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Chemosensory proteins as putative semiochemical carriers in the desert isopod *Hemilepistus reaumurii*

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ABSTRACT

The order Isopoda contains both aquatic and terrestrial species, among which *Hemilepistus reaumurii*, which lives in arid environments and is the most adapted to terrestrial life. Olfaction has been deeply investigated in insects while it has received very limited attention in other arthropods, particularly in terrestrial crustaceans. In insects, soluble proteins belonging to two main families, Odorant Binding Proteins (OBPs) and Chemosensory Proteins (CSPs), are contained in the olfactory sensillar lymph and are suggested to act as carriers of hydrophobic semiochemicals to or from membrane-bound olfactory receptors. Other protein families, namely Nieman-Pick type 2 (NPC2) and Lipocalins (LCNs) have been also reported as putative odorant carriers in insects and other arthropod clades. In this study, we have sequenced and analysed the transcriptomes of antennae and of the first pair of legs of *H. reaumurii* focusing on soluble olfactory proteins. Interestingly, we have found 13 genes encoding CSPs, whose sequences differ from those of the other arthropod clades, including non-isopod crustaceans, for the presence of two additional cysteine residues, besides the four conserved ones. Binding assays on two of these proteins showed strong affinities for fatty acids and long-chain unsaturated esters and aldehydes, putative semiochemicals for this species.

1. Introduction

Olfaction has received limited attention in Pancrustacea outside insects, including isopods, which entail species living in very diverse habitats, ranging from the sea to the desert, and showing different degrees of terrestrialization. Knowledge about chemoreception in isopods is very scanty, while more information is available for decapods. In this latter taxon, the organs named aesthetascs located on the lateral flagellum of the first pair of antennae are considered the main olfactory organ, while the chemo/mechanosensory sensilla located on the legs mediate "distributed chemoreception" (Derby et al., 2016). In terrestrial isopods, the first pair of antennae show a drastic reduction when compared to other malacostracans and also to aquatic isopods, while the second pair are considered the main cephalic sensory organs. Although these appendages bear mainly mechanosensory sensilla, the terminal segments present a structure named "apical organ" (Hoese, 1981), which is thought to detect both mechanical and chemical stimuli (Schmalfuss, 1998). Nothing is known for isopods about the role of legs in chemoreception.

Hemilepistus reaumurii (Milne-Edwards, 1840) lives in and around the deserts of North Africa and the Middle East and is regarded as the crustacean species (considered in the traditional meaning) best adapted to terrestrial life (Wägele, 1989). Adaptation to arid environments is also based on behavioural traits, among which is parental caring. Monogamous reproductive couples rear their offspring within deep burrows, where temperatures are moderate and air humidity relatively high, and discriminate, probably based on chemical cues, family members from alien individuals (Ayari et al., 2016; Linsenmair, 1984, 1985). Information about potential semiochemicals in *H. reaumurii* is very poor and limited to a single paper (Schildknecht et al., 1988) reporting the presence of some fatty acids and their esters in the exuvial washes.

Several families of soluble semiochemical-binding proteins are

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adopted by arthropods to ferry pheromones and odorants to olfactory receptors (Pelosi et al., 2014, 2018). In particular, OBPs (Odorant-binding proteins), with a conserved 6-cysteine motif and a typical folding, are found only in Hexapoda (Pelosi et al., 2018; Vogt and Riddiford, 1981); however, proteins with some structural similarity to insect OBPs (and therefore first named OBP-like) have been discovered in Chelicerata (Amigues et al., 2021; Eliash et al., 2017, 2019; Iovinella et al., 2018; Renthal et al., 2017; Vizueta et al., 2017; Zhu et al., 2021a) and Myriapoda (Vizueta et al., 2018). A further family of candidate carrier proteins (CCPs) has been reported for spiders (Vizueta et al., 2017). NPC2 (Niemann-Pick C2) proteins, instead, have been reported in all phyla and subphyla of panarthropoda (Iovinella et al., 2018; Pelosi et al., 2014), with a role in chemosensing supported by experimental evidence (Mani et al., 2022; Nganso et al., 2021; Zheng et al., 2018; Zhu et al., 2018). Proteins belonging to the lipocalin superfamily, which includes the OBPs of vertebrates (Pelosi et al., 1982; Pelosi and Knoll, 2022), are also ubiquitous among arthropods, and have been suggested to act as semiochemical carriers (Zhu et al., 2021b). Finally, CSPs (chemosensory proteins), another family of small soluble proteins endowed with several roles, including semiochemical transport, are expressed in Pancrustacea, but not in Chelicerata (Angeli et al., 1999; Pelosi et al., 2018).

In insect species a highly variable number of CSPs (from 4 to 70) have been discovered (Zhou et al., 2013) and shown to be involved in different functions such as embryonic maturation for the honeybee CSP5 (Maleszka et al., 2007), limb regeneration for protein p10 in cockroaches (Kitabayashi et al., 1998; Nomura et al., 1992), cuticle development for the fire ant CSP9 (Cheng et al., 2015) and phase shift in locust for CSP1 (Guo et al., 2011), but also active in chemical communication as specific carriers of semiochemicals (Pelosi et al., 2018). In Pancrustacea outside insects, until recently only one or two CSPs had been reported accompanied by a couple of isoforms. However, thanks to recent genomic and transcriptomic projects, relatively large numbers of CSP-encoding genes, in the order of a dozen, can be found at least in some species of Malacostraca, suggesting that the scanty information available for other species might be the result of limited annotation.

