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The opsin repertoire of the Antarctic krill *Euphausia superba*

Alberto Biscontin [a], Elena Frigato [b], Gabriele Sales [a], Gabriella M. Mazzotta [a], Mathias Teschke [c], Cristiano De Pittà [a], Simon Jarman [d,f], Bettina Meyer [c,e], Rodolfo Costa [a,*], Cristiano Bertolucci [b,**]

[a] *Dipartimento di Biologia, Università degli Studi di Padova, Padova, Italy*
[b] *Dipartimento di Scienze della Vita e Biotecnologie, Università di Ferrara, Ferrara, Italy*
[c] *Section Polar Biological Oceanography, Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research, Bremerhaven, Germany*
[d] *Australian Antarctic Division, Kingston, Tasmania, Australia*
[e] *Institute for Chemistry and Biology of the Marine Environment, Carl von Ossietzky University of Oldenburg, Oldenburg, Germany*
[f] *CIBIO-InBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Universidade do Porto, 4485-661 Vairão, Portugal*

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ABSTRACT

The Antarctic krill *Euphausia superba* experiences almost all marine photic environments throughout its life cycle. Antarctic krill eggs hatch in the aphotic zone up to 1000 m depth and larvae develop on their way to the ocean surface (development ascent) and are exposed to different quality (wavelength) and quantity (irradiance) of light. Adults show a daily vertical migration pattern, moving downward during the day and upward during the night within the top 200 m of the water column. Seawater acts as a potent chromatic filter and animals have evolved different opsin photopigments to perceive photons of specific wavelengths. We have investigated the transcriptome of *E. superba* and, using a candidate gene approach, we identified six novel opsins. Five are r-type visual opsins: four middle-wavelength-sensitive (*EsRh2*, *EsRh3*, *EsRh4* and *EsRh5*) and one long-wavelength-sensitive (*EsRh6*). Moreover, we have identified a non-visual opsin, the *EsPeropsin*. All these newly identified opsin genes were significantly expressed in compound eyes and brain, while only *EsPeropsin* and *EsRh2* were clearly detected also in the abdomen. A temporal modulation in the transcription of these novel opsins was found, but statistically significant oscillations were only observed in *EsRrh3* and *EsPeropsin*. Our results contribute to the dissection of the complex photoreception system of *E. superba*, which enables this species to respond to the daily and seasonal changes in irradiance and spectral composition in the Southern Ocean.

1. Introduction

Solar light is a complex environmental signal characterized by daily and seasonal changes in daylight, irradiance, wavelength composition, direction and polarization (Björn, 2002). In the aquatic environment, chemical and physical factors modulate the different properties of light (Jerlov, 1968). The wavelength composition varies with depth: longer wavelengths are absorbed rapidly, while shorter wavelengths become predominant until the aphotic zone (Loew and McFarland, 1990). These variations are extreme at high latitudes where the photoperiod changes from continuous daylight in summer to constant darkness in winter.

The Antarctic krill *Euphausia superba* is a crustacean species with a circumpolar distribution that is exposed to almost all marine photic environments during its life cycle. *E. superba* have a long and complex larval development including three nauplius, three calyptopis and six furcilia stages, before the metamorphosis into juvenile (Hempel and Hempel, 1986). Eggs sink to deep oceanic layers up to 1000 m and after hatching they start their developmental ascent (Marr 1962). Nauplius stages are found in the aphotic zone below 250 m, whereas the calyptopis I is the first feeding stage to enter to the photic zone. Adults show a daily vertical migration pattern moving downward during the day and upward during the night within the 200 m water column, from photic to aphotic and vice versa (Godlewska, 1996; Quetin and Ross, 1991).

To perceive photons of specific wavelengths animals have evolved several types of opsin photopigments. Opsins are G-coupled protein receptors localized to the membranes of photoreceptors. Phylogenetic analysis has shown that opsins of bilaterians are grouped in three major clades: *c-opsin* expressed in photoreceptors with a ciliary morphology as vertebrate rods and cones; *r-opsin* expressed in photoreceptors with rhabdomeric morphology as the ommatidia of arthropods; and *Group 4* opsin containing relatively poorly characterized non-visual opsin types from both chordates and invertebrates as peropsins and neuropsin (opsin-5) (Porter et al., 2012). Recently, next-generation sequencing technologies have increased significantly the available

* Correspondence to: R. Costa, Dipartimento di Biologia, Università di Padova, Via U. Bassi, 58/B, 35121 Padova, Italy.
** Correspondence to: C. Bertolucci, Dipartimento di Scienze della Vita e Biotecnologie, Università di Ferrara, Via L. Borsari, 46, 44121 Ferrara, Italy.
E-mail addresses: rodolfo.costa@unipd.it (R. Costa), bru@unife.it (C. Bertolucci).

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amount of transcriptomic and genomic data for several animal species. Interestingly, crustacean species have genomes with a highly variable number of opsin genes. For instance, transcriptome analysis of the Ostracoda *Skogsbergia lerneri* and the Branchiura *Argulus siamensis* revealed 8 opsin genes (Oakley and Huber, 2004; Sahoo et al., 2013). In contrast, the genomes of the Stomatopoda *Pseudosquilla ciliate* and the Cladocera *Daphnia pulex* encode 33 and 46 opsin genes, respectively (Colbourne et al., 2011; Porter et al., 2013). The role of this large number of opsins involved in visual and non-visual photoreception is still largely unknown. However, we could speculate that adaptation to complex marine and freshwater photic environments has occurred through an increase of the number of opsin genes (Feuda et al., 2016; Liegertová et al., 2015).

