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Research paper

Ectoparasite *Caligus rogercresseyi* modifies the lactate response in Atlantic salmon (*Salmo salar*) and Coho salmon (*Oncorhynchus kisutch*)

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ABSTRACT

Although *Caligus rogercresseyi* negatively impacts Chilean salmon farming, the metabolic effects of infection by this sea louse have never been completely characterized. Therefore, this study analyzed lactate responses in the plasma, as well as the liver/muscle lactate dehydrogenase (LDH) activity and gene expression, in *Salmo salar* and *Oncorhynchus kisutch* infested by *C. rogercresseyi*. The lactate responses of Atlantic and Coho salmon were modified by the ectoparasite. Both salmon species showed increasing in plasma levels, whereas enzymatic activity increased in the muscle but decreased in the liver. Gene expression was overexpressed in both Coho salmon tissues but only in the liver for Atlantic salmon. These results suggest that salmonids need more energy to adapt to infection, resulting in increased gene expression, plasma levels, and enzyme activity in the muscles. The responses differed between both salmon species and over the course of infection, suggesting potential species-specific responses to sea-lice infection.

1. Introduction

Ectoparasites can substantially affect hosts by impacting physiological, behavioral, and morphological traits, as well as by damaging the integument (Bunkley-Williams and Williams, 1998; Lehmann, 1993; Vargas-Chacoff et al., 2016; Wagner et al., 2003; Wendelaar Bonga, 1997). Parasitic copepods of the Caligidae family, referred to as sea lice, are responsible for fish mortalities and secondary infections in marine aquaculture as a result of mechanical damage, especially in marine aquaculture, especially under conditions of intensive salmonid production (Roth et al., 1993; Roth, 2000; Mustafa et al., 2000; Carr and Whoriskey, 2004; Johnson and Fast, 2004; Boxaspen, 2006). *Caligus rogercresseyi* (Boxshall and Bravo, 2000), an ectoparasitic Caligidae copepod, is of particular concern in the Chilean salmon industry. Infection begins with copepodid attachment, i.e., of the free-swimming planktonic stage, to host scales and fin rays. Once attached, *C. rogercresseyi* sea lice mature into adults (González and Carvajal, 2003) and solely feed on host mucus and skin as an energy source (Carvajal et al., 1998; Bravo, 2003; Bravo et al., 2010; Hamilton-West et al., 2012; Jaramillo et al., 2015, 2016).

Many factors in aquaculture systems impact fish growth rates, including abiotic and biotic factors (e.g. temperature, salinity, stocking density, fish nutritional status, and parasitism). These variables can activate endogenous stress systems and induce changes in fish physiology and metabolic responses (Wendelaar Bonga, 1997; Barton, 2002; Vargas-Chacoff et al., 2009a, 2014a). In particular, teleost fish exposed to parasitism stress can mobilize energy metabolites to modulate immunological responses to pathogens (Wendelaar Bonga, 1997; Mommsen et al., 1999; Barton, 2002), with glucose and lactate being the most important. While many studies have focused on the relationship between glucose and glycogen during stressful conditions (e.g., Vargas-Chacoff et al., 2014a,b,c, 2015, 2016), lactate consumption

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remains understudied.

Lactate, a glycolysis product under anaerobic conditions, is created by cytoplasmic lactic dehydrogenase (LDH), which facilitates the transfer of pyruvate to lactate (Brooks, 1998, 2000; Dubouchaud et al., 2000). Mammalian muscle fibers can consume, oxidize, and use lactate as an energy source. These actions are associated with concomitant decreases in glucose utilization by muscle, thus allowing other tissues to use this metabolite. Lactate use by muscle tissue is therefore a successful mechanism for responding to pathogens without limiting other metabolic functions (Brooks, 2000; Hamann et al., 2001; Kelley et al., 2002). In teleost muscle, lactate production only becomes significant in white muscle when glycogen is used as fuel during anaerobiosis or sudden attack/escape movements. While many vertebrates produce lactate through the Cori cycle in the liver, this does not occur in fish, which primarily generate lactate from muscle glycogen.