In particular, the genomes of the terrestrial Isopoda *Armadillidium vulgare* and *A. nasatum* are endowed with 12 and 7 sequences, respectively, suggesting a potential role of these proteins in semiochemical transport, although information about their expression in chemosensory organs is currently missing. The genome of another terrestrial isopod *Trachelipus rathkii* and that of the aquatic isopod *Ligia exotica* are available, but their genes have not yet been annotated.

No information is available for Ionotropic Receptors in isopods. These membrane proteins, organised in complexes of conserved co-receptors and tuning IRs and derived from Ionotropic Glutamate Receptors (Benton et al., 2009), are present in all protostomes. In insects, antennal IRs with olfactory functions as well as gustatory IRs have been reported (Croset et al., 2010). Most Crustacean (s.l.) IRs have been found to maintain glutamate binding domains similar to coreceptor IR25a (Corey et al., 2013), although a high diversity has been observed in the crustacean lineages so far investigated (Derby et al., 2016; Krieger et al., 2021). Unlike winged insects where a further type of olfactory receptors (ORs) expressed in the antennal olfactory neurons have evolved, other classes of olfactory receptors have not been identified in Pancrustacea outside insects; therefore IRs are considered their basic olfactory receptors (Derby et al., 2016; Harzsch and Krieger, 2018; Krieger et al., 2021).

In this work we have identified 13 genes encoding CSPs as a result of a transcriptomic project on the antennae and legs of the terrestrial isopod *H. reaumurii* (Milne-Edwards, 1840), we have expressed two of them in a bacterial system and found that they are tuned to fatty acids and long-chain aldehydes and esters, potential semiochemicals for this species.

Moreover, we also searched *H. reaumurii* transcripts for Ionotropic Receptor (IR) sequences.

2. Material and methods

2.1. Chemicals

Chemicals for ligand-binding experiments were of analytical grade, except for methanol used to dilute odorants, which was of spectroscopic grade (Uvasol) and purchased from Merck KGaA, Darmstadt, Germany. Acrylamide was from Bio-Rad, Vienna, Austria. Long-chain unsaturated aldehydes and alcohols were from Bedoukian (Danbury, CT, USA). Oligonucleotides and genes were custom synthesized at Eurofins Genomics (Ebersberg, Germany). All enzymes and kits for DNA purification were from New England Biolabs, Germany.

2.2. Specimen collection

Eight couples of *H. reaumurii* individuals (8 male and 8 females) were collected from their burrows in June 2019 in the area of Bchachma (35°49′N-10°10′E) near Kairouan (Tunisia), at an altitude of about 23 m. This period corresponds to the first phase of the reproductive season. Samples were flash-frozen in liquid nitrogen, carried frozen to the Department of Biology, University of Firenze, Italy, and stored at −80 °C.

2.3. RNA isolation and sequencing

Both pairs of antennae and the first pair of legs were dissected from six females and six males and pooled according to sex, for a total of 4 pools (Female Antennae: FAnt, Male Antennae: MAnt, Females legs: FLeg, Male Legs: MLeg). RNA was isolated from each pool using TRI Reagent (Sigma), following the manufacturer's protocol. RNA purity and concentration were measured on an Infinite PRO 200 reader (TECAN) by calculating the OD 260/280 ratio, and on a Qubit nanodrop, following the instruction of the Qubit® RNA HS Assay Kits. RNA integrity was evaluated using an Agilent bioanalyzer with an RNA 6000 Nano Kit. RNA samples were sequenced at Novogene Biotechnology Company (NOVOGENE, China). Libraries were generated using NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB, Ispawich, USA) according to the manufacturer's recommendations. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads (Life Technologies, CA, USA). Libraries were enriched using NEB Universal PCR Primer and Index primer in a 10 cycles PCR reaction. The library was 2 × 150 paired-end sequenced on an Illumina HiSeq 2000 platform.

2.4. Transcriptome Assembly and Differential Expression Analysis

De novo transcriptome assembly was performed using Trinity (Grabherr et al., 2011) with default parameters. Clustering of transcripts into unigenes was performed using Corset (Davidson and Oshlack, 2014).