Given its phylogenetic position and its capacity to inhabit different photic environments, we have investigated the transcriptome of the Antarctic krill *E. superba* in order to identify novel opsin paralogues. Previous investigations have identified three opsin genes in *E. superba* (Porter et al., 2007; De Pittà et al., 2013). Here, adopting a qualitative candidate gene approach, we analysed sequences from a *de novo* assembled transcriptome of krill and we identified 6 novel opsins. Among them five are r-type visual opsins. Crustacea visual pigments are clustered in three main clades, related to their spectral sensitivity: long-wavelength-sensitive (LWS), middle-wavelength-sensitive (MWS), and short-wavelength/UV-sensitive (SWS/UVS) (Porter et al., 2007). We found four MWS opsins named *EsRh2*, *EsRh3*, *EsRh4* and *EsRh5* and one LWS opsin named *EsRh6*. In addition, we have identified a *Peropsin* homologue. *Peropsin* is a non-visual opsin member of Group 4 opsins previously described in vertebrates, Cephalochordata and Artropoda and a retinal-photoisomerase activity has been proposed as its main function (Sun et al., 1997; Koyanagi et al., 2002; Nagata et al., 2010; Eriksson et al., 2013; Battelle et al., 2015, Lenz et al., 2014; Henze and Oakley, 2015). Furthermore, using qPCR on RNA isolated from compound eyes, brain and abdomen we detected the tissue expression of the newly identified opsins. Finally, prompted by the different expression levels observed among tissues, we investigated the daily fluctuations of opsin mRNA expression in the head (brain, eye and eyestalk) of krill sampled under natural conditions during the Antarctic summer.

2. Material and methods

2.1. Specimen collection

For the tissue localization experiments Antarctic krill (*E. superba*) were caught in East Antarctica (65° 31′ S, 141° 23′ E, 15 Jan. 2015) during the voyage V2 14/15 with RSV *Aurora Australis* by oblique hauls of several Rectangular Midwater Trawls, using a pelagic net (RMT 8), in the upper 200 m of the water column. Immediately after hauling, krill were transferred as quickly as possible into 200 L tanks located in a temperature constant room at 0 °C and dim light. Every day 50% of the water was substituted with fresh pre-chilled seawater to ensure a continual turnover of food and nutrients. Twice a day, dead animals and moults were removed from the tanks (Kawaguchi et al., 2010). After arriving in Hobart, Tasmania (January 25, 2015), krill were delivered directly to the Australian Antarctic Division (AAD) aquarium and kept in a 1670 L holding tank. The holding tank was connected to a 5000 L chilled sea water recirculation system. The sea water was maintained at 0.5 °C and was recirculated every hour through an array of filtration devices. Water quality was monitored continuously. A detailed description of the holding tank system at the AAD and of krill maintenance in the laboratory is described elsewhere (King et al., 2003). Lighting was provided by fluorescent tubes. A PC controlled timer system was used to set a natural photoperiod corresponding to that for the Southern Ocean (66°S at 30 m depth). Krill were fed daily with an algal mixture consisting of the cultured pennate diatom *Phaeodactylum tricornutum* (1.3×105 cells mL^{-1}), the cultured flagellates *Gemingera* sp. (1.9×103 cells mL^{-1}) and *Pyramimonas geldicola* (4.9×103 cells mL^{-1}), as well as *Pavlova* sp. (2.8×104 cells mL^{-1}), *Isochrysis* sp. (1.1×105 cells mL^{-1}) and *Thalassiosira* sp. (7.6×103 cells mL^{-1}), which are concentrated bulk feeds of instant algae mixed with seawater (Reed Mariculture, CA, USA), and a commercial larval food (EZ-Larva mixed with seawater at 0.04 mL L^{-1}). During sampling (February 26, 2015) the holding tank was exposed to a light:dark regime of 24 h light and 0 h darkness with a maximum of 100 lx light intensity at the surface of the tanks during midday. Sampling was done around midday in the middle of the light phase by catching krill randomly out of the holding tank. Animals were dissected on a cooling element and three different tissues (eyestalks, brain, and abdomen) were immediately transferred to RNA stabilization solution (RNA-later, Life Technologies, CA, USA) for subsequent molecular analyses.

For the temporal expression analysis Antarctic krill were collected in the Ross Sea (longitude: 167°28′81″ E – 179°54′68″ W, latitude 68°40′ 54″ S – 77°01″81″ S) in January 2004 during the XIX Italian Antarctic Expedition with procedures described in Mazzotta et al. (2010). Adult specimens were caught at different times throughout the 24-hour cycle (local times: 01:00, 06:00, 10:00, 15:00, and 18:00) and stored at −80 °C in RNA-later (Life Technologies, CA, USA). Specimens caught at each time point were dissected and total RNA was extracted from the head (including the brain and the compound eyes).

2.2. Screening of transcriptome for opsins

To identify new putative opsins we manually inspected a *de novo* unpublished transcriptome of *E. superba*, provided by the Australian Antarctic Division (Kingston, Tasmania). Total RNA extracted from Calyptosis I and Furcilia V larval stages was used to produce the cDNA libraries for massive sequencing. The Illumina reads were used for clustering and *de novo* assembly using Trinity RNA-seq software (Grabherr et al., 2011). We used the default k-mer size of 25 nt, we imposed a minimum k-mer coverage of two and it enabled the Jaccard clipping to mitigate falsely fused transcripts. After eliminating adapter sequences and filtering out the low-quality reads, including too short ones (20 nt) and repeats, a total of 223.193 contigs longer than 200 bp were obtained. Assembled contigs ranged in size from 200 bp to 13.460 bp, with a median size of 328 bp. Each putative transcript was searched locally against the NR database downloaded from the NCBI FTP site on the 8.10.2015 by using Blast-N (against NCBI nucleotide database) and Blast-X (against NCBI protein database and TrEMBL) with an e-value cut-off of 1e-6 (De Pittà et al., 2013). The first five matches from each search were grouped and priority was given to Blast-N results. Alignments characterized by less than 50% of identity or coverage were discarded, as they were considered poorly informative. All the putative transcripts resulting in at least an opsin description among the first five high scoring pairs were chosen. Blast results were manually inspected to select only the sequences unambiguously identified as putative opsins and not those encoding for others members of the G protein-coupled receptors family.

