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Transcriptome analysis of the couch potato (CPO) protein reveals an expression pattern associated with early development in the salmon louse *Caligus rogercresseyi*

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ABSTRACT

The couch potato (CPO) protein is a key biomolecule involved in regulating diapause through the RNA-binding process of the peripheral and central nervous systems in insects and also recently discovered in a few crustacean species. As such, ectoparasitic copepods are interesting model species that have no evidence of developmental arrest. The present study is the first to report on the cloning of a putative *CPO* gene from the salmon louse *Caligus rogercresseyi* (*CrCPO*), as identified by high-throughput transcriptome sequencing. In addition, the transcription expression in larvae and adults was evaluated using quantitative real-time PCR. The *CrCPO* cDNA sequence showed 3261 base pairs (bp), consisting of 713 bp of 5′ UTR, 1741 bp of 3′ UTR, and an open reading frame of 807 bp encoding for 268 amino acids. The highly conserved RNA binding regions RNP2 (LFVSGL) and RNP1 (SPVGFVTF), as well the dimerization site (LEF), were also found. Furthermore, eight single nucleotide polymorphisms located in the untranslated regions and one located in the coding region were detected. Gene transcription analysis revealed that *CrCPO* has ubiquitous expression across larval stages and in adult individuals, with the highest expression from nauplius to copepodid stages. The present study suggests a putative biological function of *CrCPO* associated with the development of the nervous system in salmon lice and contributes molecular evidence for candidate genes related to host–parasite interactions.

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1. Introduction

The couch potato (CPO) protein was first isolated and cloned from *Drosophila* embryos, and its expression in neuronal precursors and their daughter cells evidences its pivotal role in the development and proper biological functioning of the peripheral and central nervous systems (Bellen et al., 1992; Glasscock and Tanouye, 2005). In addition to the expression of CPO in the nervous system, this protein is also expressed in larval ring glands, suggesting a biological function likely involved with neuroendocrine activity during larval development (Harvie et al., 1998). Regarding its molecular characteristics, CPO belongs to an RNA-binding protein family that has complex structural features such as a molecular size over 100 kb that encodes for at least three transcripts with alternative splicing, a lack of the AUG initiation codon, and the probable encoding of three different proteins (Bellen et al., 1992).

The *CPO* gene gains its name from several partial loss-of-function alleles that cause hypoactive behavior, such as abnormal geotaxis, phototaxis, and flight behavior, in adults of *Drosophila*, thus evidencing a mutant genotype in insects with life strategies that involve diapause (Saunders et al., 1989). Diapause is a classic adaptation to seasonality in arthropods, and its expression can result in an extreme extension of lifespan as well as an enhanced resistance to environmental challenges. So far, experiments using quantitative trait mapping, as carried out by Schmidt et al. (2008), have revealed the RNA-binding protein encoding CPO to be major genetic locus that modulates the diapause phenotype in *Drosophila melanogaster*. Furthermore, sequence analysis from natural populations demonstrated that variation for the diapause phenotype is caused by a single Lys/Ile substitution, and the polymorphisms also show geographic variation according to latitudinal cline (Tyukmaeva et al., 2011).

Aquatic organisms are constantly exposed to environmental stimuli or anthropogenic pollutants. As such, diapause is of critical importance for the life history of some crustaceans, including calanoid and cylopoid copepods (Hirche, 1996; Bron et al., 2011). For instance, a prolonged

Abbreviations: CPO, couch potato protein; UTR, untranslated region; RNP1, RNA binding region 1; RNP2, RNA binding region 2; RNA-seq, RNA sequencing; EST, expressed sequence tag; NCBI, National Center for Biotechnology Information; RACE, rapid amplification of cDNA ends; NJ, neighbor-joining method; SNPs, single nucleotide polymorphisms; PSVs, paralogous sequence variants; CDS, coding sequence; mRNA, messenger RNA; HKG, housekeeping gene; qPCR, quantitative PCR; PCR, polymerase chain reaction; ANOVA, analysis of variance; bp, base pairs; RRM, RNA recognition motif; blast, Basic Local Alignment Search Tool; GluCl, glutamate-gated chloride channel; GABA-Cl, γ-aminobutyric acid chloride channel; ABC, ATP-binding cassette transporters; Pgp, P-glycoprotein.