Analysis of the GO annotation of the assembled unigenes was accomplished using the Blast2GO version 2.5 program (Conesa et al., 2005). The completeness of the transcriptome was assessed with the software BUSCO (Seppey et al., 2019) implemented in the platform Galaxy (Afgan et al., 2018), using the Arthropoda dataset lineage (arthropoda_odb10). GO functional classifications of the unigenes were performed using the WEGO version 1.0 software (Ye et al., 2006). Annotation of the unigenes was based on data from the NCBI non-redundant protein sequences (Nr) database, and NCBI non-redundant nucleotide sequences (Nt) database, Clusters of Orthologous Group of proteins (KOG/COG) database, KEGG ortholog (KO) database, a manually annotated and reviewed protein sequence (SwissProt protein) database, Gene Ontology (GO) database, and protein family (Pfam) database. The COGs protein database phylogenetically classifies the complete complement of proteins encoded in a genome. Each COG is a group of three or more proteins that are inferred to be

orthologs. All the unigenes were submitted to the KEGG pathway database (Kyoto Encyclopedia of Genes and Genomes). The FPKM (Fragments per kb per million fragments), calculated using a conventional method (RNA-seq reads mapped to the genome), was used as a proxy of unigene expression. Differential expression analysis of antennae (FAnt, MAnt) and legs (FLeg, MLeg) samples was performed using the DESeq2 R package (version 1.16.1; Bioconductor), considering male and female samples as replicates. The resulting P values were adjusted using Benjamini and Hochberg's approach for controlling the false discovery rate. Unigenes with an adjusted P value < 0.05 and $\log_2$ (fold change) > 1 or < -1 were considered differentially expressed.

2.5. Identification of olfactory genes

The assembled transcriptome was searched for putative olfactory soluble proteins belonging to the families of CSPs, NPC2, OBP-like, lipocalins and CCPs. Sequences (NCBI protein database) of *Armadillidium vulgare* were used as queries for CSPs; *Daphnia pulex*, *Ixodes scapularis*, *Triops cancriformis* and *Hypsibius dujardini* for NPC2 (Pelosi et al., 2014); *Varroa destructor* for OBP-like (Amigues et al., 2021); *Metaseiulus occidentalis*, *Parasteatoda tepidariorum* and *Stegodyphus mimosarum* for CCPs (Vizueta et al., 2018), and *Penaeus vannanamei* for lipocalins (Zhu, et al., 2021b). Moreover, the transcriptome was also searched for Ionotropic Receptors (IRs) using as queries the sequences of two *Armadillidium* species (in NCBI respectively 9 and 13 for *A. nasatum* and *A. vulgare*, among which one single co-receptor, IR 25a, for each species) as well as the 22 sequences reported by Groh-Lunow et al. (2015) in a study of putative olfactory proteins in the terrestrial hermit crab *Coenobita clypeatus* (including the co-receptors IR25a and IR93a). All these sequences, apart from the coreceptors are annotated as "Ionotropic Glutamate Receptor".

2.6. Proteomic analysis

Four protein extracts were prepared by crushing the antennae and the legs of two males and two females (FAant, MAant, FLeg, MLeg) in a mortar under liquid nitrogen with a solution of 6 M Urea/2 M Thiourea in Tris-Cl 50 mM pH 7.4. The protein extracts were centrifuged at 13,000 rpm at 4 °C for 30 min and the supernatants were collected. Protein concentration was measured on a Qubit nanodrop using the Qubit Protein Assay kit. For shotgun proteomics, protein digestion and subsequent purification were carried out on 15 μg of protein extracts (as reported by Cini et al., 2020). The eluates were concentrated and reconstituted to 20 μL in 0.5% acetic acid, before HPLC-MS analyses.

Samples were analysed by nano LC-ESI-HRMS/MS using an EASY-nLCTM 1200 system coupled with a Linear Trap Quadrupole (LTQ)-OrbitrapTM mass spectrometer (Thermofisher Scientific, Waltham, MA, USA) using an Acclaim PepMap C18 column (50 μm × 15 cm, 2 μm, 100 Å). One μL of the digested protein extract was injected directly and eluted with a flow rate of 300 nl/min. The elution mobile phases were: aqueous 0.1% formic acid (phase A) and water/0.1% formic acid in acetonitrile 20/80 (phase B). The elution program was: 0–2 min: 2% B; 240 min: 45% B; 243 min: 45% B; 246 min: 75% B; 249 min: 75% B. Mass spectra were acquired in positive ion mode, setting the spray voltage at 1.7 kV, the capillary voltage at 42 V, the temperature at 175 °C, the tube lens voltage at 120 V. Data were acquired in data dependent mode with dynamic exclusion enabled (repeat count 2, repeat duration 15 s, exclusion duration 30 s); survey MS scans were recorded in the Orbitrap analyser in the mass range 350–2000 m/z with a 60,000 nominal resolution at m/z = 400. Up to seven most intense ions in each full MS scan were fragmented (isolation width 2 m/z, normalized collision energy 35) and analysed in the IT analyser. Monocharged ions did not trigger MS/MS experiments.

The identification of proteins was performed using Mascot 2.4 search engine (Matrix Science Ltd., London, UK) against the assembled transcriptome. Searches were performed allowing: (i) up to four missed cleavage sites, (ii) 10 ppm of tolerance for the monoisotopic precursor ion and 0.5 mass unit for monoisotopic fragment ions, (iii) carbamidomethylation of cysteine and oxidation of methionine as variable modification. The peptide significance threshold was set at 0.01 and only peptides with scores higher than this threshold, which indicates identity or extensive homology were considered.