2.3. Phylogenetic analysis

E. superba opsin cDNA sequences were converted into amino acid sequences using the translate tool by ExPASy Proteomics (http://www.expasy.org/tools/dna.html) and then aligned with other arthropod opsin sequences obtained from UniProtKB (http://www.uniprot.org) using ClustalW2 (Thompson et al., 1994). A phylogenetic tree was generated using a neighbour-joining algorithm based on the Jones-Taylor-Thornton (JTT) model (MEGA 5.0; Tamura et al., 2007). Confidence in nodes was estimated by 1000 bootstrap replicates. A pairwise deletion algorithm was also used to eliminate any alignment gaps present in the sequence. The tree was rooted using the *Homo sapiens* VIP receptor 1 as outgroup.

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2.4. Cloning and sequencing of E. superba opsins cDNA

To confirm the presence of *E. superba* opsins mRNA sequences identified in the transcriptome, we cloned and sequenced the complete coding sequences of *EsRh2*, *EsRh3*, *EsRh4*, *EsRh5*, *EsRh6* and *EsPeropsin*. The sequences were deposited in GenBank (acc. nos *EsRh2*: KU682720, *EsRh3*: KU682721, *EsRh4*: KU682722, *EsRh5*: KU682723, *ESRh6*: KU682724 and *EsPeropsin*: KU682725). First-strand cDNA was synthesized from 1 μg of total RNA from head using the SuperScript™/First-Strand cDNA Synthesis Kit (Invitrogen, CA, USA), according to the manufacturer's instructions. The first strand cDNA was used as template for PCR with oligonucleotides designed on krill opsins sequences identified in the transcriptome. The oligos used to obtain full-length opsin transcripts are listed in Table 1 and the following programme was used: 94 °C for 2 min, followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min, with the addition of a final polymerization step at 72 °C for 7 min. All primers used to clone opsins were designed using the Primer3 design tool (Untergasser et al., 2012). All the PCR reactions were performed with high-fidelity DNA polymerases in order to minimize PCR-induced errors. The PCR products were cloned using StrataClone PCR Cloning Kit (Agilent Technologies, CA, USA). Full-length *E. superba* opsins cDNA sequence were sequenced with a modified Sanger dideoxy terminator cycle sequencing chemistry, the ABI BigDye kit version 3.1, on an ABI 3730 48-capillary sequencer and 36 cm capillaries (BMR Genomics Sequencing Service, Italy).

2.5. Quantitative RT-PCR

Quantitative RT-PCR was used to localize the expression of opsins. 1 μg of total RNA from head, eye and abdomen of single specimens was used to perform independent cDNA syntheses in a final volume of 20 μL, using a mix of random hexamers and oligodT and SuperScript II reverse transcriptase (Life Technologies, CA, USA). Three biological replicates were analysed. One μL aliquot of 1:4 diluted first-strand cDNA was PCR amplified in 10 μL volume using the SYBR Green chemistry according to the manufacturer's recommendations (SsoFast™ EvaGreen® supermix, Bio-Rad Laboratories, CA, USA) with a CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, CA, USA). Thermal cycling conditions were as follows: 3 min denaturation at 95 °C, followed by 40 cycles of a 5 s denaturation step at 95 °C and an annealing-elongation step for 20 s at 60 °C. After amplification, melting curve analysis to confirm the specificity of the amplicon was performed from 60 to 95 °C, with increments of 0.5 °C 10 s^{-1}. All samples were run in triplicate. Gene-specific primers designed using the Primer3 design tool (Untergasser et al., 2012) were used to amplify fragments of 120–150 bp in length (Table 1). We verified the efficiency of the primers by producing standard curves for all genes investigated. Moreover, the dissociation curve was used to confirm the specificity of the amplicon. The relative levels of each RNA were calculated by the $2^{-\Delta\Delta CT}$ method (where CT is the cycle number at which the signal reaches the threshold of detection) (Livak and Schmittgen, 2001). As previously reported (Mazzotta et al., 2010; De Pittà et al., 2013), 18S was used as housekeeping gene (QuantumRNA™ 18S Internal Standards, Ambion, Austin, TX, USA). Each CT value used for these calculations is the mean of three replicates of the same reaction. To ascertain that the apparent changes in the expression of opsins mRNA were not artefacts of normalizing to the *18 s* housekeeping gene, a control experiment was conducted normalizing to another reference gene, the *ubiquitin carboxyl-terminal hydrolase 46* (*USP46*, ID Transcript: N18758 in Meyer et al., 2015; F: 5′-AAATCGTCAGAAACGGGCTA-3′, R: 5′-TTAG CGGTTATGGAACATTACG-3′, Efficiency = 99.2%, $R^2 = 0.989$). We used *USP46* as a reference gene based on a microarray experiments conducted in our laboratory (data not shown). The expression levels of USP46 were constant during a 24 h experiment in both LD and DD conditions. Furthermore, we tested the reliability of *USP46* as housekeeping gene by using three independent algorithms: geNorm (Pattyn et al., 2013), Bestkeeper (Pfaffl et al., 2004), and Normfinder (Andersen et al., 2004). Nearly identical expression profiles were observed when opsins transcript levels were normalized to *18* s or *USP46* mRNA. This method was also used to report on changes in expression of opsins in krill heads (brain and eyes) collected at different time of day (01:00, 06:00, 10:00, 15:00, 18:00). Because data were not normally distributed (D'Agostino-Pearson normality test, $p < 0.05$), non-parametric Kruskal-Wallis one-way ANOVA and Dunn's multiple comparison test were used to determine significant differences ($P < 0.05$) between groups (GraphPad Prism 4.0, GraphPad Software, CA, USA). The RAIN non-parametric test with the default settings (independent) was used to detect rhythmicity in 24-h time series of opsins expression (Supplementary Table 1). This R package is capable of detecting both sawtooth-shaped and sinusoidal rising and falling patterns (Thaben and Westermark, 2014). We set the sampling interval to 1 h and the number of replicates for each time point, using the argument named "measure. sequence", in order to detect the periodicity in our irregular time series.