Atlantic salmon (*Salmo salar*) and Coho salmon (*Oncorhynchus kisutch*), the main aquaculture species in Chile, are differently susceptible to sea lice infection. Generally, Coho salmon appear to be less susceptible to sea lice than Atlantic salmon (Fast et al., 2002), but the metabolic consequences of infection have only recently become a subject of investigation (González et al., 2015, 2016; Vargas-Chacoff et al., 2016). Plasma glucose levels in Atlantic salmon increase 1–3 days post-infection (dpi) with *C. rogercresseyi*, and amino acid metabolism in the liver and muscle of *O. kisutch* increase during infection, indicating a higher use of gluconeogenesis than *S. salar* (González et al., 2015, 2016). Additionally, amino acid and carbohydrate enzymes in *O. kisutch* activate soon after initial infection, thereby allowing this species to harness energy substrates earlier than *S. salar* (Vargas-Chacoff et al., 2016).

Ectoparasite infection also generally leads to osmotic stress and poorer fish condition, resulting in susceptibility to secondary infections (Dawson et al., 1999; Wells et al., 2006). The present work is the first to experimentally assess the effects of *C. rogercresseyi* infection on the lactate responses of Atlantic and Coho salmon over time. This investigation furthers current understandings in aquaculture by comparing the effects of sea lice infection on two species with different susceptibility to the caligid.

2. Materials and methods

All experiments were performed under the guidelines for the use of laboratory animals established by the Chilean National Commission of Scientific and Technological Research (CONICYT) and the Universidad Austral de Chile.

2.1. Fish and experimental design

The same specimens and experimental procedures used in Vargas-Chacoff et al. (2016) were applied in the present study. Briefly, groups of juvenile post-smolt Atlantic salmon (166.4 ± 17.5 g body weight, n = 250) and Coho salmon (161.2 ± 15.8 g body weight, n = 250) were purchased from the Puerto Phillipi and Chaparano fish farms, respectively. All fish were verified pathogen-free by accredited laboratories. The salmon were transported to the Lenca Laboratory of Fundación Chile (Quillaipe, Chile) and distributed into seawater (35 psu) tanks (500 L) with a continuous flow-through system, 12:12 h light:dark photoperiod cycle, and a water temperature of 12 ± 2 °C. The fish were acclimated for two weeks and maintained under the same conditions for a further three weeks. During these acclimation and maintenance stages, fish were fed *ad libitum* using EWOS Transfer 100 pellet feed, without boost.

2.2. Experimental conditions

The metabolic responses of Atlantic and Coho salmon to *C. rogercresseyi* infection were evaluated. Triplicate infested (50 fish per tank) and duplicate control (50 fish per tank) groups were established for each salmonid species. Each experimental tank was infested with 35*C. rogercresseyi* copepodids per fish. Sea lice were obtained from previously collected specimens maintained at the Fundación Chile Laboratory (Puerto Montt, Chile) according to the protocols defined by González et al. (2015). The control tanks were not subjected to parasite infectation. Tissue samples were taken at time 0 (prior to infection; these samples were not assessed for gene expression), and at 1, 3, 7, and 14 dpi. Ten fish were sampled from each tank at each sampling timepoint, including from the non-infected control tank.

Table 1
Primer sequences used in the experiments.

Primer	Nucleotide Sequence (5′ → 3′)
LDH − Forward	TGTGCGATGAGCTGTGCCTAAT
LDH − Reverse	GTAGTCCTTATCGCCCACGATCTT
18S − Forward	GTCCGGGAAACCAAAGTC
18S − Reverse	TTGAGTCAAATTAAGCCGCA

2.3. Sampling procedure

Fish were netted, submitted to a lethal dose of AQUI-S™ clove oil (50 mg L^{-10}; Bayer Company), and euthanized by spinal sectioning before tissue removal. The fish, water, and collection trays were inspected for detached parasites, which were counted and classified by developmental stage (González and Carvajal, 2003). Fish blood was collected from the caudal peduncle in 1 mL heparinized syringes (25,000 U of ammonium heparin, 3 mL of 0.6% NaCl saline solution). Plasma was separated from cells by centrifuging whole blood for 5 min at 2000 × *g* at 4 °C. The collected plasma, muscle tissue, and the complete liver were each snap-frozen in liquid N_2 and stored at −80 °C until analysis.

2.4. Plasma parameters

Plasma lactate was measured using the commercial Lactate Ref. 1001330 kit (Spinreact) adapted to 96-well microplates (Vargas-Chacoff et al., 2009a,b). The assays were performed with a Multiskan GO Microplate Reader (Thermo Fischer Scientific) using SkanIt v.3.2.

