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Molecular Characterization and Transcription Analysis of *P-Glycoprotein* Gene from the Salmon Louse *Caligus rogercresseyi*

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Abstract

Despite the efforts to manage the infestation *Caligus rogercresseyi* on salmon species, overuse of chemical antiparasites such as avermectins, organophosphates and pyrethroids have increasingly generated drug resistance, impacting on the efficacy of salmon lice control measures. So far, previous reports have evidenced that the ATP-binding cassette transporter P-glycoprotein (Pgp) is a candidate gene implicated in the response of salmon lice to neurotoxins. However, transcription patterns of *Pgp* in presence of pyrethroids and the expression pattern during the ontogenetic stages are still not complete elucidated. Herein, this study characterizes the Pgp mRNA from *C. rogercresseyi* (*Cr-Pgp*) and evaluates the transcription expression in during its lifecycle, and also in adults exposed to the antiparasitic drug deltamethrin (AlphaMax®). The molecular characterization of *Cr-Pgp* showed a complete sequence of 4,730 bp, containing a 5'UTR of 56 bp, 3'UTR of 833 bp, and open reading frame (ORF) of 3,840 bp encoding for 1,280 amino acids. Interestingly, eleven SNPs were identified, being two of them nonsynonymous polymorphisms. *Cr-Pgp* transcription expression was evaluated in conjunction with cytochrome P450 due its well-established role as key molecule in drug detoxifications. Herein, *Cr-Pgp* transcription was mainly associated to adult females than males exposed to deltamethrin, which was also linked with cytochrome P450 expression in adult females at 2 ppb of deltamethrin. This study suggests that *Cr-Pgp* gene is involved in pyrethroid detoxification and evidences specific expression patterns related to developmental stages, as well as provides novel SNPs that could be associated with resistance/susceptibility to delousing drugs.

Keywords: *Caligus rogercresseyi*; P-glycoprotein; qPCR; Pyrethroid; P450 enzyme; SNP

Introduction

One of the most important defense mechanisms of ectoparasites against chemotherapeutic or xenobiotic agents is related with the ATP-binding cassette (ABC) transporter family [1] he ABC transporters have been associated to multidrugs resistance (MDR) in different organisms. ABC proteins are able to recognize different chemical substrates and to control their transport on a cellular level [2]. Through this, the ABC proteins have a pivotal role in blocking high concentrations neurotoxins, which intend to enter into the nervous system of parasitic invertebrates [3,4]. Within this family of proteins are the multidrug resistance-associated protein, the half-transporters, and the P-glycoprotein (Pgp). Pgp is a highly conserved membrane-bound protein in eukaryotes and prokaryotes, and it functions as an ATP-dependent efflux pump, reducing the concentration of drugs in the cells and conferring resistance [5-7]. In mammals, Pgp has been reported as one of the principal barriers against the entrance of drugs from the blood stream into the nervous system [8].

Caligus rogercresseyi [9] constitutes the main cause of economical losses in farmed salmon Chilean industry. Infection by the salmon louse does not generally cause host death, but its interaction with the salmon causes a high level of stress which depresses the immune system and increases susceptibility to bacterial and viral pathogens [10,11]. In order to control the prevalence of *C. rogercresseyi*, xenobiotics like pyrethroids [11], organophosphates [12], and avermectins [13] have been used. The overuse of these chemical agents has been shown to diminish their efficiency in treating parasite infestations due to resistance [14-16]. The time required by the parasite to acquire resistance depends on various factors, among which are environmental conditions and the concentration and frequency of the treatment [12,17-19]. Numerous studies have shown behavioral differences of the salmon louse when it is exposed to parasiticides depending on its developmental [20-22]. Avermectins used for the salmon louse control, such as emamectin benzoate (EMB), have been shown to be effective during the development stages of copepods [23]. Herein, Pgp has been observed to be the main defense of the salmon louse against xenobiotics, particularly to EMB, which joins to the gamma–aminobutyric acid (GABA) receptors, causing paralysis [24]. The authors Heumann et al. [17] identified *Pgp* in *Lepeophtheirus salmonis* [18] that were overexpressed in resistant strain after 24 h of exposure to EMB, suggesting their participation in the response to the antiparasite. Other xenobiotic used for sea lice control is the pyrethroid named deltamethrin (Alphamax®). Deltamethrin is applied by topical treatments and interact with sodium channel depolarized the nerve ending [26]. Similarly, it has been observed that the family of cytochrome P450 enzymes participates in pyrethroid detoxification process in insects [27-29].