2.6. Ethics statement

All animal work has been conducted according to relevant national and international guidelines. Animals were collected both at the Australian Antarctic Division (Hobart, Australia) and during the XIX Italian Antarctic Expedition (2003–2004). Krill fishing was conducted in accordance with the Convention for the Conservation of Antarctic Marine Living Resources (CCAMLR, 1980) under permissions from the Italian Scientific Commission for Antartica (CSA) and the Consortium of Programma Nazionale di Ricerche in Antartide (PNRA, Project N. 2003/1.3 "Molecular neurogenetics of circadian rhythmicity in *E. superba*"). Export of krill specimens from Australia to Italy was made in accordance with the Department of the Environment, Australian Government (registration number IT111A).

Table 1
Primers used for cloning and quantitative PCR.

	Primers for cloning	Primers for qPCR	%	R^2
EsRh2	F: 5′-TGGTCAGAAGCTCTGCACTG-3′ R: 5′-TTTTCATGATTGCTGGGACA-3′	F: 5′-TCATCATGGCAAACATCACA-3′ R: 5′-TGTGCCACATTGGATTGACT-3′	106.4	0.997
EsRh3	F: 5′-GTGGCACGAGGATCTCAAAT-3′ R: 5′-TGTTTGCTGGGTTATCATGC-3′	F: 5′-GTGGCACGAGGATCTCAAAT-3′ R: 5′-GATGGAGGTTCCCTCTGGAT-3′	100.8	0.935
EsRh4	F: 5′-ATTGCCCATTGGTCATTCAT-3′ R: 5′-GCAGTGGTATCAACGCAGAG-3′	F: 5′-GCTCATACACCTTCATAGTC-3′ R: 5′-CTCTTGACATTCATCTTCTC-3′	94.9	0.950
EsRh5	F: 5′-TGGCTAGCAGTCAACCAACA-3′ R: 5′-GGCCACAATGTTGCATTTTA-3′	F: 5′-TGGCTAGCAGTCAACCAACA-3′ R: 5′-GTTGTATCCCCCTTCGGAAC-3′	104.3	0.974
EsRh6	F: 5′-GGAAGAGAGGATACGGACCA-3′ R: 5′-CCTGTTCCCTCAAATCTGGA-3′	F: 5′-ATTTGTGACTACAAAGAACC-3′ R: 5′-GAAAAGACACAGAAGTCATC-3′	107.5	0.997
EsPeropsin	F: 5′-TGGATCCACTAGAAAAACAATGG-3′ R: 5′-TCGGCTTCTAGGCTATGTGG-3′	F: 5′-GCTATTACCAGAGCGCCAAG-3′ R: 5′-CCACAGCATACCATGACCAG-3′	102.7	0.999

%: percentage of efficiency.

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3. Results

3.1. Identification of opsin genes in the trascriptome of E. superba

Our screening of *E. superba* transcriptome for opsins resulted in 57 highly redundant contigs. Fifty-four contigs are members of the MWS clade, the other 3 are dispersed among LWS and Group 4 opsin (1 and 2 contigs, respectively). The transcriptome did not contain SWS/UV transcripts. In order to reduce this redundancy we have manually clustered very similar sequences (>85% identity) obtaining 8 unique putative transcripts: 6 newly and 2 previously identified (Rh1a and Rh1b; De Pittà et al., 2013) opsin genes. We successfully cloned sequences of all the new opsin transcripts, which we named according to the clade in which they are located in our phylogenetic analyses (MWS opsins: *EsRh2*, *EsRh3*, *EsRh4*, *EsRh5*, LWS opsin: *ESRh6* and *EsPeropsin*; Fig. 1). One of the newly found opsins in the *E. superba* transcriptome is a member of the Peropsin group. It has 48–49% of amino acid identity with Chelicerata peropsins and 35–39% with deuterostome homologues (Fig. 2). MWS opsins (*ESRh2-Rh5*) are >50% identical in their amino acid sequences (Fig. 3). *EsRh6* and *EsPeropsin* show 35–38% and 25–28% of amino acid identity to MWS opsins, respectively.

All these novel opsins are predicted to be seven transmembrane (TM) domain proteins and possess all the key elements necessary for phototransduction (Gartner and Towner, 1995; Townson et al., 1998): 1) the lysine (K) responsible for covalent binding to the chromophore through a Schiff's base interaction in the VIITM domain; 2) two conserved cysteine (C) residues in the I and II extracellular domains, responsible for disulphide linkage stabilization; 3) a glutamic acid (E) to serve as Schiff's base counterion in the II extracellular domain; 4) a potential G-protein binding site (DRY) in the II intracellular domain; 5) the NPXXY motif in the VIITM domain, which is a highly conserved motif among G-protein coupled receptors; 6) a HPR(K) motif in the C-terminus, characteristic of opsins that activate the Gq/11 class of GTP-binding proteins (Figs. 2 and 3). Furthermore, *E. superba* opsins nested within MWS and LWS clade have two residues (Y and S in the IIITM domain) functionally related to the long/middle-wavelength spectral sensitivity (Chang et al., 1995) (Fig. 3).

3.2. Localization and temporal expression of E. superba opsin trascripts

To investigate the expression at the mRNA level of opsin genes in different tissues of *E. superba*, we performed qPCR using RNA from dissected brain, eye and abdomen (Fig. 4). The newly identified opsins were all expressed, albeit with some differences in the expression levels, in compound eyes and brains, while only *EsPeropsin* and *EsRh2* were clearly detected in the abdomen (Fig. 4C).