2.7. Gene cloning

The genes encoding the mature sequences of HreaCSP1 and HreaCSP6 were custom synthesized at Eurofins Genomics (Ebersberg, Germany), and subcloned into pET30a plasmid, using *Nde*I and *Eco*RI as restriction enzymes at the 5′ and 3′ ends, respectively. As a consequence, the recombinant proteins contained a starting methionine as the only addition to their mature sequences.

2.8. Protein expression and purification

To express the recombinant proteins, competent BL-21 cells were transformed with plasmids containing the genes encoding HreaCSP1 and HreaCSP6. Protein synthesis was induced by the addition of 0.4 mM IPTG after the culture had reached an OD at 600 nm around 0.8, and then the cells were grown for three additional hours at 37 °C. The bacterial pellet was sonicated and centrifuged, yielding both recombinant CSPs in the pellet as inclusion bodies. The proteins were solubilized in 8 M urea and 5 mM DTT for 1 h at room temperature, then refolded by extensive dialysis (three times) against 50 mM Tris-HCl buffer, pH 7.4. Purification of the solubilized proteins was accomplished by anion-exchange chromatography on High-Prep Q columns (Cytiva, Austria).

2.9. Ligand-binding assays

Binding of potential ligands was measured by displacement of a fluorescent probe from its complex with the protein at room temperature. A PerkinElmer FL 6500 spectrofluorometer in a right-angle configuration was used with quartz cuvettes of 1 cm path. The fluorescent probe *N*-phenyl-1-naphthylamine (1-NPN) was excited at 337 nm, and emission spectra were recorded between 380 and 450 nm. To evaluate the affinity of 1-NPN to each protein, fluorescence signals were measured after treating a 2 μM solution of the protein in 50 mM Tris-HCl pH 7.4 with aliquots of 1 mM 1-NPN in methanol to final concentrations of 2–16 μM. Intensity values were recorded at the peak maximum, around 412 nm. The dissociation constants of the complexes protein/1-NPN were calculated using Prism software (https://www.graphpad.com/scientific-software/prism/). The affinities of other ligands were evaluated in competitive binding assays by adding aliquots of 1 mM methanol solutions of each ligand to final concentrations of 2–16 μM, to a mixture of the protein and 1-NPN, both at the concentration of 2 μM in 50 mM Tris-HCl buffer, pH 7.4. Dissociation constants of other ligands were calculated from the corresponding $[IC]_{50}$ values (the concentration of each ligand halving the initial value of fluorescence), using the equation: $K_D = [IC]_{50}/1 + [1\text{-NPN}]/K_{NPN}$, where [1-NPN] is the concentration of free 1-NPN and K_{NPN} the dissociation constant of the complex OBP/1-NPN.

2.10. Protein modelling and docking

The three-dimensional model of HreaCSP6 was generated with the online software Swiss Model (Bertoni et al., 2017; Studer et al., 2021; Waterhouse et al., 2018) using the AlphaFold model of *Armadillidium nasatum* CSP (Uniprot KAB7504823.1, 26% identity), as a template. Figures were created with Chimera software (Pettersen et al., 2004). Docking of ligands was simulated with SwissDock software (Grosdidier et al., 2011a, 2011b).

3. Results

3.1. Transcriptome analysis of Hemilepistus reaumurii antennae and legs

The transcriptome analysis of crude mRNA extracted from antennae and legs of *H. reaumurii* generated a total of 183 million paired-end raw reads, of which more than 43 million reads from female antennae (FAnt), 45 million from male antennae (MAnt), 48 million from female legs (Fleg) and 45 million from male legs (MLeg). All raw-sequence reads data have been deposited in the NCBI Sequence Read Archive (SRA) database (BioProject PRJNA1022787). Following quality control and low-quality data filtration, 41,048,843, 42,205,928, 47,560,146 and 44,190,015 clean reads were obtained from FAnt, MAnt, FLeg, MLeg samples, respectively. More than 90% of clean reads in all the samples exceeded Q30 indicating a high quality of the sequencing data. Quality and duplication level of the transcriptome assessed using BUSCO set comprised of arthropod single copy orthologs showed a gene presence of 92.7% with 33.5% of duplicate genes, 4.1% of fragmented genes and 3% of missing genes (Fig. S1 in File S1). Using Trinity for *de novo* assembly, 132,949 transcripts were obtained with an average length of 757 bp. These transcripts were clustered into 132,891 unigenes (File S2) with an equal average length of 757 bp and an N_{50} value (the length of the shortest contig for which longer and equal length contigs cover at least 50% of the assembly) of 1074 bp.

3.2. Functional annotation and Differential Expression Analysis

The unigenes were scanned against the databases of NR, GO, KEGG, Pfam, String and Swiss-Prot using BLASTX and BLASTN. For more than one-third of them (48,151 out of 132,891, 36%) we found significant matches in one or more databases. The BLAST results against NT, NR and Swiss-Prot are reported in Supplementary files S3, S4, S5, respectively. The species with the most similar unigenes in the NR database were the amphipod *Hyalella azteca* (26%), the termites *Cryptotermes secundus* (4%), and *Zootermopsis nevadensis* (2%), and the arachnid *Nephila clavipes* (4%) and *Centruroides sculpturatus* (2%). The annotation analysis of the unigenes into the three major GO categories: Biological Process, Cellular Component and Molecular Function is reported in Fig. S2 of File S1, while Fig. S3 summarises the KEGG pathways.