In Chile, the most widely used xenobiotic for the salmon lice control is deltamethrin [30]. However, there are no studies addressed to evaluate the transcription expression of *Pgp* from salmon louse exposed to deltamethrin. Moreover, studies are limited in regards to the functions that detoxification genes like *Pgp* have during the development of salmon lice, and it is only possible to find

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comparative studies on the effects that EMB has on expression levels in adult copepods. Given this, the aims of the present study were to perform a molecular characterization of the P-glycoprotein in *Caligus rogercresseyi*, evaluate their expression in adults treated with deltamethrin and assay its expression on a transcriptomic level during larval and adult stages.

Materials and Methods

Samples

Samples from the copepodid and chalimus stages and of adult male and female sea lice were collected from a commercial farm located in region X in Chile (41°40'48.5"S; 73°02'31.34"O¨). The permissions for the sea lice collections were authorized by Marine Harvest S.A, Ruta 226, Km. 8, Camino El Tepual, Puerto Montt, Chile. The specimens were used to characterize the *Pgp* mRNA. A pool of each stage was fixed in RNAlater® RNA Stabilization Reagent (Ambion, USA) and stored at -80ºC for subsequent RNA extraction.

Bioassay

Formulations of deltamethrin were made using "AlphaMax®" (10 mg deltamethrin/mL) diluted with seawater to six concentrations (0, 1, 2, 3 ppb). A stock solution of 10 ppm was prepared for each bioassay by diluting 1 ml AlphaMax in 999 ml seawater. The other concentrations were made by serial dilutions. Thirty adults (fifteen females and five males) of sea louse were exposed to each concentration of the deltamethrin formulation in petri plate containing 50 ml of seawater (three biological replicates were also evaluated). The exposure period was 40 min according to the recommended treatment time for AlphaMax. During the exposure, organisms were maintained at 12°C. After 24 h the sea louse were fixed in RNAlater® RNA Stabilization Reagent (Ambion, USA) and stored at -80ºC for subsequent RNA extraction.

P-glycoprotein gene isolation

A partial sequence of the *Pgp* gene was identified from a *C. rogercresseyi* EST-database from Illumina MiSeq library generated for *C. rogercresseyi* in the Laboratory of Biotechnology and Aquatic Genomics, Interdisciplinary Center for Aquaculture Research (INCAR), University of Concepción. The sequence was used as a template for primer design with the Primer3 tool [30] (Table 1) included in the Geneious Pro software [31]. 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 synthetized 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 $MgCl_2$, 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 5.1.6 software [31]. The resulting partial sequence, termed *Cr-Pgp*, 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 electrocompetent bacteria in LB/amp/IPTG/Xgal plates overnight at 37°C. The positive clones were selected and purified to obtain plasmid by using the E.Z.N.A® Plasmid DNA Mini Kit II (Omega Bio-tek, Doraville, GA, USA). The plasmids obtained were sequenced in both directions, and their sequences were then assembled using the software Geneious 5.1.3. [31].

Primer name	Primer Sequence
Cr-Pgp_F	ACACCTTTCCTGGATGGAGTCACTC
Cr-Pgp_R	CTTGCGTTCCATTTGTCCAT
Cr-Pgp_Race5_R1	AATGAGACCAATGATGGCCG
Cr-Pgp_Race5_R2	AAAGCGAGTGACTCCATCCA
Cr-Pgp_Race3_F1	TTGAATCAAGGACGAGTGGC
Cr-Pgp_Race3_F2	AAGTCACGACTCACTCCTCT
Cr_P-gp_qF	AATCTTCCGAGGTGTCCTCA
Cr_P-gp_qR	ACAGAGTTCTCGGTCGTCTA
Cr_P450_qF	CACCAGGATTTTCAGCTCCA
Cr_P450_qR	TACTTCCCGGAACCCAATGA
Cr_1266_F	TGATGGTGGATGATGTGGAC
Cr_1266_R	AAAACTGGCTCTTGGCCTAC
Cr_1353_F	TCGGGATAATATCTGCGTCG
Cr_1353_R	AAAATCGTACGCATTCGCAG

Table 1: Sequences of primers used in this study.