As we had previously observed a daily variation of *EsRh1a* and *EsRh1b* mRNA expression in the head of Antarctic krill collected under natural conditions (De Pittà et al., 2013), we investigated the temporal expression of the novel opsins detected in the *E. superba* transcriptome. qPCR was performed on specimens collected in the Ross Sea at different times of the day (01:00, 06:00, 10:00, 15:00, and 18:00) during the Antarctic summer (Mazzotta et al., 2010), when they were exposed to an almost continuous 24 h photoperiod characterized by daily variations in solar irradiance (Fig. 5). qPCR showed a general high inter-individual variability in opsin levels, consistently with the fact that no significant temporal patterns of expression were detected by ANOVA (*EsRh2*: $H_5 = 2$, $p > 0.7$; *EsRh3*: $H_5 = 9.1$, $p = 0.06$; *EsRh4*: $H_5 = 4.8$, $p > 0.3$; *EsRh5*: $H_5 = 4.9$, $p > 0.3$; *EsRh6*: $H_5 = 4.3$, $p > 0.3$; *EsPeropsin*: $H_5 = 9.2$, $p > 0.05$). However, the overall data suggest the

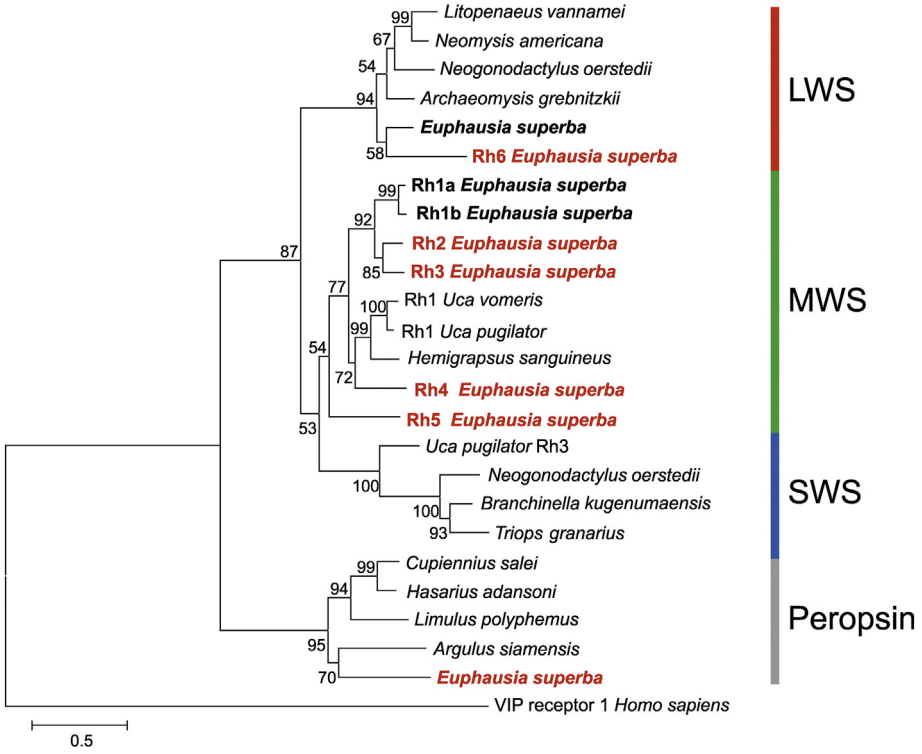

Fig. 1. Phylogenetic relationships of *Euphausia superba* opsins. Phylogenetic analysis of selected opsins shows that EsRh2, EsRh3, EsRh4, and EsRh5 opsin proteins fall in the MWS clade, whereas EsRh6 is a member of the LWS clade and EsPeropsin clusterizes with other peropsin orthologs. Bootstrap confidence values based on 1000 replicates are shown at nodes. Scale bars indicate amino acid substitutions per site. LWS, long-wavelength sensitive; MWS, middle-wavelength sensitive; SWS, short-wavelength sensitive. Accession numbers: *Litopenaeus vannamei*: ABH00987; *Neomysis americana*: ABI48886; *Neogonodactylus oerstedii*: ACU00212; *Archaeomysis grebnitzkii*: ABI48867; *Euphausia superba*: ABI48874; *Uca pugilator* Rh2: ADQ01810; *Hemigrapsus sanguineus*: Q25158; *Uca vomeris*: ACT31580; *Uca pugilator* Rh1: ADQ01809; *Uca pugilator* Rh3: E5G6F2; *Neogonodactylus oerstedii*: AIF73508; *Branchinella kugenumaensis*: BAG80984; *Triops granaries*: BAG80978; *Cupiennius salei*: CCP46949; *Hasarius adansoni*: BAJ22674; *Limulus polyphemus*: AIT75833; *Argulus siamensis*: 461527168, VIP receptor 1 *Homo sapiens*: P32241.

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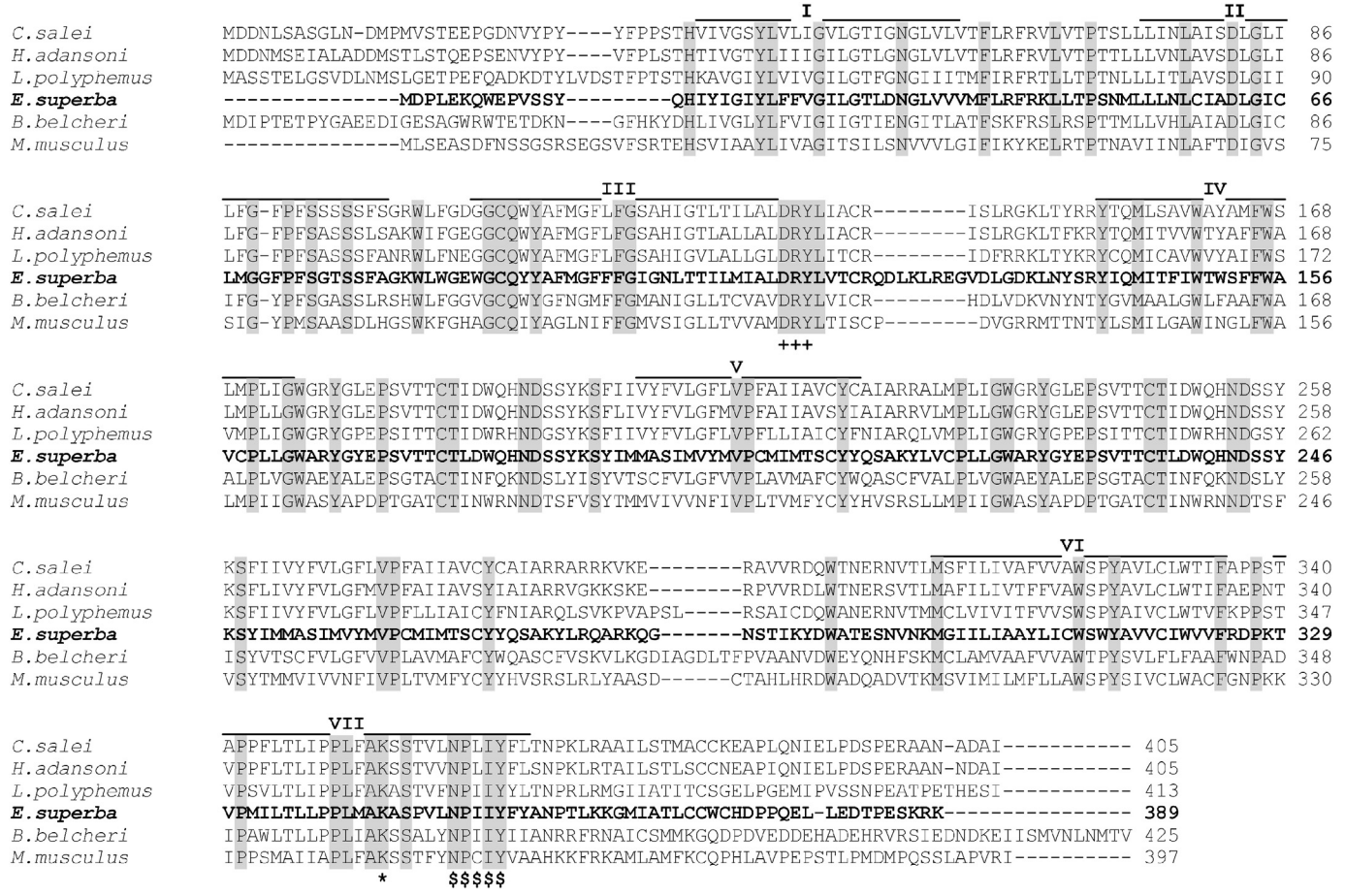

