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Transcriptome survey of the lipid metabolic pathways involved in energy production and ecdysteroid synthesis in the salmon louse *Caligus rogercresseyi* (Crustacea: Copepoda)

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ABSTRACT

The goal of this study was to identify and analyze the lipid metabolic pathways involved in energy production and ecdysteroid synthesis in the ectoparasite copepod *Caligus rogercresseyi*. Massive transcriptome sequencing analysis was performed during the infectious copepodid larval stage, during the attached chalimus larval stage, and also in female and male adults. Thirty genes were selected for describing the pathways, and these were annotated for proteins or enzymes involved in lipid digestion, absorption, and transport; fatty acid degradation; the synthesis and degradation of ketone bodies; and steroid and ecdysteroid syntheses. Differential expression of these genes was analyzed by ontogenic stage and discussed considering each stage's feeding habits and energetic needs. Copepodids showed a low expression of fatty acid digestion genes, reflected by a non-feeding behavior, and the upregulation of genes involved in steroid biosynthesis, which was consistent with a pathway for cholesterol synthesis during ecdysis. The chalimus stage showed an upregulation of genes related to fatty acid digestion, absorption, and transport, as well as to fatty acid degradation and the synthesis of ketone bodies, therefore suggesting that lipids ingested from the mucus and skin of the host fish are metabolized as important sources of energy. Adult females also showed a pattern of high lipid metabolism for energy supply and mobilization in relation to reproduction and vitellogenesis. Adult females and males revealed different lipid metabolism patterns that reflected different energetic needs. This study reports for the first time the probable lipid metabolic pathways involved in the energy production and ecdysteroid synthesis of *C. rogercresseyi*.

1. Introduction

Sea lice are copepods from the Caligidae family and are responsible for severe parasitic infestations in salmonid farming. Economic losses from the sea lice genera *Caligus* and *Lepeophtheirus* are estimated at US$480 million per year (Costello, 2009). Among sea lice species, *Caligus rogercresseyi* is the dominant species in Chile, and its epidemiological impact is a serious concern for the aquaculture industry (Hamilton-West et al., 2012). *C. rogercresseyi* was first identified in 1997 while parasitizing Atlantic salmon (*Salmo salar*) in Chile (Boxshall and Bravo, 2000). Its life cycle includes the following eight development stages: three planktonic stages which include two nauplius phases and an infective copepodid phase, and five parasitic stages, which include four chalimus phases and an adult phase.

The planktonic stages live on endogenous reserves, and when the copepodid settles on the host, it molts and extrudes a frontal filament with which it attaches itself to the host (González and Carvajal, 2003). The chalimus stage develops attached to the host by feeding on the mucus and skin of the area confined by the frontal filament. In turn, mature adults, which are no longer attached to the host, move freely on the skin's surface, thus increasing the area available for feeding.

The food regime of *C. rogercresseyi* is thought to be similar to the dominant European sea louse *Lepeophtheirus salmonis*, which consists of the mucus, skin, and blood of the host (Kabata, 1974; Brandal et al., 1976). The activities associated with feeding cause irritation, increased mucus secretion, abrasions, the loss of scales, skin disruptions, and wounds, all of which affect the barrier function of the skin and fish osmoregulation (Tveiten et al., 2010), ultimately facilitating secondary infections either indirectly or directly as a vector (Oelckers et al., 2014). Additionally, the presence of parasitic sea lice on fish acts as a chronic stressor that compromises homeostatic balance, immunocompetence, and overall health (Bowers et al., 2000; Costello, 2006).

One of the life cycle processes essential for a sea louse's success is molting, or ecdysis. Ecdysis is controlled by ecdysteroids and derivatives, which are synthesized from cholesterol (reviewed by Mykles, 2011). Cholesterol and other sterols are essential nutrients for crustaceans since they lack the ability to synthesize the steroid ring de novo

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(Grieneisen, 1994; Rabid et al., 1999). As such, sources of cholesterol are restricted to diet and endogenous reserves. Apart from being the precursor to ecdysteroid synthesis, cholesterol also forms the basis for the synthesis of sex hormones and other components. It is also an important membrane component with fundamental roles in the maintenance of membrane stability, permeability and fluidity regulation supporting environmental adaptations such as thermal adaptation, an important feature especially in ectothermic organisms like copepods (Hassett and Crockett, 2009). In addition, membrane cholesterol also plays a role in the absorption and transport of fatty acids (revised by Sánchez-Paz et al., 2006). Hence, knowledge on cholesterol and the associated lipid metabolism is of great interest for understanding sea lice development and for improving current management strategies toward achieving sustainable salmonid aquaculture.

Despite the existing information for the sea lice life cycle and epidemiology, information regarding metabolism is rather scarce and is mostly based on research in insects or crustaceans like decapods and other marine copepods such as *Calanus* genus members. However, recent advances in molecular biology and high-throughput sequencing techniques have increased the ability to acquire information on metabolic pathways and physiological mechanisms (Eichner et al., 2008; Bron et al., 2011; Sutherland et al., 2012).

The aim of this study was to screen and identify fatty acid metabolic pathways involved in energy production and ecdysteroid synthesis in *C. rogercresseyi* using high-throughput transcriptome sequencing. To accomplish this, the differential expression of candidate genes was investigated in silico during the distinct life stages of *C. rogercresseyi*, including the infectious copepodid, parasitic chalimus, and adult male and female stages.