Amino acid sequence analyses

Protein alignments were conducted using MUSCLE, and phylogenetic trees were constructed using the neighbor-joining method with 1,000 bootstrap repetitions. Both analyses were carried out in Geneious 5.1.6 [31]. For identifying the conserved domains and active sites, the PROSITE [32] and SMART [33] platforms were used.

SNPs mining and validation

Using the assembly obtained for the *Pgp* sequence, SNPs mining was performed using the Genomics Workbench 5.0.1 software (CLC bio, Denmark). The parameters used were as follows: window length=11, maximum gap and mismatch count=2, minimum average quality of surrounding bases=15, minimum quality of central base=20, maximum coverage=100, minimum coverage=8, minimum variant frequency (%)=35.0, and maximum expected variations (ploidy)=2.

For SNPs validation, high resolution melting analysis (HRMA) was performed using primers designed with the Primer3 tool [30] (Table 1) included in the Geneious Pro software [31]. Total DNA was isolated using the E.Z.N.A DNA extraction kit (Omega) from 20 adult salmon lice (10 females and 10 males). The PCR was carried out in 10 µl reaction with 13 ng of template DNA using the Fast EvaGreen® qPCR Master Mix (Biotum). For HRMA, thermal cycling was performed with an ECO Real Time PCR System (Ilumina Inc, USA) as follows: 2 min for enzyme activation, 40 cycles at 95°C for 5 s, 56°C for 5 s, and 60°C for 25 s. HRMA data were collected between 60 and 95°C with a temperature interval of 0.3%. Genotyping was analyzed for the presence of a discrete melting curve using the software EcoReal Time System (Illumina Inc. USA).

Expression profiles of *Cr-Pgp*

Transcripts levels were evaluated for the copepodid and chalimus stages and for adult females and males of *C. rogercresseyi*. On other hand, an expression level of *Cr-Pgp* in sea lice exposed to deltamethrin were evaluated and compared with P450 gene expression. The primers

for qPCR reactions were designed from the sequences obtained from *Cr-Pgp* and from partial sequence of *Cr-P450* identified in Illumina MiSeq library for *C. rogercresseyi* (Table 1). The qPCR runs were performed with StepOnePlus™ (Applied Biosystems, Life Technologies, USA) using the comparative ΔCt method. Each reaction was conducted with a volume of 10 µL using the Maxima® SYBR Green/ROX qPCR Master Mix (Thermo Scientific, USA). The amplification conditions were as follows: 95°C for 10 min, 40 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. *Beta-tubulin* was selected as the housekeeping gene (HKG) due to its stable value as inferred through the NormFinder algorithm. The other HKGs tested were *alpha-tubulin, elongation factor alpha*, and *beta-actin*. Statistical analyses were performed using the software Statistical 8.0. The data obtained were analyzed through the Kruskal-Wallis test with the Statistica software (Version 7.0, StatSoft, Inc.). Statistically significant differences were accepted with a $p<0.05$.

Results

Molecular characterization of *Cr-P-glycoprotein*

The sequence for the characterized *Pgp* gene was obtained by assembling partial sequences identified in the Illumina cDNA library for *C. rogercresseyi* and the fragment amplified by RACE-PCR. The full