Fig. 2. Alignment of amino acid sequences of *Euphausia superba* Peropsin with orthologs of mouse (*Mus musculus*), amphioxus (*Branchiostoma belcheri*), Crustacea (*Argulus siamensis*) and three arthropods (the limulus *Limulus polyphemus* and the spiders *Cupiennius salei* and *Hasarius adansoni*). Amino acid residues conserved are highlighted on a grey background and alignment gaps are indicated by dashes (-). The seven transmembrane domains are indicated by lines above the sequence and labelled with Roman numerals. The lysine residues which bind to the retinal chromophore is indicated by an asterisk. The "DRY" and "NPXXY" motifs, which are highly conserved among G-protein coupled receptors, are indicated by symbols + and $, respectively.

existence of a temporal modulation of the amplitude and phase of expression for all the novel opsins (Fig. 5). Indeed, *EsRh2*, *EsRh3* and *EsRh5* showed maximum levels at 06:00 (Fig. 5A–B, D), whereas *EsRh4* and *EsRh6* (Fig. 5C, E) reached the peak in the second half of the daylight (15:00–18:00). Conversely, *EsPeropsin* shows its peak in the middle of the night, at 01:00, when solar irradiance reaches the lowest levels (Fig. 5F). A further analysis performed with RAIN, a robust non-parametric method for the identification of rhythms in biological time series (Thaben and Westermark, 2014), showed that a significant sinusoidal pattern of oscillation actually exists in the case of *Peropsin* and *EsRh3* ($p = 0.0195$, Phase: 01:00 and $p = 0.0016$, Phase: 06:00, respectively).

4. Discussion

Using a transcriptomic approach, we significantly expanded our knowledge of the opsin repertoire in the Antarctic krill *E. superba*. Based on phylogenetic affiliations, we identified 5 visual r-opsins belonging to the MWS and LWS clades and a non-visual opsin member of the Peropsin group. The expression of many opsins in the photoreceptor system seems to be common in crustaceans where many species express more opsins than expected based on their photoreceptor physiology. For instance, Stomatopoda and Cladocera species have more than 30 opsins, belonging to different classes, expressed in their transcriptomes. Visual opsins form three evolutionarily distinct groups that generally correlate with the maximum wavelength absorption (LWS, MWS and SWS/UVS; Porter et al., 2007, 2012). Krill opsins identified until now belonged to LWS and MWS clades. Interestingly, none of the contigs that we identified was member of the SWS/UVS clade. This is quite an unexpected result as maintenance of blue sensitivity is likely to be dependent on the photic environment of the species and adult Antarctic krill show a daily vertical migration pattern along the water column below 30 m, where only the blue wavelengths penetrate (Godlewska, 1996). However, another crustacean species phylogenetically closely related to the Antarctic krill, the stomatopod *Hemisquilla californiensis*, living the blue wavelengths range (10–50 m), showed in the transcriptome only one transcript annotated as member of SWS/UVS clade (Porter et al., 2013). We expect that an increased depth of coverage of sequencing would identify additional opsin/s in *E. superba*, with the maximum wavelength absorption in the short wavelengths spectrum.