2.5. Tissue metabolites and enzymatic activities

Frozen liver and muscle tissues were finely minced in an ice-cooled petri dish and divided into two aliquots to assess enzyme activity and metabolite levels. Tissues used for metabolite concentration assessments were homogenized by ultrasonic disruption with 7.5 vols of ice-cooled 0.6 N perchloric acid, neutralized using 1 M potassium bicarbonate, and centrifuged for 30 min at 13,000*g* in an Eppendorf 5415R (Sigma-Aldrich). The supernatant was then used to assay tissue metabolite levels. Tissue lactate levels were spectrophotometrically determined using the commercial Lactate Ref. 1001330 kit (Spinreact). The tissue aliquots used for assessing enzymatic activities were homogenized through ultrasonic disruption with 10 vols of ice-cold stopping buffer (pH 7.5) containing 50 mM HCl, 1 mM 2-mercaptoethanol, 50 mM NaF, 4 mM 165 EDTA, 250 mM sucrose, and 0.5 mM *p*-methylsulphonyl fluoride (Sigma Chemical Co.), which were added as dry crystals immediately before homogenization. The homogenate was centrifuged for 30 min at 13,000*g*, and the resulting supernatant was used in enzyme assays for lactate dehydrogenase-oxidase (LDH-O, EC 1.1.1.27). Liver and muscle enzymatic activities were determined using a Multiskan GO microplate reader (Thermo Fischer Scientific) and SkanIt v.3.2 software. Enzyme reaction rates were determined through changes in absorbance of NAD(P)H at 340 nm. The reactions were started by adding homogenates (15 µL) at a pre-established protein concentration, omitting the substrate in control wells (final volume