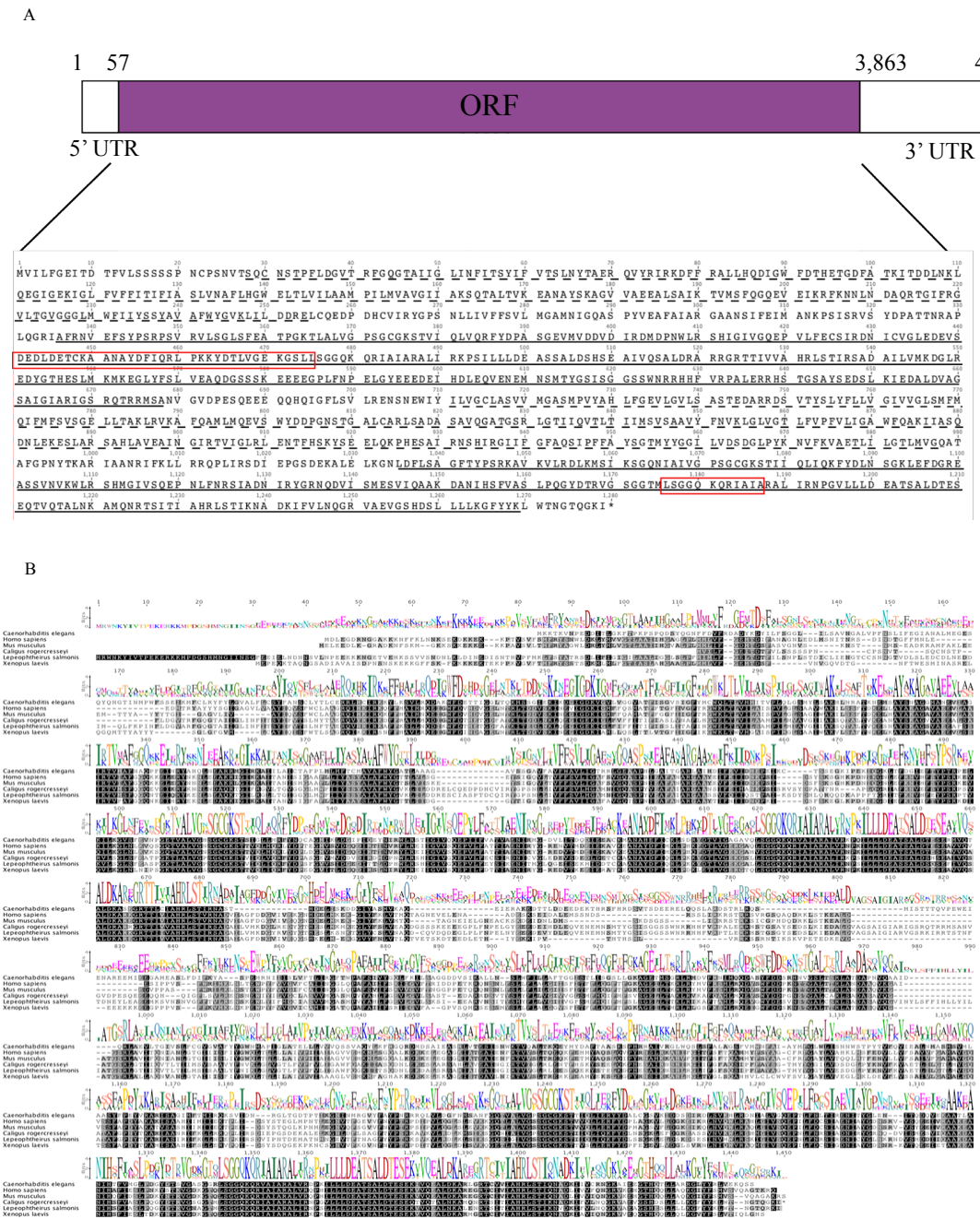

Figure 1: *Cr-Pgp* molecular characterization. (A) Full-length cDNA sequence and predicted amino acid sequence for *Cr-Pgp* (GenBank access nºKF704367). Transmembrane domain in discontinuous underline; Nucleotide binding domain in underlined; ABC transporter signature motif in red box. (B) Multiple alignment of *Cr-Pgp*, in black the conserved amino acid residues.

sequence for the *Cr-Pgp* gene was deposited into the NCBI Genbank database under GenBank Accession No. KF704367. The complete cDNA sequence for *Cr-Pgp* had 4,730 bp with an open reading frame (ORF) of 3,840 bp encoding for 1,280 amino acids, while the 5'UTR was 56 bp and the 3'UTR was 833 bp (Figure 1A). The amino acid sequence had a predicted molecular weight of 140.3 kDa and an isoelectric point of 6.07. ABC transporters minimally consist of two conserved regions: a highly conserved ATP binding cassette (ABC) and a less conserved transmembrane domain (TMD). These can be found on the same protein or on two different ones. Herein, the translation of the *Cr-Pgp* cDNA sequence detected ABC-transporter transmembrane domains (TMDs) and nucleotide binding domains (NBD), arranged in an N-terminal to C-terminal order of TMD-NBD-TMD-NBD, the typical domain architecture of ABC full transporters (Figure 1A). Further, two TMDs with six transmembrane helices each, and two cytosolic domains were predicted.