Differential Expression Analysis showed that 3644 genes were significantly more expressed in antennae than in legs, whereas 9833 were more expressed in legs (Fig. S4 in File S1; File S6).

3.3. Proteomic analysis of antennae and legs extracts

Digested protein obtained from crude extracts of antennae and legs of both sexes of *H. reaumurii* were subjected to nano-HPLC-MS and MS/MS. The search of the MS data against the unigene database produced a total of 796 identified clusters (563 hits, grouped into 525 protein families for FA; 635 hits, 595 protein families for MA; 483 hits, 443 protein families for FL, 405 hits, 366 protein families for ML; File S7). Only 210 of the identified proteins were assigned to a GO molecular function, the best represented categories being catalytic activity, binding and hydrolase activity (Fig. S5 in File S1).

3.4. Identification of chemosensory proteins

A BLAST search of the contigs obtained from the transcriptome of *H. reaumurii*, using representative sequences of the major classes of semiochemical-binding proteins, returned 13 sequences that could be confidently assigned to the family of Chemosensory proteins (CSPs). We could also detect three sequences similar to NPC2 proteins and 9 lipocalins, but none similar to arthropod OBPs (File S8). Moreover, seven CSPs and one NPC2 member were also identified at the protein level in the shotgun analyses (CSPs: Clusters 41527.10923, 41527.12412, 41527.13504, 41527.37870, 41527.38572, 41527.38573, 41527.6016; NPC2: Cluster 41527.11594; File S7).

The amino acid sequences of the 13 CSPs identified in the transcriptome analysis are aligned in Fig. S6 (in File S1), while Fig. S7 shows a tree built on the *H. reaumurii* members (HreaCSPs) and CSPs from two other isopod species, *Armadillidium vulgare* and *A. nasatum*. The amino acid sequences of the three species, which were used to generate the phylogenetic tree, are reported in File S8. The CSPs of the three isopods are strongly divergent within the same species, with amino acid identities ranging, apart from a couple of exceptions, between 13 and 50%. This fact may indicate a differentiation under environmental pressure and suggests a function of these proteins in chemical communication.

All the CSPs of *H. reaumurii* present the typical motif of four conserved cysteines observed in the sequences of all Hexapoda. Moreover, 11 of them contain two additional conserved cysteines in their N-terminal region. When isopod (*H. reaumurii and A. nasatum*) and insect CSPs (a member of *Bemisia tabaci* representative of CSPs of different orders of insects) are aligned while keeping the four conserved cysteines in the same positions, then the isopod members exhibit a longer N-terminus containing the two additional cysteines. This segment is not present in other non-isopod crustacea, such as *Daphnia pulex, Hyatella azteca, Penaeus japonicus* and *P. vannamei*, as shown in the alignment of Fig. 1. For protein CSP6, a tryptic peptide (MSADQLINEAPPDWSYGCLSK) containing the second cysteine residue was identified in the proteomic shotgun experiment, indicating that the N-terminal sequence is present in the mature protein.

The BLAST search of the *Armadillidium* IR sequences produced many hits with both high bitscore (>50) and low e-value (<0.000001). The selection of these proteins produced a list of 12 candidate IR protein sequences (File S9). On the contrary, the BLAST search of *C. clypeatus* IR sequences gave one single hit with a significant e-value (and a bitscore of 41). The sequence translation produced short and interrupted reading frames and was therefore not included among the putative *H. reaumurii* IR sequences. The BLAST search of these proteins against "Crustacea" non-redundant protein database in NCBI found that two of them share a high homology with the coreceptor IR25 reported for two species of the genus *Penaeus* (File S9).

None of the sequences identified to code for putative chemosensory proteins was significantly more expressed in antennae, while on the contrary 12 (4 CSP; 2 lipocalins, 1 NCP2 and 5 IR) were significantly more expressed in the first pair of legs (Fig. S4 in File S1).

We have selected two CSPs of *H. reaumurii*, both detected in the antennae in our proteomic study, for expression and characterization: CSP1 (Cluster 41527.10923) and CSP6 (Cluster 41527.38573), which are representative of two well distinct clades (Fig. S7). Their amino acid sequences, aligned in Fig. 1, share only 30% of their residues.

3.5. Bacterial expression

The two selected CSPs were expressed in bacteria in high yields. After sonication, the recombinant proteins were found in the pellet as inclusion bodies. Both proteins could be easily solubilized by denaturation in 8 M urea and 5 mM DTT and refolded by extensive dialysis. Both CSPs were then purified by anion-exchange chromatography on HiPrep-Q column, following standard protocols, and used in ligand-binding experiments. Samples of bacterial pellets before and after induction, of supernatant and pellet obtained after sonication and of the purified proteins were analysed by SDS-PAGE (Fig. S8 in file S1).