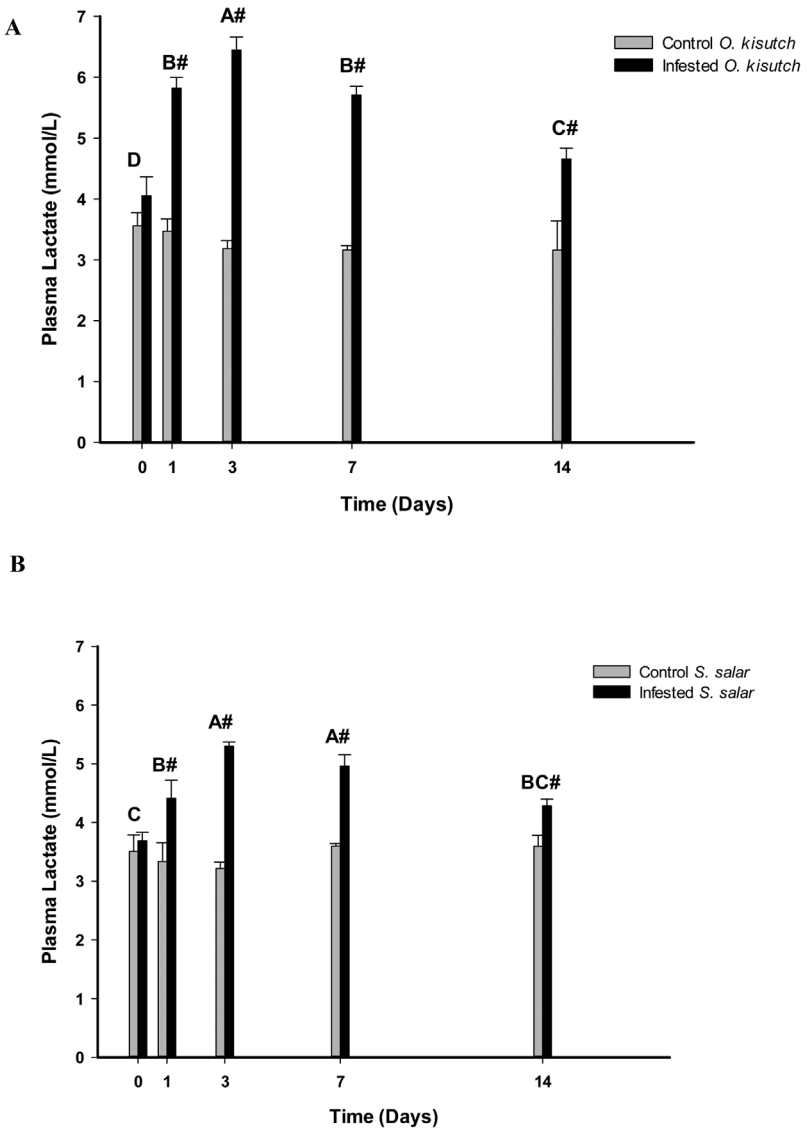

Fig. 1. Lactate plasma levels in (A) *O. kisutch* and (B) *S. salar*. Control (uninfected) and *C. rogercresseyi*-infected fish were sampled at day 0, and then at 1, 3, 7, and 14 days post-infection (dpi). Values are expressed as the mean ± SEM (n = 10). Different letters indicate significant differences between sampling days. Hash tags (#) indicate significant differences between control and infested groups (two-way ANOVA, post-hoc Tukey's test, $P < 0.05$).

275–295 μL), and allowing the reactions to proceed at 37 °C for pre-established times (5–15 min). Protein levels were assayed in triplicate using the Pierce BCA Protein Assay Kit #23225 (Thermo Fischer Scientific). Enzyme assays were carried out under initial velocity conditions. The conditions for enzyme assays for hepatic muscle LDH-O (Vargas-Chacoff et al., 2009b, 2014b,c, 2016) were 50 mM imidazole–HCl (pH 8.5) and 2.5 mM NADP, with 6.25 mM lactic acid being used as the substrate.

2.6. Total RNA extraction

Liver and muscle samples were taken aseptically and used for total RNA extraction. Total RNA was extracted using the Total RNA Mini Kit (Geneaid), and the samples were treated with amplification-grade DNase I (1 U/μg RNA, Invitrogen). RNA was quantified in a spectrophotometer (NanoDrop Technologies), and the quality was verified through electrophoresis on 1% agarose gels. First-strand cDNA was synthesized by M-MLV Reverse Transcriptase (200 U/μL, Invitrogen) from 1 g of total RNA using the oligo-dT18 primer at 50 °C for 50 min.

2.7. RT-qPCR analysis of gene expression

Reactions were carried out using an AriaMx Real-Time PCR System. RT-qPCR analyses used cDNA diluted to 100 ng as a template and the Brilliant SYBR® Green qPCR Master Mix (Stratagene). Primers were designed for LDH and 18 s (housekeeping gene). All reactions were performed in triplicate and in a total volume of 14 μL, which contained 6 μL SYBR® Green, 2 μL cDNA template, 1.08 μL of each primer, and 4.92 μL PCR-grade water. The applied PCR protocol was as follows: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 1 m, and finally 95 °C for 15 s. Melting curve analysis of amplification products was performed at the end of each PCR to confirm the detection and amplification of only one product. LDH was analyzed using the comparative C_t (ΔΔCt) method (Livak and Schmittgen, 2001). The used primers (Table 1) displayed an efficiency of 100% with an R^2 of 0.997. Data are expressed as fold-differences in normalized mRNA expressions relative to values obtained for uninfected control fish. All data are given in terms of relative expression and expressed as the mean ± standard error (SE).

2.8. Statistical analyses

Assumptions of normality and homogeneity for the variances were tested. For each metabolic variable, a two-way analysis of variance was performed. The factors of variance were the infested fish as compared to control fish, and time. A post-hoc Tukey-test was used to identify significantly different groups. Differences were considered significant at $P \leq 0.05$