From a multiple sequence alignment of *Cr-Pgp,* a 75.7% identity was observed with sequences described for *Lepeophtheirus salmonis* and a 43.3% identity was observed with the homologues sequences described for *Homo sapiens* and *Drosophila menalogaster* (Figure 1B). After performing a phylogenetic analysis through the neighbor-joining method, two clades were observed, one of these being for vertebrates and the other for invertebrate species. The *Cr-Pgp* sequence was grouped, as expected, within the sequences of *Cr-Pgp* reported for *L. salmonis* (Figure 2).

Expression analysis

To determine the effect of deltamethrin over *Cr-Pgp* expression we evaluated individuals obtained from bioassays and compared with a gene involved in detoxification process as P450. From the qPCR analysis higher expression levels of *Cr-Pgp* and *Cr-P450* were observed in samples exposed to 2 ppb of deltamethrin (Figure 3). Furthermore, *Cr-Pgp* in female shows higher transcription levels

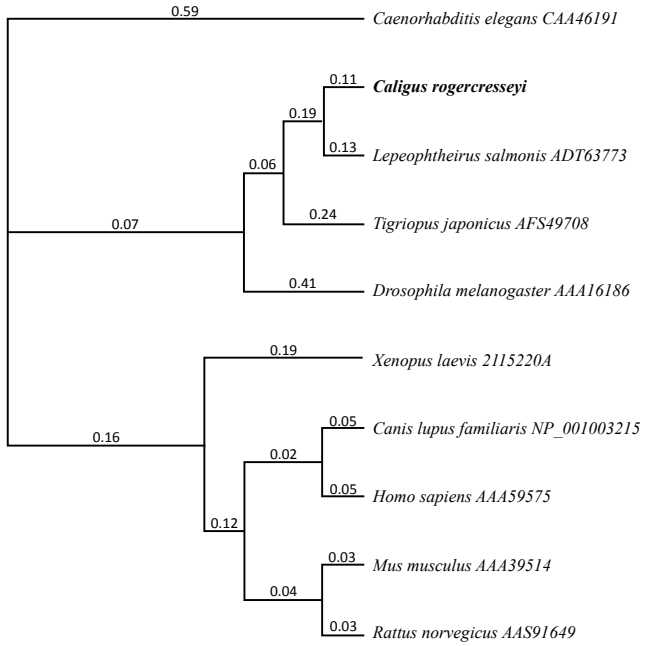

Figure 2: Phylogenetic analysis of *Cr-Pgp* with vertebrate and invertebrate P-gp orthologous. The phylogenetic tree was constructed using the full amino acid sequences with MUSCLE software and 1000 bootstrap repetitions.

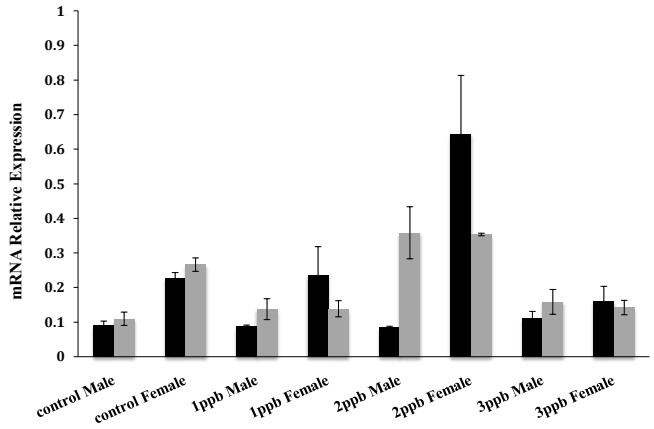

Figure 3: Relative expression level of *Cr-Pgp* and comparison with *Cr-P450* in adults exposed to deltamethrin. Black bar: *Cr-P-gp*gene expression, Grey bar: P450 gene expression. All data have significantly differences ($p<0.05$).

