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Short Communication

Histopathological changes induced by *Caligus rogercresseyi* in rainbow trout (*Oncorhynchus mykiss*)

Verónica Rojas[1], Delany Sánchez[1], José A. Gallardo[3] & Luis Mercado[1,2]
[1]Laboratorio de Genética e Inmunología Molecular, Instituto de Biología
Facultad de Ciencias Básicas, Pontificia Universidad Católica de Valparaíso, Chile
[2]Núcleo de Biotecnología de Curauma (NBC), Pontificia Universidad Católica de Valparaíso, Chile
[3]Escuela de Ciencias del Mar, Pontificia Universidad Católica de Valparaíso, Chile
Corresponding author: Verónica Rojas (veronica.rojas@pucv.cl)

ABSTRACT. The purpose of this study was to characterize histopathological changes induced by the sea louse *Caligus rogercresseyi* in juvenile individuals of rainbow trout (*Oncorhynchus mykiss*). Specific effects were determined for mucus-secreting cells, mast cells/eosinophilic granule cells (MCs/EGCs) and activation of pro-inflammatory caspase-1, which are involved in the inflammatory response. As expected, *C. rogercresseyi* eroded the skin to varying degrees, with increased mucus-secreting cells in the epidermis and melanophores in the dermis. Gill responses included epithelial hyperplasia, secondary lamellae fusion, an increased quantity of mucus-secreting cells in the epithelium, and MCs/EGCs in the connective tissue. Other histological changes included abundant MCs/EGCs and secretory cells in the intestine, as well as numerous melano-macrophage centres in head kidney stroma cells. Finally, the observed inflammatory response was associated with active caspase-1 detection in the gills, but not in the other assessed organs. In conclusion, *C. rogercresseyi* induced significant histopathological alterations in *O. mykiss* skin and gills and minor histological changes in the intestine and head kidney, which may reflect the overall inflammatory response of rainbow trout to infestation with sea lice.

Keywords: *Caligus rogercresseyi*, rainbow trout, inflammatory response, caspase-1, sea lice.

Sea lice are ectoparasitic copepods that feed on host mucus, epidermal tissue, and blood, thereby causing sub-lethal effects, such as stress, appetite loss, and immune system depression, as well as lethal effects in heavily infected fish (Tully & Nolan, 2002; Fast *et al.*, 2006; Burka *et al.*, 2012). *Caligus rogercresseyi* (Boxshall & Bravo, 2000) is the main ectoparasite affecting salmon and trout aquaculture in Chile (Yatabe *et al.*, 2011). It causes extensive skin erosion and hemorrhaging in salmonids (Bravo, 2003), negatively affects fish physiology during the advanced stages of parasite development (González *et al.*, 2015) and reduces resistance to other diseases, such as *Piscirickettsiosis* (Lhorente *et al.*, 2014). In consideration of these impacts, the purpose of this study was tocharacterizee the histopathological changes induced by the sea louse *C. rogercresseyi* in rainbow trout *(Oncorhynchus mykiss),* a salmonid species highly susceptible to this parasite (González *et al.*, 2000). Therefore, we analyzed the histopathological effects on barrier tissues, such as skin, gills, intestine, and on a hematopoietic organ, the head kidney. Specifically, changes in mucus-secreting cells were evaluated due to their role as protective epithelial cells, mast cells/eosinophilic granule cells (MCs/EGCs). Mucus-secreting cells have been associated with inflammatory reactions and parasitic infection in salmonids (Reite & Evensen, 2006), and pro-inflammatory caspase-1 activation as a proxy for immunological activation due to the fundamental role this molecule plays in vertebrate innate immunity (López-Castejón *et al.*, 2008).

All experimental procedures were carried outfollowingh regulations for laboratory animal use defined by the Bioethics Committee of the Pontifical Catholic University of Valparaíso, Chile. Thirty-one rainbow trout specimens were challenged with the disease (Bethke *et al.*, 2012). Fish were sampled from

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two groups, an infected group (n = 11; body weight = 147 ± 54 g; body length = 26.2 ± 2.4 cm) and a control group (n = 20; body weight 171 ± 57 g; body length 27.0 ± 2.6 cm). Using Fisher's Exact Test, no significant differences were found between infected and control groups for either weight ($F = 1.05$; $P > 0.05$) or length ($F = 0.48$; $P > 0.05$).