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dormancy phase during the life cycle of *Calanus finmarchicus* is a strategy, which allows it to avoid the unfavorable environmental conditions typical of the upper ocean from late summer to early spring in the subarctic North Atlantic (Carmichael et al., 2013; Olsvik et al., 2013). Furthermore, the functional genomics approach has been used to identity putative CPO homologs in *C. finmarchicus* (Christie et al., 2013). Mining of 454 pyrosequencing datasets and bioinformatics analyses from published databases resulted in the identification of two full-length CPO proteins and two additional putative sequences in the parasitic copepod *Lernaeocera branchialis* and the shrimp *Penaeus monodon*. According to the authors, no convincing CPO-encoding transcripts were identified for crustacean species with very large datasets, such as *Litopenaeus vannamei*, *Daphnia pulex*, and *Lepeophtheirus salmonis*, suggesting that CPO transcription has a low expression or is absent in some crustacean species.

The salmon louse *Caligus rogercresseyi* is a marine ectoparasite that is the cause of high economic losses for the salmon aquaculture industry in Chile. This parasite produces significant physiological and pathological consequences for infected fish (Bartsch et al., 2013). For instance, heavy infections lead to the erosion of the epidermis, exposure of the dermis, and in severe cases, exposure of skeletal muscle (Skilbrei et al., 2013). However, subclinical and physiological effects, which include stress, increased plasma cortisol concentrations, and changes in blood glucose, electrolyte, and plasma sex-steroid concentrations, are the more common impacts of sea lice (Saksida et al., 2013). Salmon lice infestations have been managed by antiparasitic agents such as organophosphates (Jones et al., 1992; Roth et al., 1996), pyrethroids (Sevatdal and Horsberg, 2003), hydrogen peroxide (Bravo et al., 2010), and avermectins (Duston and Cusack, 2002; Bravo et al., 2008). However, the overexposure of parasites to chemical control agents tends to promote drug resistance in wild populations (ffrench-Constant et al., 2004). In order to address this concern and that of the scarce genomic knowledge on the molecular functions affected by anti-sea louse treatments, investigations of novel candidate genes that could be related to the invertebrate nervous system are required. In this context, CPO is of interest to the current study as there is evidence related to its functional role during early developmental stages in *C. finmarchicus*. Interestingly, the model species *C. rogercresseyi* differs in comparison to other sea lice in regard to its life cycle strategy and association with its host fish and in its lack of a diapause stage.

This study is the first to report a putative *CPO* gene from the salmon louse *C. rogercresseyi*, as identified by high-throughput transcriptome sequencing. Additionally, transcription expression was performed in copepodid and chalimus larval stages and in female and male adults so as to evaluate expression patterns associated to ontogenetic development.

2. Materials and methods

2.1. Laboratory conditions for salmon lice culturing

Ovigerous specimens of *C. rogercresseyi* were collected from recently harvested fish at the AquaChile salmon processing plant in Puerto Montt, Chile. Individuals were transported back to the laboratory on ice, and their egg strings were then removed and placed in culture buckets supplied with seawater flow (12 °C) and gentle aeration. Eggs were allowed to hatch and develop until the infectious copepodid stage, at which point they were harvested for RNA extraction and cDNA library construction. The culture procedure was carried out according to Bravo (2010).