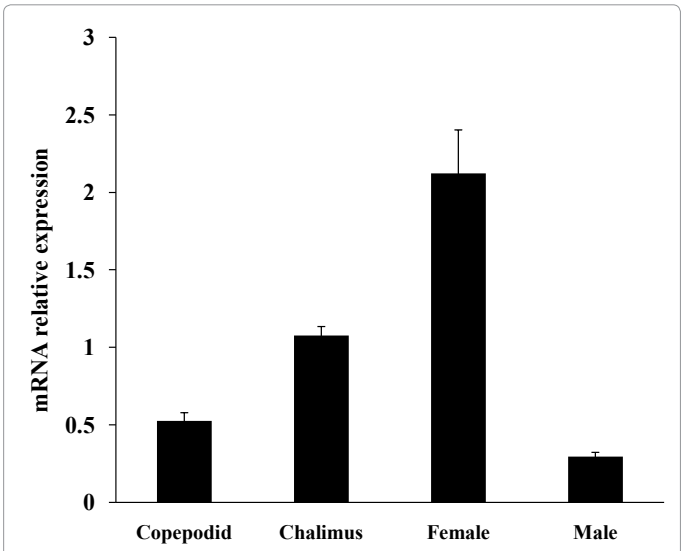

Figure 4: Relative expression level of *Cr-Pgp* in different developmental stages. Each bar represents the mean normalized expression levels of replicates (N=3). All data have significantly differences ($p<0.05$).

Deltamethrin (ppb)	Female	Male
0	100	100
1	53,3	13,3
2	26,7	20,0
3	26,7	0,0

Table 2: Percentage of survival of sea louse exposed to deltamethrin.

than males, which is reflected in the higher percentage of survival in female individuals (Table 2). Contrary, *Cr-P450* not significant differences in gene expression between male and female samples were observed (Figure 3). Through qPCR, the transcript levels for the characterized gene were analyzed in different stages of development copepodid, chalimus, and male and female adults. Relative expression calculated using housekeeping gene *beta-tubulin* presented a stability value as calculated by the NormFinder algorithm. Comparing the four samples, the highest expression levels of *Cr-Pgp* were observed in adult females, and an overregulation was registered for adult males (Figure 4). Comparing the copepodid and chalimus stages, an up-regulation

Position	Variation	Frequency	Substitution
1091	G/A	27,27	synonymous
1121	C/T	26,97	synonymous
1196	C/T	47,22	synonymous
1266	A/T	36,84	nonsynonymous
1353	G/A	33,8	nonsynonymous
1415	G/A	28,57	synonymous
2603	C/T	43,75	synonymous
2666	C/G	42,99	synonymous
2933	G/A	30	synonymous
3458	G/A	36,51	synonymous

Table 3: SNPs variation identified in *Cr-Pgp* sequence.