Histopathologic analyses were performed on the fish at 20 days' post infection. Briefly, skin samples (8×8 mm) were taken from the mid-body between the dorsal fin and lateral line, gill samples from the second-gill arch, gut samples from the middle intestine, and kidney samples from the head kidney. For histological analysis, samples were fixed in paraformaldehyde (diluted at a 1:9 ratio with 96% ethanol), and for immunohistochemistry, in Bouin's solution (71% saturated picric acid, 24% formaldehyde, 5% acetic acid). Then, they were dehydrated through an ascending ethanol series and embedded in Histosec (Merck). Sections were cut to 5-6 µm using a rotary microtome (Leica RM 2235), mounted on glass slides, cleared, hydrated and stained. The stains corresponded to: hematoxylin and eosin, for general histology analysis; Alcian blue, Toluidine blue and Masson's trichrome, to identify ECGs cells and characterize the content of their granules, and performic acid and Schiff reagent (PAS), to detect mucopolysaccharides.

The tissues were examined under a Leica DM5000B microscope equipped with a Leica DFC450C digital camera. Analyses were performed on tissue sections from five different fish to ensure consistent reproducibility. For transmission electron microscopy (TEM) analysis, the samples (measuring 5×5 mm) were fixed for 4-8 h in 2.5% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.2), post-fixed for 1 h in 1% osmium tetroxide, dehydrated by incubation in a graded ethanol series, and infiltrated with Epon 812 (Shell Chemical Co.). Ultrathin sections were analyzed with a Zeiss-EM-109 transmission electron microscope. Moreover, histological sections were evaluated by indirect immunofluorescence to detect active caspase-1, as described by Rojas *et al.* (2015). For immune-histochemistry analysis, samples were incubated with an anti-active caspase-1 antibody (0.3 µg μL^{-1} in 1% PBSA for 90 min), and them with a secondary antibody conjugated to fluorescein isothiocyanate (Sigma-Aldrich); moreover, nuclei were stained with propidium iodide (10 µg mL^{-1} for 5 min).

The infected group presented 100% prevalence and parasite abundance of 9 ± 4.02, all collected from the skin. Of the total of 99 parasites, 62 were mobile parasites (reproductive adults, and 37 were sessile (Chalimus 4). No parasites were observed in the control group. Histological skin analyses revealed differences between the epidermis of the control and infected fish. In particular, attachment sites were characterized by varying degrees of epithelium erosion and disorganization, no epidermal hyperplasia was observed, although this isa typicaln response of fish epidermis to sea lice infection (Burka *et al.,* 2012). Lesions extended into the underlying dermis, showing hyperplasia, inflammation of the *stratum compactum* connective tissue, disorganization of collagen fibre arrangement, and an increased number of melanophores (Figs. 1a, 1b, 1d *vs.* control 1c). Furthermore, an increased quantity of goblet cells was interspersed between epithelial cells (Figs. 1f *vs.* control 1e). No metachromatic reaction with toluidine blue or cells with active caspase-1 was detected in the skin of the infected fish (data not shown). Using TEM, the ultra-structural analysis of the skin from infected fish also showed collagen fiber disruption and an evident increase in subdermal melanophores (Figs. 1g-1h).

Although no parasites were detected on the gills of the infected fish, the histological analysis showed an inflammatory response. The secondary lamellae showed evidence of hyperplasia, base and tip thickening, and complete or partial lamellar fusion (Figs. 2b, 2d). In comparison, control gills presented ordered organisation, with numerous parallel threadlike secondary lamellae arranged at nearly right angles to the primary lamellae (Figs. 2a, 2c). Moreover, at the primary lamellae of the infected fish, the connective tissue showed an increased quantity of MCs/EGCs, monocytes/macrophage, and lymphocytes (Figs. 2e-2f). Furthermore, infected-fish gills presented an increase in mucus-secreting or goblet cells in the epithelium of secondary lamellae (Figs. 3a-3b) and the distal tip of the gill filaments (Figs. 3c-3d).

Finally, by immunohistochemistry, a notable quantity of gill cells showed activated pro-inflammatory caspase-1 in the challenged fish as compared with control fish, caspase-1 activation was found between the secondary lamellae (Figs. 4a-4b) as well as at the gill base (Figs. 4c-4d). Considering the location, this protease might have been activated in chloride cells, which are responsible for ionic exchanges in fish gills. Nevertheless, more specific staining for these cells, such as the Von Kossa method (Pereira & Caetano, 2009), should be used in future studies. This protease is also activated in rainbow trout infested by the pathogen *Aeromonas salmonicida* (Rojas *et al.*, 2015), and in fish such as *Sparus aurata* (López-Castejón *et al.*, 2008), *Danio rerio* (Masumoto *et al.*, 2003), and *Dicentrarchus labrax* (Reis *et al.*, 2012) in response to dangers or pathogenic signals. This inflammatory response was similar to that reported in coho salmon during infestation by the ectoparasite *L. salmonis* (Fast

