 57: 915–929.

Pesenti, M. E., S. Spinelli, V. Bezzirard, L. Briand, J.-C. Pernollet, V. Campanacci, M. Tegoni, and C. Cambillau. 2009. Queen bee pheromone binding protein pH-induced domain swapping favors pheromone release. J. Mol. Biol. 390: 981–990.

Phillips, J. C., R. Braun, W. Wang, J. Gumbart, E. Tajkhorshid, E. Villa, C. Chipot, R. D. Skeel, L. Kale, and K. Schulten. 2005. Scalable molecular dynamics with NAMD. J. Comput. Chem. 26: 1781–1802.

Quiroz, A., E. Fuentes-Contreras, C. C. Ramirez, G. B. Russell, and H. M. Niemeyer. 1999. Host-plant chemicals and distribution of Neuenquenaphis on Nothobagrus. J. Chem. Ecol. 25: 1043–1054.

Quirós, A., R. Palma, P. Etcheverría, V. Navarro, and R. Rebollo. 2007. Males of Hylemorphe elegans Brunner (Coleoptera: Scarabaeidae) are attracted to odors released from conspecific females. Environ. Entomol. 36: 272–280.

Ratcliffe, B. C., and D. F. Ocampo. 2002. A review of the genus Hylamorpha (Coleoptera: Scarabaeidae: Rutelinae). J. Integrative Agriculture. 14: 1356–1366.

Rodrı´guez, M., A. France, and M. Gerding. 2004. Evaluation de dos cepas del hongo Metarhizium anisopliae var. Anisopliae (Metls.) para el control de larvas de gusano blanco Hylemorphe elegans Burm. (Coleoptera: Scarabaeidae). Agríc. Téc. 64: 17–24.

Sali, A., and T. L. Blundell. 1993. Comparative protein modelling by satisfaction of spatial restraints. J. Mol. Biol. 234: 779–815.

Sanner, M. F. 1999. Python: a programming language for software integration and development. J. Mol. Graph Model 17: 57–61.
Shen, M.-Y., and A. Sali. 2006. Statistical potential for assessment and prediction of protein structures. Protein Sci. 15: 2507–2524.

Simkin, A., B. Underwood, M. Auldridge, K. Shibuya, E. Schmelz, D. Clark, and H. Klee. 2004. Circadian regulation of the PhCCD1 carotenoid cleavage dioxygenase controls emission of β-ionone, a fragrance volatile of petunia flowers. Plant Physiol. 136: 3504–3514.

Stajich, J. E., D. Block, K. Boulez, S. A. Brenner, S. Chervitz, C. Dagdigian, G. Fuellen, J.G.R. Gilbert, I. Korf, H. Lapp, et al. 2002. The Bioperl toolkit: Perl modules for the life sciences. Genome Res. 12: 1611–1618.

Tomaselli, S., O. Crescenzi, and D. Sanfelice. 2006. Solution structure of a chemosensory protein from the desert locust Schistocerca gregaria. Biochemistry 45: 10606–10613.

Trott, O., and A. J. Olson. 2009. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. J. Comp. Chem. 31: 455–461.

Venthur, H., A. Mutis, J.-J. Zhou, and A. Quiroz. 2014. Ligand binding and homology modelling of insect odorant-binding proteins. Physiol. Entomol. 39: 183–198.

Venthur, H., J.-J. Zhou, A. Mutis, R. Mella-Herrera, G. Larama, A. Avila, P. Iturriaga-Vásquez, M. Faundez-Parraguez, M. Alvear, and A. Quiroz. 2016. β-Ionone as putative semiochemical suggested by ligand binding on the odorant-binding protein 1 of Hylamorpha elegans (Burmeister) and electroantennographic recordings. Entomol. Sci. (in press).

Vergara-Jaque, A., J. Comer, L. Monsalve, F. D. González-Nilo, and C. Sandoval. 2013. Computationally efficient methodology for atomic-level characterization of dendrimer-drug complexes: a comparison of amine- and acetyl-terminated PAMAM. J. Phys. Chem. B 117: 6801–6813.

Vieira, F. G., A. Sánchez-Gracia, and J. Rozas. 2007. Comparative genomic analysis of the odorant-binding protein family in 12 Drosophila genomes: purifying selection and birth-and-death evolution. Genome Biol. 8:R235.

Wang, B., L. Guan, T. Zhong, K. Li, J. Yin, and Y. Cao. 2013. Potential co-operations between odorant-binding proteins of the scarab beetle Holotrichia oblita Faldermann (Coleoptera: Scarabaeidae). PLoS One 8: e84795.

Wang, B., L. Li, T. D. Hurley, and S. O. Meroueh. 2013. Molecular recognition in a diverse set of protein-ligand interactions studied with molecular dynamics simulations and end-point free energy calculations. J. Chem. Inf. Mod. 53: 2659–2670.

Wogulis, M., T. Morgan, Y. Ishida, W. S. Leal, and D. K. Wilson. 2006. The crystal structure of an odorant binding protein from Anopheles gambiae: evidence for a common ligand release mechanism. Biochem. Biophys. Res. Commun. 339: 157–164.

Wojtasek, H., B. S. Hansson, and W.S. Leal. 1998. Attracted or repelled?—A matter of two neurons, one pheromone binding protein, and a chiral center. Biochem. Biophys. Res. Commun. 250: 217–222. doi:10.1006/bbrc.1998.9278.

Yuan, H.-B., Y.-X. Ding, S.-H. Gu, L. Sun, X.-Q. Zhu, H.-W. Liu, K. H. Dhuloo, Y.-J. Zhang, and Y.-Y. Guo. 2015. Molecular characterization and expression profiling of odorant-binding proteins in Apolygus lucorum. PLoS One 10: e0140562.

Zhuang, X., Q. Wang, B. Wang, T. Zhong, Y. Cao, K. Li, and J. Yin. 2014. Prediction of the key binding site of odorant-binding protein of Holotrichia oblita Faldermann (Coleoptera: Scarabaeidae). Insect Mol. Biol. 23: 381–390.