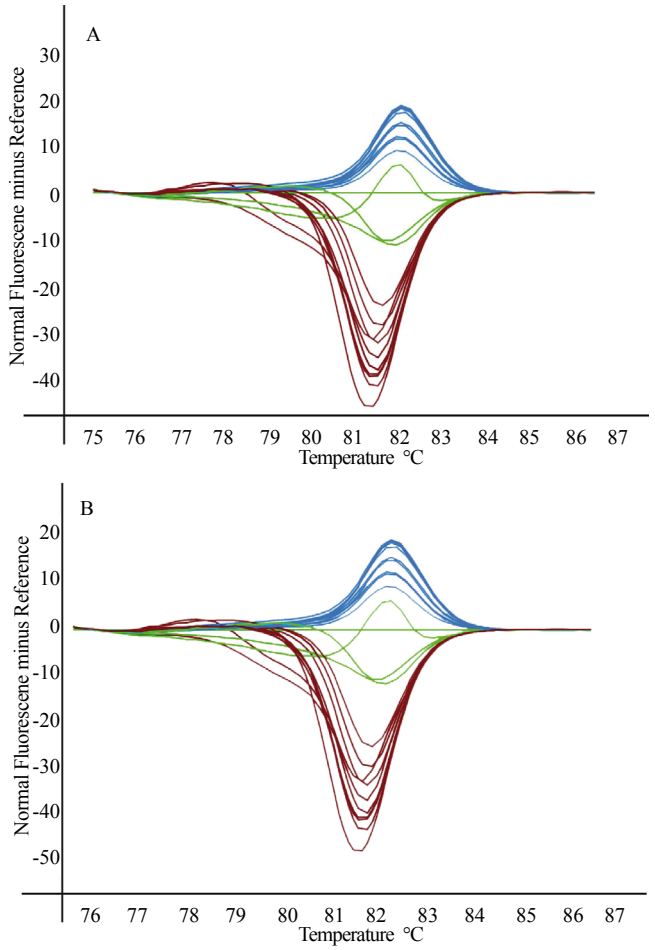

Figure 5: High Resolution Melting curves. A: difference curve for visual identification of allele A/T position 1,266; B: difference curve for visual identification of allele G/A position 1,353.

was observed in chalimus samples. The expression analysis showed that *Cr-Pgp* it was strongly regulated in females, suggesting a better detoxification mechanism to pyrethroid.

SNPs identification and validation

From the characterization of nucleotide sequence, a total of eleven SNPs present in the ORF of *Cr-Pgp* obtained from 10,921 reads were identified. Two SNPs located at +1,266A/T and +1,353 G/A were nonsynonymous and involved in amino acid changes between Met/Leu and Val/Ile (Table 3). Furthermore, 20 adults of *C. rogercresseyi* were genotyped by HRMA analysis and showed three profiles of melt curves, evidencing the different allelic variants for these SNPs (Figure 5). However, it is necessary evaluates the SNPs in groups with different susceptibility to pyrethroids in order to associate putative mutations with resistance to xenobiotics.

Discussion

ABC transporters are transmembrane proteins responsible for the translocation of various compounds in prokaryotic and eukaryotic organisms [8]. In mammals and fish, proteins belonging to this family have a role in the metabolism of xenobiotics [8]. In bivalves which are constantly exposed to toxins, the proteins of this family have been observed to act as a protection barrier against xenobiotics in the gills, thus restricting the transport of toxic substances to other tissues [34]. One member of this family is the P-glycorpotein, which is a highly conserved protein in vertebrate and invertebrate organisms and which is formed by two nucleotide-binding domains (NBD) and two transmembrane (TMB) domains. Moreover, Pgp has two drug binding sites which act as a substrate and which restrict the quantity of drugs incorporated into the cells through ATPasa activity [35]. One of the substrates with the greatest affinity for Pgp are the avermectins, which are used for controlling parasites, among which are nematodes and copepods, by acting as a barrier to the nervous system [3,4,36].

The overuse of xenobiotics for controlling infestations of salmon lice without an adequate control and management system has generated an increased resistance in natural populations. This study characterized the full cDNA of *P-glycoprotein* in *C. rogercresseyi*, evidencing a coding sequence of 1,280 amino acids that contain two highly conserved TMB and NBD domains [8,35,36]. The phylogenetic analysis showed that *Cr-Pgp* had an identity of 76.7% with its homologue describe in *L. salmonis* [24] and 77.6% of identity with SL-PGY1 describe by Heumann [17]. Moreover, this gene showed a high homology with sequences described for *Homo sapiens* and *Drosophila melanogaster*, confirming *Cr-Pgp* as a member belonging to the ABC transporter family.

In cell lines of mammals, the accumulation of xenobiotic agents has been observed to increase chemical resistance, which is associated with changes in the expression of *Pgp* [6]. As observed in nematodes treated with avermectins, *Pgp* increased its transcripts levels and conferred resistance, making treatment with avermectins an inefficient method [3,7]. In *L. Salmonis* [17,25] and *C. rogercresseyi* [21], adult copepods exposed to EMB evidences an increase of their Pgp transcriptional activity that in turn affect the efficiency of EMB as chemical antiparasite.