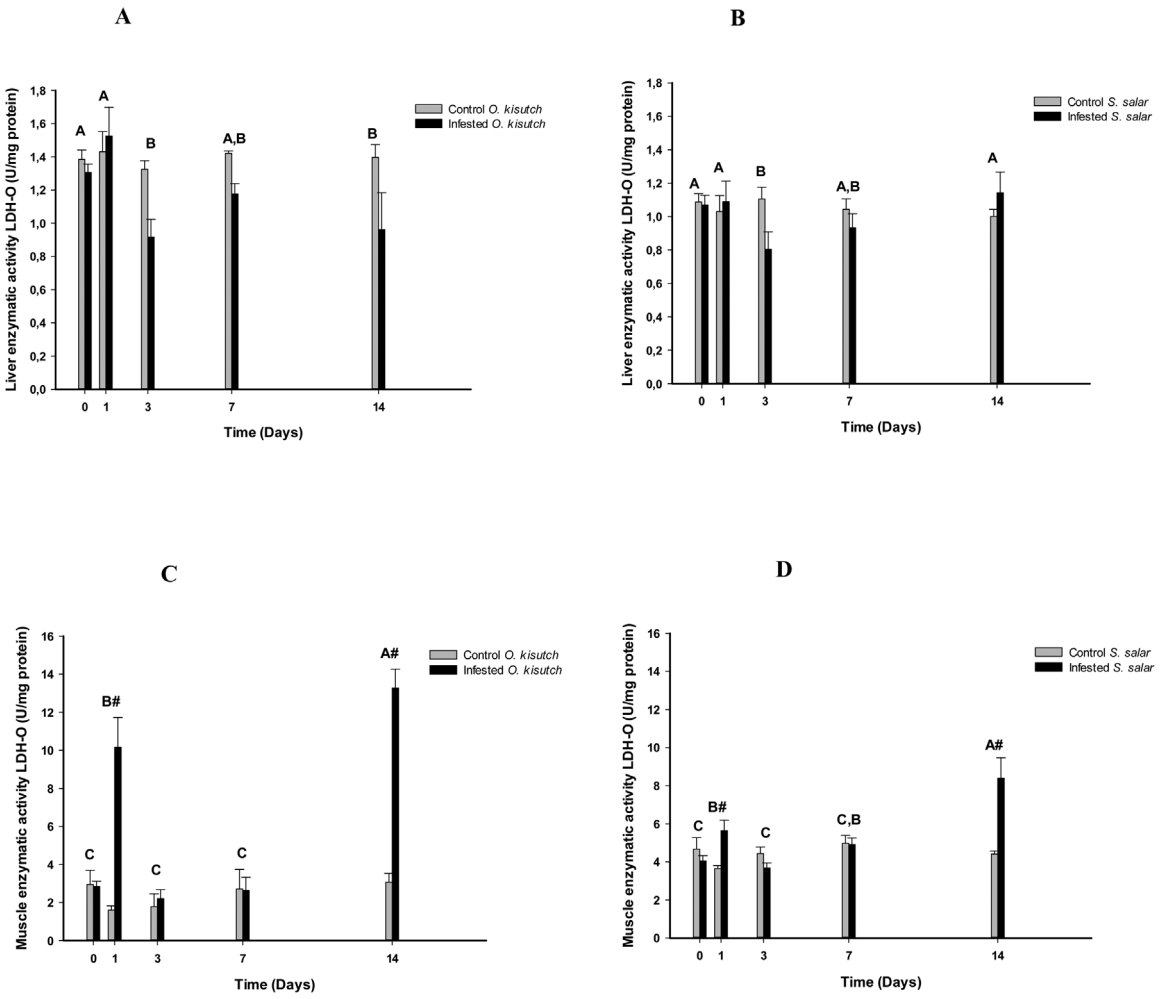

Fig. 2. LDH–O hepatic enzymatic activity in (A) *O. kisutch* and (B) *S. salar*. LDH–O muscle enzymatic activity (C) *O. kisutch* and (D) *S. salar*. Control (uninfected) and *C. rogercresseyi*-infected fish were sampled at day 0, and then at 1, 3, 7, and 14 days post-infection (dpi). Values are expressed as the mean ± SEM (n = 10). Further details are provided in Fig. 1 legend.

3. Results

No fish mortalities were observed in any group throughout the acclimatization, maintenance, or experimental periods.

3.1. Lactate response

Coho and Atlantic salmon displayed increased lactate levels in plasma during the experiment. Both species presented similar lactate level patterns, and while the highest lactate levels were observed at 3 dpi, statistical differences as compared to the control group were found for all time-points (Fig. 1A,B).

LDH-O activity in the liver was similar in both species, presenting the lowest activities at 3 and 7 dpi. Results for LDH-O were not significantly different to the control group in either species, but a decreasing trend existed (Fig. 2A,B). Enzymatic LDH-O activity in the muscle was significantly different from the control group. Furthermore, activity was more intense in Coho salmon than Atlantic salmon, although enzymatic activity was highest in infested fish at 1 and 14 dpi for both species. The increase in Coho salmon was 5-fold at 1 dpi and 4-fold at 14 dpi, while the Atlantic salmon increase was 0.5-fold at 1 dpi and 1-fold at 14 dpi (Fig. 2C,D).