3.6. Binding assays

HreaCSP1 and HreaCSP6 bind the fluorescent probe *N*-phenyl-1-naphthylamine (1-NPN) with dissociation constants of 17.7 and 5.85 μM, respectively (Fig. 2A), thus enabling the use of competitive binding assay to evaluate the affinities of other chemicals. The choice of potential ligands fell on long-chain fatty acids, esters, aldehydes and alcohols, which are widely represented among insect pheromones.

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HreaCSP1              MKFVILVLFLSKCLLLTSS
HreaCSP6              MQRISLAVLLAVFLLSASA
Anas_KAB7504823.1       GILLTVLLPVLLLSASA
Btab_CAH0778299.1     MSRVTLLLVAVALVGFVAG
Dpul_ABH88166.1      MKSTVVILIVLAVLAAVASA
Hazt_XP_018017107.1  MFPRASVLAALLACVVVVVA
Pvan_XP_027231336.1   MRALAALLVLAVAAAVSNA
Pjap_XP_042870055.1  MKLTVILVFVLIAGAVRVGG

HreaCSP1               QSTKDLCTVDITNMTIDEFVNNPPSGVTTACIDKPELEKFLVHKEHLTAVLECVLSDPPVCPKEG
HreaCSP6               QNFDAKCDPKIEKMSADQLINEAPPDWSYGCLSKRIITEMINNKEHVKGIVDCLHPNDPICTKES
Anas_KAB7504823.1         QKCTYNVENMSADQIINEAPPDWSFECMNKRIIEETVNSNNHIKGIVDCLHPDHPICAKDA
Btab_CAH0778299.1                        APAPLEQSDLEKFENMDLSSILSNKRLRTAYVNCMVDKGP-CTADA
Dpul_ABH88166.1                             QQTSWETLETMDVDNVLKNTKLVKRYLDCLLDRGR-CEKNG
Hazt_XP_018017107.1                       ASNPRLRGLEEIEEAELDAILNDELELKFYTDCVLDKGS-CDRSG
Pjap_XP_042870055.1                             QALDVDSRIVLRELDSPRKVEYYVSCASGQGS-CDTRG

HreaCSP1              YQIITDMIVPKIGQTLQCKDCSEKENDLGIYLLSYFQDNYPGYYRRLLAHFAK
HreaCSP6              YRIIAEEIFQRSDSSGRCPTCSEKINALVDYALKLLQKLEPRELRRGLGYLG
Anas_KAB7504823.1     YRVIAEEIFRRTDAGGRCPACSPETAALIDYTLRLLQQRQPRELRRGLGYLG
Btab_CAH0778299.1     AEFK--KILPDLTET-QCADCSAKFKELIKKSVTTFQKDYPEDWKTLMAHFDPDNKRAADLEKFMSS
Dpul_ABH88166.1       KDWKG-MLPRILNEG--CSGCTPKQVEKSDQIVNFMKANHSEDWAAIEAKYKTG
Hazt_XP_018017107.1   RAIKETLADMRFTKD-ICKNCSELQRRRAIKILKTLQSKPVYDEILRYYKLIL
Pjap_XP_042870055.1   --LQLRTYIPIVARGESCFRCSPRENRNLRLMVSTMQRRYPRCWQILVLAAQDLPTPSARGCAN
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Fig. 1. Alignement of *H. reamuri* CSP1 (HreaCSP1) and CSP6 (HreaCSP6) with representative CSPs from another isopod, *Armadillidium nasatum* (Anas), an insect species, *Bemisia tabaci* (Btab) and some aquatic crustacean species, *Daphnia pulex* (Dpul), *Hyalella azteca* (Hazt), *Penaeus vannamei* (Pvan) *and P. japonicus* (PjapP). Cysteine residues are highlighted. Four cysteines are conserved in CSPs of all three groups, but *H. reamuri* CSPs contain two additional cysteines at their N-terminal region.