Non-visual opsins have been detected in invertebrates. For instance a non-visual c-opsin, named pteropsin, has been identified in the brain of the honey bee *Apis mellifera* (Velarde et al., 2005). Neuropsin (opsin-5) orthologs have been found in the genomes of the tardigrade *Hypsibius dujardini* (Hering and Mayer, 2014) and of the echinoderm *Amphiura filiformis* (Delroisse et al., 2014). In the present investigation we have detected in the transcriptome of *E. superba* a transcript of the non-visual opsin *Peropsin* gene. Peropsin has been originally described in human retina (Sun et al., 1997) and further experiment in lancelets demonstrated its photoisomerase activity (Koyanagi et al., 2002). *Peropsin* orthologs have been identified and characterized in different Chelicerata (spiders *Hasarius adansoni* and *Cupiennius salei*, horseshoe

```
                                                                                  I
Rh2    MANITGPQAMAYGGGPMEVTFGYPEGTSIIDIQHDYMKPLIDPHWRNFPPVNPMWHNLLGVLYTFIVFFAVTGNSMVVWLFNKHAPLRTP    90
Rh3    MANITGPQAMAYGGG-QEFTFGYPEGTSIIDIQPDYMKAIIDPHWAKFPPVNPMWHYVLGLLYIFIVFFAVTGNFLVIWLFNKHAPLRTP    89
Rh4    ---MAAPG------------FGYPEGTKITDFVPAEILPLVHEHWYQFPPVNPMWHFLLGCIYIVMGFWSILGNGVVVYLFFKSDTLKTP    75
Rh5    MNFTEGPMAEAYGSE-GGYNMGYPAGVTIIDMVPDHIKHMVHPHWANYPPVNPMWHYLLGMVYVILGFFSFTGNGLVMYLFFNHANLRTP    89

                  II                                       III
Rh2    SNFLVVNLALSDLIMMTTNCPFFVYNCFSGGRWSLPAEYCNIYACLGAITGVCSIWTLAMISADRYNIICNGFNGPKLSKGKAAAMSLFA   180
Rh3    SNFLVVNLALSDLIMMTTNCPFFIWNCFHGGVWSMSIEYCSVYAALGAVTGVCSIWTLACISADRFNIICNGFNGPKLTKGKASVMALWC   179
Rh4    SNFLVVNLALSDLIMLLTNFPFFAYNCFSGGVWMSSAFYCELYAALGAVTGVCSIWTLAMISADRYNVICNGFNGPKLTKGKAGVMSLTC   165
Rh5    ANKLIFNLTFSDFCMMLSQFPWFAYNCFNGGVWSFSPFACELYACLGSITGLCSLWTLAFISFDRYNVIVNGMQATPLSNKGAMARLGFC   179
                                                £                     +++

           IV                                 V
Rh2    WFMAIGIALCPLIGWGGYGPEGILTSCSFDYLS-QDIGTITYNLFMIIFDYFVPLFVIVGSYAMIVKAIFAHEEAMRAQAAKMNVKSLR-   268
Rh3    WTLSCTIASLPFFGWGSYGPEGILTSCSFDYIS-QDIGTITYNLFMFIFNYCCPLLIIVGSYAMIVKAIFAHEEAMRAQAAKMNVKSLR-   267
Rh4    WAMAIGWAIPPFVGWGKYIPEGILDSCSYDYLT-LDTNTRSYNMCIFAFDFFVPLFIIVGSYTFIVKAICAHEAAMRKQAEKMNVKSLR-   253
Rh5    WGYATMMAVPPFFGWGKYIPEGILDSCSFDYLTRDDIMIRTHGMALIVFNFCIPVTIIAGAYVMIVKAVTAHESAMRAQAAKMNVKDLRG   269
                         #    £

                         VI                                    VII
Rh2    TQEANEQRAEIRIAKTAIFNIGLWIVCWTPYTVITYHGCIGDFEGLKPLTTTLPALFAKSASCYNPFVYAIGHPKFRQAMTIHMPWFCVH   358
Rh3    SAEANEQRAEIRIAKTAIFNIALWIICWTPYAAITLQGCVGRFDHLKPLTTTLPALLAKSASCYNPFVYAIGHPRFRQAMTIHMPWFCVH   357
Rh4    SGDDSAESAEIRIAKTAIFNICLWLCCWTPYALVTLMGVLGRHDNITPLMTMLPALLAKSASCYTPFVYAVGHPKFRQGITIHMPWFCIT   343
Rh5    NADANKMSAEVRIAKVAICNVTLWLICWTPYAKIVAQGVFFDQSSITPIASMLPALIAKSASGYNPVVYAINHPRFRLAMTKSFPGFCVH   359
                                                                       *     $$$$$   §§§

Rh2    EATKT----ADNES-----STTAATEEKA   378
Rh3    ENTPQ----ADNQS-----AAT-ATEEKA   376
Rh4    EDTPKKLAPTDNKSDGDNSTQPGDTAEKA   372
Rh5    ENEPDN---ASTAT-----EANTTQEEKA   380
```

Fig. 3. Alignment of the 4 novel krill opsin sequences clustering in the MWS clade showing conserved amino acid residues (grey shaded). Black shading indicates two residues in the IIITM domain functionally related to the long/middle-wavelength spectral sensitivity. Lines above sequences indicate the transmembrane domains. Symbols mark residues and motifs necessary for phototransduction (for more details see results and legend to Fig. 2).

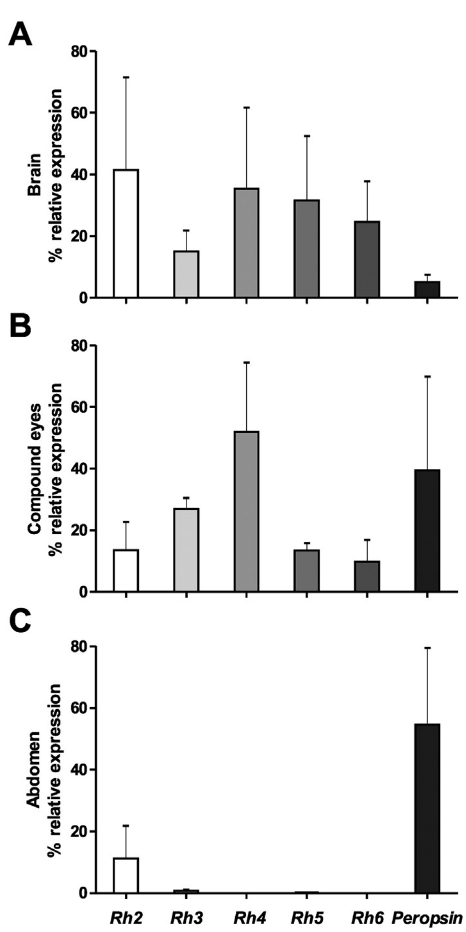

Fig. 4. Relative expression levels of *Euphausia superba* opsins in brain, compound eyes and abdomen.