The gene expression profiles of Coho and Atlantic salmon LDH were assessed in the liver and muscle to determine relationships with the infection response. These tissues showed high LDH expression levels in Coho salmon at 3 and 14 dpi, presenting a 20-fold increase in the liver and 80-fold increase in muscle at 3 dpi (Fig. 3A,C). Both tissues presented statistical differences. In comparison, Atlantic salmon LDH expression increased 15-fold in the liver but showed no statistical increase in muscle at 3 dpi (Fig. 3B,D).

4. Discussion

The ectoparasite *C. rogercresseyi* modified the lactate response of Atlantic and Coho salmon via an energy metabolism pathway in the liver and muscle. The responses in both salmon species were different over the course of infection, which is consistent with studies conducted in the Northern Hemisphere for other salmonids and sea lice (Dawson et al., 1999; Wells et al., 2006). Plasma lactate dehydrogenase levels in *S. salar* infected with *Lepeophtheirus salmonis* significantly decreased at 21 dpi (Dawson et al., 1999). By contrast, infected *Salmo trutta* showed plasma lactate levels in the infected group that were increased at 21 dpi, as compared to controls (Wells et al., 2007).

Atlantic and Coho salmon plasma lactate levels increased in the present study. Values were higher than in the control group at all sampling time-points, but maximum levels were found at 3 dpi (Fig. 1A,B). Lactate metabolite production is a secondary stress response, with high plasma lactate levels described in *S. salar* at 22 dpi with *C. rogercresseyi* (González et al., 2015). Non-salmonid aquaculture fish also respond to stress with lactate production. *Sparus aurata* responds to adverse temperature and salinity conditions with high lactate levels (Vargas-Chacoff et al., 2009a,b). High lactate levels were also described in *Solea senegalensis* after changes in salinity, with this lactate used to fuel osmoregulation (Arjona et al., 2007). In the present study,