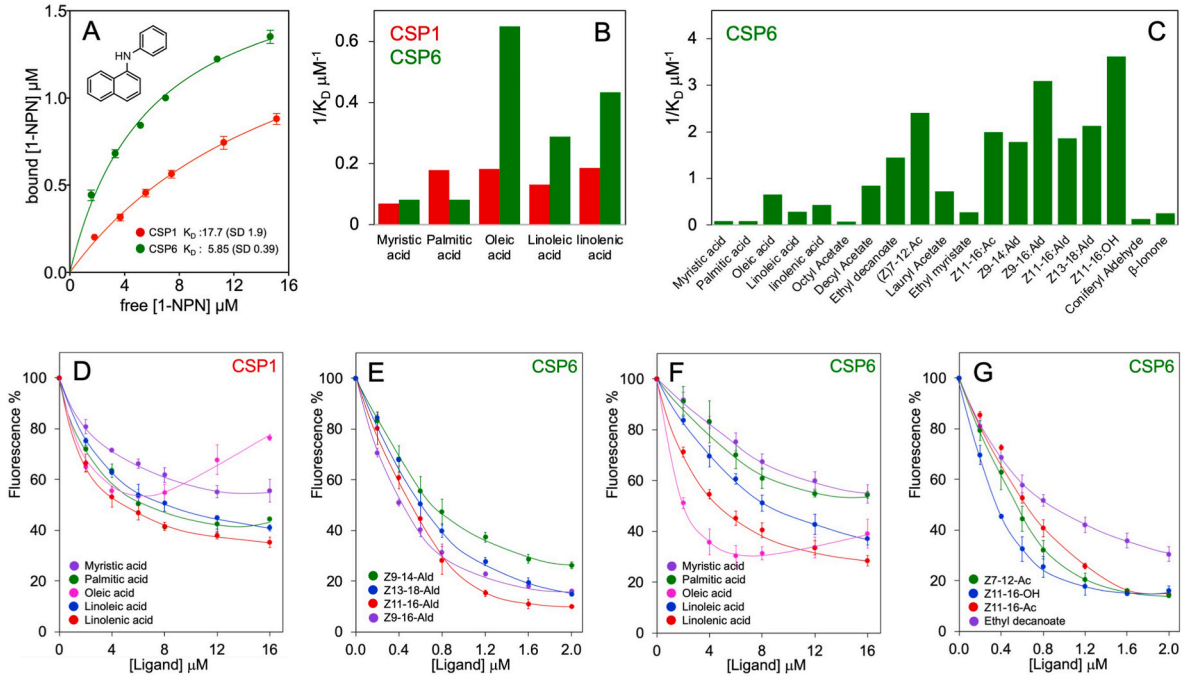

Fig. 2. Ligand-binding properties of two representative members of *H. reaumuri* CSPS. (**A**) Binding of the fluorescent reporter *N*-phenyl-1-naphthylamine (1-NPN) to the recombinant proteins. Aliquots of 1 mM 1-NPN in methanol were sequentially added to a 2 µM solution of the proteins in 50 mM Tris buffer, 1 M NaCl, pH 7.4, and emission intensities were recorded at 412 nm, upon exciting the fluorescent probe at 337 nm. Measurements were performed in triplicates and standard deviations are reported. Data were analysed with Prism software. (**B**) Graphical comparison of the affinities of HreaCSP1 and HreaCSP6 to a series of long-chain fatty acids. (**C**) Graphical representation of the affinities of best ligands to HreaCSP6. Linear long-chain aldehydes and a structurally related alcohols bind the protein much better than fatty acids, with dissociation constants lower by one order of magnitude. All dissociation constants are reported in Table S1. (**D-G**). Representative displacement curves of selected ligands.

Moreover, a single paper available on this subject (Schildknecht et al., 1988) reports on the identification of long-chain fatty acids and their methyl esters in the cuticle wash of *H. reaumurii*. Using a selection of these chemicals we measured good affinity for long-chain unsaturated fatty acids for both proteins with dissociation constants in the lower micromolar range. However, while HreaCSP1 seems to be specific for such ligands, HreaCSP6 also binds with even lower dissociation constants long-chain unsaturated esters and aldehydes. The affinities of strong and moderate ligands are reported in the histograms of Fig. 2B and C as the reciprocal of their dissociation constants. Examples of competition curves obtained with the best ligands are shown in Fig. 2D–G, while their dissociation constants are reported in Table S1 (in File S1). In addition, the following chemicals did not appreciably bind either of the two proteins: octanoic, nonanoic, decanoic, undecanoic and dodecanoic acids, salicylic acid, vanillin, vanillyl alcohol, vanillic acid, linalool and β-pinene. It should be noted that the chemicals marked by

an asterisk (*) in Table S1 have been used at concentrations above their solubility in water, therefore, the relative measured dissociation constants may not be accurate.

A model of HreaCSP6 (Fig. 3) was built based on the AlphaFold model of *A. nasatum* CSP (Uniprot KAB7504823.1, 26% identity). In this model, the first two cysteines, present in the N-terminal region are connected by a disulphide bridge, while the other four reproduce the pairing observed in insect CSPs. Docking of the best ligand, (*Z*)-11-hexadecenol, shows this molecule well inside the binding pocket of HreaCSP6 (Fig. 3). The energy predicted for the complex by the docking programme SwissDock (Grosdidier et al., 2011a, 2011b), was −8.36 kcal/mol. Two other good ligands, (*Z*)-9-hexadecenal and (*Z*)-11-hexadecenal, are also predicted to strongly bind the same protein with energies of −8.35 and −8.41 kcal/mol, respectively. The values of the dissociation constants calculated from the ΔG predicted by the docking programme are 2–3 times higher than those measured in our binding assays. This is not surprising and can be acceptable, considering the fact that most docking softwares do not take into account the flexibility of the protein.

4. Discussion

We identified in the transcriptome of antennae and legs of *H. reaumirii* sequences coding for proteins belonging to three different families of putative soluble proteins, i.e., Chemosensory proteins, Lipocalins and Niemann-Pick type C2, and for putative chemosensory receptors belonging to the Ionotropic receptor family, including two putative coreceptors.

Interestingly none of these genes was found to be more expressed in the antennae, while on the contrary for each family of putative olfactory proteins, some members were found to be more expressed in the legs. This result questions the role of isopod antennae as olfactory organs while suggesting that, similarly to decapods, isopod legs are involved in chemoreception (Derby et al., 2016).