The metabolism of pyrethroids in many species has been associated to overexpression of cytochrome enzyme P450 [36-38]. In *Helicoverpa armigera* at least five CYP genes that encode a P450 enzyme were overexpressed in a resistance strain, suggesting that multiple P450 enzymes are involved in deltamethrin resistance [19]. Besides, an increased sensibility to deltamethrin was observed in *L. salmonis* after pre-treatment with piperonyl butoxide (PBO), which is a P450 inhibitor, suggesting the role of these enzyme to pyrethroid metabolism in copepods [39]. Bariami et al. [12] in *Aedes aegypti* identified twenty genes from the P450 superfamily which were overexpressed in resistant strains, also the authors observed an increased level of transcripts of ABC transporter gene, which is orthologous to *ABCB4* describe for *Anopheles gambiae* [39]. These results indicate that ABC transport proteins could be involved in pyrethroid detoxification process. Our results evidenced an overexpression of *Cr-Pgp* and *P450* genes in sea louse exposed to 2 ppb of deltamethrin, suggesting a relation between both genes and a potential resistance to pyrethroid in *C. rogercresseyi*.

However, these results are different to some previous investigations. In several studies in mouse cell lines, between Pgp and various pesticides demonstrate a low interaction among pyrethroids and Pgp protein [40]. Furthermore, in the parasite *Rhipocephalus microplus* exposed to ABC transporter inhibitors was observed higher mortality in individuals treated with iveromectin (averomectin), but not in parasites treated with cypermethrin (pyrethroid) [41]. This could indicate an absence of pyrethroid efflux mediated by Pgp in these studied species.

Studies of xenobiotic resistance in salmon lice and the evaluation of Pgp expression has only been carried out in adult individuals exposed to EMB, showing a difference in expression levels between males and females [19]. Analysis from the current study shows that female specimens of *C. rogercresseyi* presented higher levels of Pgp than males, which is contrary to the information reported for *L. salmonis*. Additionally, Igboeli [18] observed that the increased expression of *Pgp* observed in males occurred at an earlier stage than in females, granting males a greater resistance to EMB. From this, it is possible to predict that the greater expression of *Pgp* observed in females of *C. rogercresseyi* in the current study could be linked to a greater resistance. This is consistent with the results obtained from *Cr-Pgp* expression in samples exposed to deltamethrin, where females present higher expression levels in relation to males. In regards to *Pgp* expression, there are no reports for the earliest developmental stages. It is known that when copepodids are exposed to EMB, development is interrupted, and in the chalimus stage, maturation is delayed [22]. Through expression analysis, it is inferred that copepodids of the analyzed population would be more susceptible to EMB given that the expression *Cr-Pgp* transcripts in this stage were lower than in the chalimus stage and adult females of *C. rogercresseyi*. An overregulation of chalimus *Cr-Pgp* was observed in comparison with expression in copepodids and males, whose expression patterns were similar to those observed in pre-adults and female adults of *L. salmonis* [19].

The expression and functionality of Pgp could be influenced by the presence of molecular polymorphisms, for instance SNPs within the sequence, which could cause differences in the efficiency of the xenobiotic in different individuals [42,43] evaluated the response of *Pgp* in humans to changes caused by a SNP (C/T) located in position 3,435, which did not alter the protein but showed an overexpression of the gene in homozygote individuals [42]. In females of *Onchocerca volvulus*, three SNPs were identified which affected the functionality of Pgp [43]. In reference to the characterization of SL-PGY1 in *L. salmonis*, no SNPs were reported [17]. However, this study reports 11 SNPs mutations as validated through HRMA. Two of these generated nonsynonymous changes in Met/Leu and Val/Ile. Future studies will evaluate the association of these polymorphisms with resistance or susceptibility to chemotherapeutic agents in the salmon louse *C. rogercresseyi* [44,45]

The present study is the first to describe the mRNA of *P-glycoprotein* for *C. rogercresseyi* and simultaneously to measure its expression in adults expose to pyrethroid, and during the larval and adult stages of *C. rogercresseyi*. The results showed a relation between *Cr-Pgp* expression and the detoxifying gene *Cr-P540*, suggesting a role of *Cr-Pgp* in pyrethroid metabolism.

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