2. Materials and methods

2.1. Salmon lice culturing

Ovigerous specimens and chalimus larvae (stages 3–4) of *C. rogercresseyi* were collected from fish (*S. salar*) obtained from different tanks of a commercial salmon farm located in Puerto Montt, Chile. Individuals were transported on ice to the laboratory, and the egg strings were subsequently removed and placed in separate culture buckets supplied with aerated seawater flow at 12 °C. Eggs were allowed to hatch and develop until the infectious copepodid stage following Bravo (2010).

2.2. High-throughput sequencing from C. rogercresseyi transcriptome

Twenty individuals from each larval (copepodid and chalimus) and adult (female and male) stage were pooled from different culture and inoculation tanks and were treated and analyzed following Gallardo-Escárate et al. (2014). For each stage individuals were pooled into two biological replicates ($n = 10$) and total RNA was extracted with the RibopureTM kit (Ambion®, Life Technologies™, USA) following the manufacturer's instructions. Concentration and purity were measured in a spectophotometer (ND-1000, Nanodrop Technologies), and RNA integrity was assessed with electrophoresis in MOPS/formaldehyde agarose gels at 12% stained with 0.001% ethidium bromide. In addition, the quality of isolated RNA was further measured in a Bioanalyzer TapeStation 2200 (Agilent Technologies Inc., Santa Clara, CA, USA) using the R6K reagent kit according to the manufacturer's instructions. The RNA extracts with 260/280 and 260/230 purity indices ≥ 2.0, integral RNA (assessed by electrophoresis) and with a Bioanalyzer assessment RIN > 8 were selected for analysis. Double-stranded cDNA libraries were then constructed using the TruSeq RNA Sample Preparation kit v2 (Illumina®, San Diego, CA, USA). Two biological replicates for each stage of *C. rogercresseyi* were separately sequenced by the MiSeq (Illumina®) platform according to maker's instructions, using sequenced runs of 2×250 paired-end reads at the Laboratory of Biotechnology and Aquatic Genomics, Interdisciplinary Center for Aquaculture Research (INCAR), Universidad de Concepción, Chile.

2.3. In silico identification of genes for target metabolic routes in C. rogercresseyi

The experimental design and workflow for this study are presented in Fig. 1. Raw reads obtained from sequencing the four types of cDNA samples which represented the different developmental stages of *C. rogercresseyi* were separately screened for quality using CLC Genomics Workbench (Version 6.5, CLC Bio, Denmark) QC integrated tool. This tool allowed trimming adapter remains, sequences with high occurrence of ambiguous bases, and low-complexity regions. In addition, reads shorter than 15 bp were removed. The size selected and trimmed reads obtained via Illumina sequencing of the four stages were de novo assembled in a unique file using the same software. 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 resulted in 84,023 contigs that were mapped based on a tBLASTx analysis against UniprotKB/Swiss-Prot database, NCBI non-redundant protein databases and tBLASTn analysis against EST-datasets downloaded from the NCBI for crustacean species. Annotations were assigned to each assembled contig based on the best hit retrieved from BLAST analysis and were accepted under a cutoff E-value $\leq 1E-05$. Assembled sequences were also annotated with Gene Ontology (GO) terms to describe biological processer, molecular functions and cellular components using the Blast2Go software (Conesa et al., 2005) under a plugin of CLC software. To identify genes that are orthologs to genes of the target metabolic pathways the consensus sequences of contigs were mapped against KEGG GENES database and were annotated by the KEGG Orthology (KO) system using the KEGG Automatic Annotation Server (KAAS) (Moriya et al., 2007), using the bi-directional best hit (BBH) method with an E-value $\leq 1E-05$. Since this system involves a pathway base definition of orthologous genes, once the KO identifiers are assigned to the contigs by the KAAS server analysis, organism-specific pathways can be identified. According to this, the lipid digestion, absorption, and transport; fatty acid degradation; the synthesis and degradation of ketone bodies; and steroid and ecdysteroid synthesis pathways were identified and the contigs annotated with the participating genes (which were attributed to the respective KO entry) were retrieved for subsequent analysis. Since the model species available in KEGG database are not genetically close to *C. rogercresseyi*, we manually searched for all the genes participating in the target pathways in GenBank databases for species with higher proximity with *C. rogercresseyi*. The obtained sequences were blasted against our transcriptomic database for *C. rogercresseyi* to retrieve contigs that were not identified via KAAS method. In addition, the contigs annotated for genes involved in the target pathways (with greatest hit and E-value lower than $1E-05$) after the tBLASTx analysis were manually screened and retrieved to complement the analysis via KAAS method. The screen for contigs related to genes involved in the target pathways (via KAAS analysis, tBLASTx and manually blasting downloaded sequences) resulted in 284 contigs annotated for genes with products related to the lipid digestion, absorption, and transport; fatty acid degradation; the synthesis and degradation of ketone bodies; and the steroid and ecdysteroid synthesis pathways.

2.4. Differential expression analysis

The differential expression of the 284 contigs annotated as genes that compose the target pathways was assessed by RNA-seq expression analysis. The reads of each *C. rogercresseyi* stage (copepodid, chalimus, adult female and adult male) were mapped against the reference contigs (selection of 284 contigs), and the expression level of each transcript was quantified in reads per kilobase of the transcript per million mapped reads (RPKM) in each of the four stages' transcriptome. Differentially