The relatively large number of CSP genes identified in the antennae of the terrestrial isopod *H. reaumirii*, their differences in the number of cysteine residues and structure compared to the CSPs so far reported and the strong divergence in their amino acid sequences suggest that gene duplication and differentiation may have occurred under environmental pressure, thus supporting the hypothesis that CSPs could act in terrestrial crustacea as semiochemical carriers.

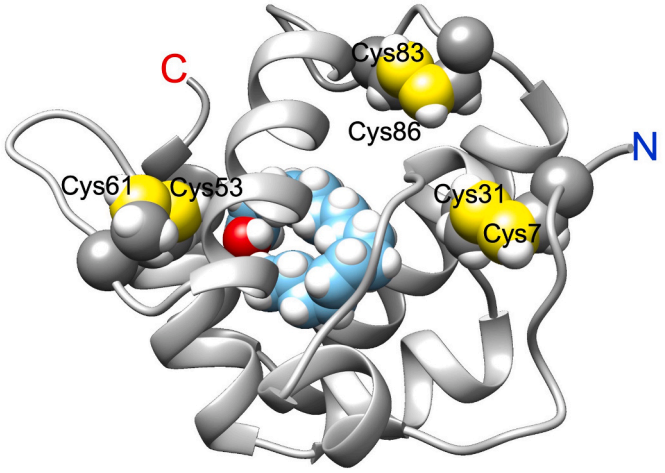

Fig. 3. (**A**) Three-dimensional model of HreaCSP6 built using the on-line software Swiss Model (Bertoni et al., 2017; Studer et al., 2021; Waterhouse et al., 2018) and the AlphaFold model of *Armadillidium nasatum* CSP (Uniprot KAB7504823.1, 26% identity) as a template. Six cysteines (shown in space-filling mode) are predicted to be linked by three disulphide bridges. The first two, located in the N-terminal region, are typical of isopods, while the other four reproduce the pairing observed in all insect CSPs. The figure was created with Chimera software (Pettersen et al., 2004). A molecule of (*Z*)-11-hexadecenol (pale blue) was docked inside the protein, using the on-line software SwissDock (Grosdidier et al., 2011a, 2011b).

In insects, CSPs have been shown to be involved in different functions, from development to nutrition and insecticide resistance, but also in the detection and release of pheromones. Insect olfaction is generally mediated by OBPs, but in some species, CSPs have adopted such a role. Examples include the paper wasp *Polistes dominulus*, where CSPs are specifically expressed in antennae instead of OBPs (Calvello et al., 2003), the Japanese carpenter ant *Camponotus japonicus*, where a CSP has been shown to mediate individual recognition (Hojo et al., 2015; Ozaki et al., 1995) and the migratory locust *Locusta migratoria*, with a CSP specifically binding the putative pheromone 3-(1-naphthyl)-propionitrile (Zhou et al., 2013). It appears that CSPs are more represented, both in number of genes and in abundance of expressed proteins, in some Orthopteroidea, such as Phasmida (Mameli et al., 1996; Marchese et al., 2000; Tuccini et al., 1996) and Orthoptera (Zhou et al., 2013), while a similar carrier function has been taken on by the more efficient OBPs in other clades. As an example, in the genome of the migratory locust, 70 genes encode for CSPs, but only 15 for OBPs (Zhou et al., 2013). By contrast, at least 60–70 genes encode OBPs in Diptera, such as *Drosophila* and *Anopheles*, but only 4–6 genes for CSPs are present in their genomes (Pelosi et al., 2014, 2018).

To investigate the potential role of CSPs in complexing and ferrying semiochemicals to chemoreceptors in the antennae of *H. reaumirii*, we decided to study the ligand-binding characteristics of two representative members (HreaCSP1 and HreaCSP6) markedly different between each other at the amino acid level. HreaCSP1 binds few linear fatty acids of 16–18 carbon atoms, both saturated and unsaturated, with high selectivity. HreaCSP6, on the other hand, while showing good affinity for the same fatty acids, binds with even better strength their corresponding esters, as well as aldehydes and alcohols of the same length (16–18 carbon atoms).

Pheromones or other semiochemicals have not yet been identified in isopods, however, it is worth citing a single paper reporting the presence in the cuticle wash of some fatty acids, such as palmitic, oleic, linoleic and linolenic, which showed good affinity for both CSPs investigated in this work (Schildknecht et al., 1988). The scanty information available on the semiochemicals of isopods does not allow further speculation on the role of CSPs in such species, while chemical communication in isopods remains an interesting field to explore from several approaches.

5. Author contributions

F.R.D., P.P., K.N-A and A.A conceived the study. F.R.D. and P.P designed the experimental work. A.A. and K.N.A. provided the biological samples. L.A. and A.I. analysed the transcriptome. F.R.D. performed the proteomic analysis and processed the data. J.Z. and P.P. expressed and purified the proteins and performed the binding experiments. W.K. contributed materials and facilities. F.R.D. and P.P. wrote the initial draft. All authors made contributions and comments to the manuscript.

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Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

All data are available as e-material; unicode sequences have been submitted to NCBI, BioProject PRJNA1022787.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2023.104012.

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