crab *Limulus polyphemus*; Nagata et al., 2010; Eriksson et al., 2013; Battelle et al., 2015) and Crustacea (the Branchiura *A. siamensis* and the Copedoda *Calanus finmarchicus*; Sahoo et al., 2013; Lenz et al., 2014) species. Interestingly, *EsPeropsin* is expressed both in eye and in abdomen. The expression in the compound eyes suggests the hypothesis that it could play a role in vision as retinal photoisomerase. However, the expression in the abdomen seems to indicate that it has not (if any) exclusively a role as a photoisomerase, but it could also work as a photoreceptor. The expression of opsins in the crustacean abdomen has been previously reported. The crayfish *Procambarus clarkii* has a well-characterized extraocular photoreceptor, called the caudal photoreceptor, located in the sixth abdominal ganglion. SWS and LWS opsin transcripts have been observed not only in the sixth abdominal ganglion, but also in all ganglia of the nerve cord (Kingston and Cronin, 2015). The functional role of the caudal photoreceptor is extended to the triggering of the tail reflex, and to the walking behaviour of the animal. Furthermore, because the abdomen of the Antarctic krill presents photophores, which are used for counterillumination, a common and successful cryptic strategy, we cannot exclude an involvement of the abdominal photoreceptors in a feedback mechanism controlling the irradiance of the light emitted by photophores (Grinnell et al., 1988).

We had previously observed daily rhythms in the expression of the krill opsins *EsRh1a* and *EsRh1b*, with a peak of expression at 06:00, in specimen exposed to natural lighting conditions, despite high levels of inter-individual variability (De Pittà et al., 2013). In the present study, the use of a robust non-parametric method specifically designed for the identification of biological rhythmicity allowed us to detect a significant sinusoidal pattern of oscillation for two out of the six newly identified opsins, namely *EsPeropsin* and *EsRh3*. Interestingly, *EsPeropsin* and *EsRh3* have different pattern of expression and peak at different time of day (01:00 and 06:00, respectively). Different daily expression profiles of opsins with different spectral sensitivity could be important for the Antarctic krill to entrain their physiological, metabolic and behavioural processes to the daily changes in irradiance and spectral composition of sunlight that occur in the Southern Ocean throughout the seasons (Gaten et al., 2008; Teschke et al., 2011; De Pittà et al., 2013).

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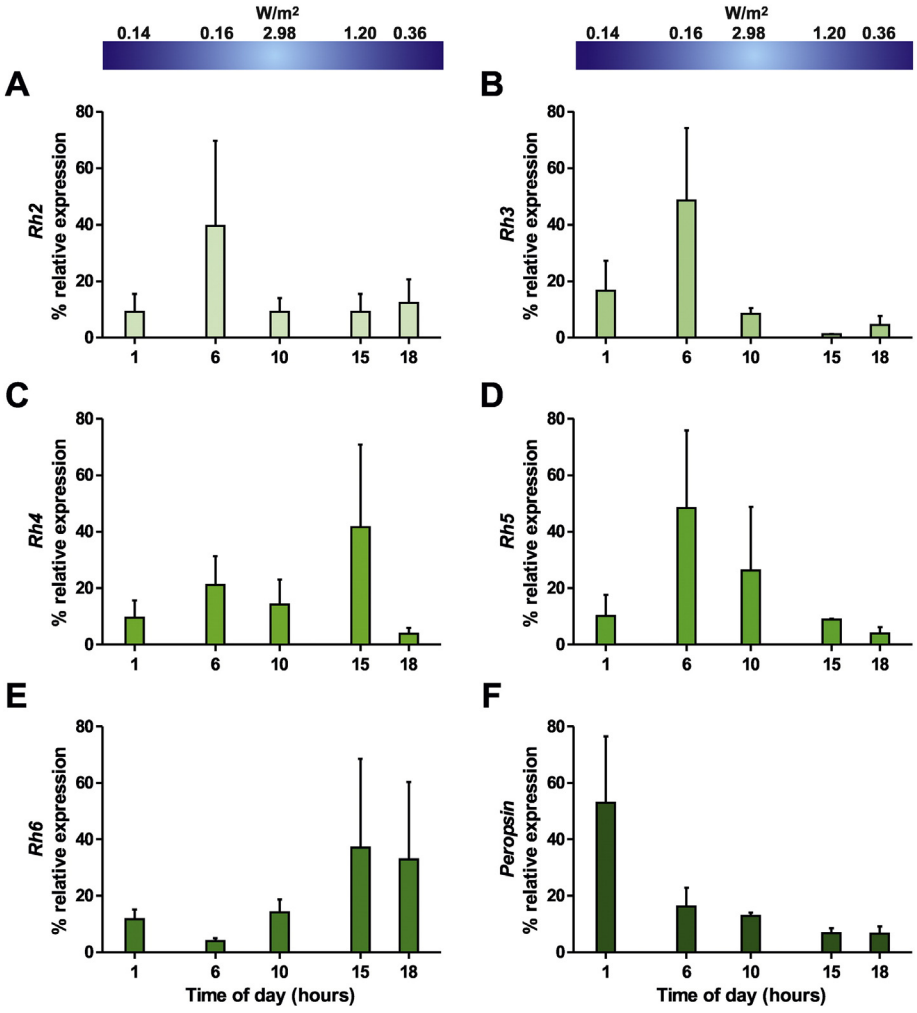

Fig. 5. Temporal patterns of expression of *Euphausia superba* opsins in the head of specimens caught under natural conditions during the Antarctic summer. Times of fishing (h) and irradiance (W/m^2) at the fishing depth are reported. Blue bars provide a representation of underwater irradiance at the fishing depth. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In summary, we have extended our knowledge on the *E. superba* opsin repertoire, including the discovery of a non-visual opsin. The future identification of other opsins as pteropsin (c-opsin), arthropsin (r-opsin) and neuropsin (group 4 opsin) could permit further reconstruction of the set of *E. superba* opsins and interpretation of the complex behavioural responses of krill to changes in illumination during the daily vertical migration and during the seasonal changes of sun irradiance.

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Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CB and RC conceived and designed the experiments. AB, EF, CDP, and GM performed the experiments. MT and SJ collected the specimens. GS, AB, CB, SJ, BM and RC analysed the data. CB, RC, MT, AB, BM and EF wrote the paper.

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