2.2. High-throughput sequencing from C. rogercresseyi transcriptome

Total RNA was extracted from pools containing 10 individuals for each larval stage (nauplius I, nauplius II, copepodid and chalimus) and adults (female and male) using the Ribopure™ kit (Ambion®, Life Technologies™, USA) following the manufacturer's instructions. Quantity, purity, and quality of isolated RNA were measured in TapeStation 2200 (Agilent Technologies Inc., Santa Clara, CA, USA) using the R6K reagent kit according to the manufacturer's instructions. Subsequently, double-stranded cDNA libraries were constructed using the TruSeq RNA Sample Preparation kit v2 (Illumina®, San Diego, CA, USA). Two biological replicates for each sample pool ($n = 12$) were sequenced by the MiSeq (Illumina®) platform using sequenced runs of 2×250 paired-end reads at the Laboratory of Biotechnology and Aquatic Genomics, Interdisciplinary Center for Aquaculture Research (INCAR), University of Concepción, Chile.

2.3. In silico identification of CPO from C. rogercresseyi

The raw data for each pool of samples were separately trimmed and de novo assembled in a unique file using CLC Genomics Workbench software (Version 6.0.1, CLC Bio, Denmark). The overlap settings for this assembly were a mismatch cost of 2, an insert cost of 3, a minimum contig length of 200 bp, a similarity of 0.8, and a trimming quality score of 0.05. This assembly yielded 125,813 contigs that were annotated according to Gene Ontology terms with the Blast2Go software (Conesa et al., 2005) by mapping against protein resources. Furthermore, tBLASTn analysis was performed against EST-datasets downloaded from the NCBI for crustacean species, and specifically for EST encoding putative CPO proteins. From this, a *CPO* homologous contig of 1643 bp from *C. rogercresseyi* transcriptome was identified with an e-value of 8.99E− 76.

2.4. Cloning of the CPO gene from C. rogercresseyi

The partial sequence of the *CPO* gene was used as a template for primer design with the Primer3 tool (Rozen and Skaletsky, 2000) (Table 1) included in the Geneious Pro software (Version 6.0, Biomatters, New Zealand). For gene amplification, total RNA was isolated using the TRI reagent® (Invitrogen, Carlsbad, CA, USA) protocol. The purity was determined (ratio A260/A280) with a Nanodrop ND1000 spectrophotometer (Thermo Fisher Scientific, Copenhagen, USA), and the integrity was determined by agarose gel under denaturant conditions. From 200 ng/μl of total RNA, cDNA was synthesized using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Glen Burnie, Maryland, USA). PCR analysis was performed using 1 μl of cDNA, 10 μM of each primer (Table 1), 1.5 mM MgCl2, and 0.06 U taq DNA polymerase (Thermo Scientific, Maryland, USA). PCR was performed in 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 45 s. The PCR product was observed by electrophoresis on 1% agarose gel and sequenced in the ABI 3730xl sequencer (Applied Biosystems, CA, USA). The sequence was analyzed using the Geneious Pro software (Version 6.0, Biomatters Ltda.). The resulting partial sequence was used for designing specific new primers (Table 1) for the amplification of the 3′ and 5′ UTR ends through the SMARTer™ RACE cDNA Amplification Kit (Clontech) according to the manufacturer's instructions. The fragments obtained for the 5′ and 3′ UTR ends were cloned in the TOPO TA Cloning Kit (Invitrogen™, Life Technologies, Carlsbad, CA, USA) and transformed into *E. coli* JM109

Table 1
Sequence of oligonucleotide primers used.

Primer name	Sequence (5′ to 3′)	Specification
Cr-CPO_3o	GAGATGATGATAAGGGAGGGA	3′-RACE
Cr-CPO_3i	ACGATACACACACGATGGAA	3′-RACE
Cr-CPO_5o	TATACTATAATAGCTTTGGATA	5′-RACE
Cr-CPO_5i	ATCTCCTCCAGGGAAGACAAA	5′-RACE
Cr-CPO_qF	CGAACACTATTTGTGAGCGG	qPCR
Cr-CPO_qR	CGAGGACGTCTTCCCATTTT	qPCR

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