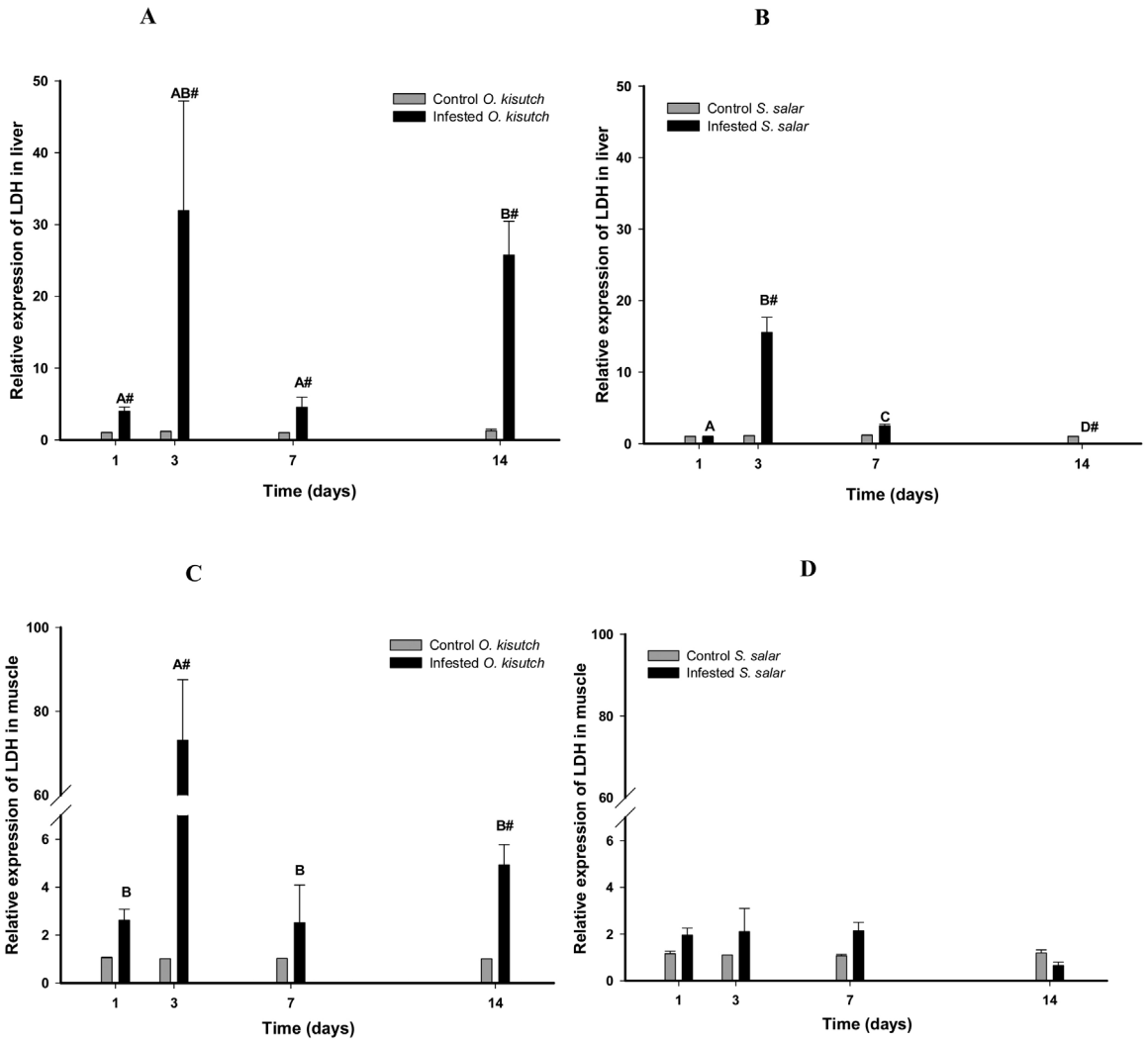

Fig. 3. Gene expression of LDH in the liver of (A) *O. kisutch* and (B) *S. salar*. Gene expression of LDH in the muscle of (C) *O. kisutch* and (D) *S. salar*. Control (uninfected) and *C. rogercresseyi*-infected fish were sampled 1, 3, 7, and 14 days post-infection (dpi). Relative expression was calculated using the $2^{-\Delta\Delta CT}$ method and with the 18 s ribosomal protein as the internal reference gene. Each value represents the mean ± S.E.M (n = 10). Further details are provided in Fig. 1 legend.

the high lactate levels might be used as fuel for the immune system or for mitochondria via the monocarboxylate transporter 1, albeit through converting this lactate to pyruvate via gluconeogenesis (Gladden, 2001, 2004).

Liver enzymatic activity decreased in all fish groups, although LDH gene expression was higher in Coho salmon than in Atlantic salmon. Under the implemented methodological approach, mRNA-level expression and active proteins can be measured in the liver, but the amount of protein is dependent on product mRNA translation and secretion rate into the system. This made LDH accumulation obvious in the liver of infested fish, especially Coho salmon. Similar gene and protein expression patterns in *S. aurata* have been previously described (Vargas-Chacoff et al., 2009c,d). Supporting this, LDH gene expression was consistent with LHD activity in the muscle, indicating that gene and protein expression are indeed synchronized.

In vertebrates, especially mammals, lactate plays many essential roles, such as oxidative fuel, a glycolytic end-product, a gluconeogenesis precursor, and an intracellular signal (Brooks, 2009; Gladden, 2004; Philp et al., 2005). In fish, lactate is an energy metabolite used to maintain energetic balance when necessary. Monocarboxylate transporters can facilitate lactate movement across membranes (Halestrap and Wilson, 2012). Several studies investigating potential mechanisms for explaining lactate retention suggest that fish white muscle may only rely on simple diffusion for transmembrane lactate transport (Wang et al., 1997; Laberee and Milligan, 1999; Sharpe and Milligan, 2003). Nevertheless, more studies are needed to verify the role and relationship of monocarboxylate transporters with lactate levels during parasite infection.

5. Conclusion

This is the first study to focus on lactate metabolism in Atlantic and Coho salmon during infection by *C. rogercresseyi* sea lice. The obtained results suggest that fish need more energy to respond to increased infection. To this end, *S. salar* and *O. kisutch* showed increased gene expression, plasmatic levels, and enzymatic activity, mainly in muscle, in response to sea lice infection.

Conflict of interest

The authors declare that there are no conflicts of interest.

Ethical approval

All experimental procedures complied with the ethical guidelines for the use of laboratory animals by the Chilean National Commission of Scientific and Technological Research (CONICYT) and the Universidad Austral de Chile.

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