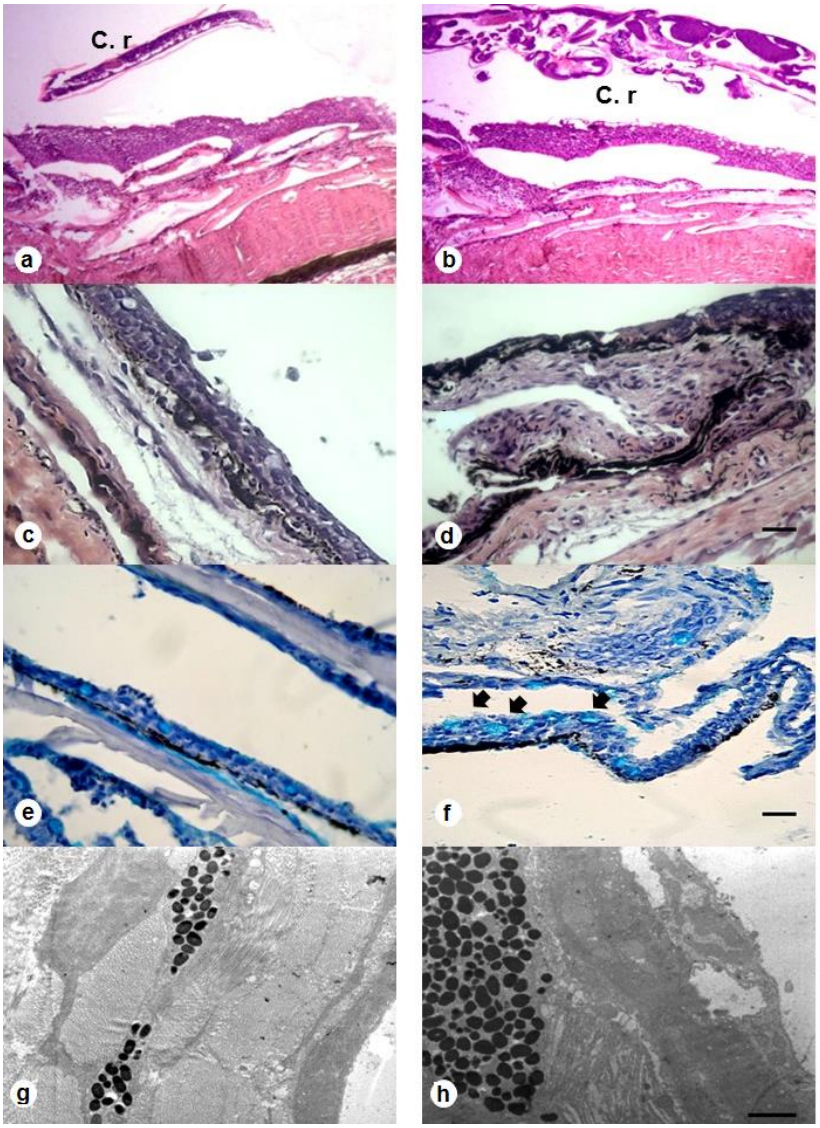

Figure 1. Skin cross-sections. a-d) Attachment sites showed epithelial erosion and disorganized dermal collagenfibers (hematoxylin and eosin), e-f) increased goblet cells (stained blue) containing acid mucopolysaccharides or carbohydrates (Alcian blue), and g-h) increased melanophores (Transmission electron microscopy). c,e,g) Control fish, and a,b,d,f,h) infected fish. Arrows: secretory cells; C.r: *C. rogercresseyi.* Scale bar: 25 µm (a-b), 50 µm (c-f), 1 µm (g-h).

et al., 2002). Changes in the gills may be associated with osmotic stress, since changes in gill thickness can restrict the spaces/channels through which water flows, subsequently affecting the gas exchange process (Evans *et al.*, 2005).

The gut of the infected fish presented inflammatory reactions as disorganized epithelium overlying the submucosa and abundant MCs/EGCs (Figs. 5a-5d); moreover, a large number of secretory cells in the epithelium were found as compared to control counterparts (Figs. 5e-5f). Nevertheless, no visible ultrastructural changes or caspase-1 activation were detected in the gut tissue of the challenged fish (data not shown). No histopathological changes, nor MCs/EGCs or active caspase-1 were found in the head kidney of the infected fish; nevertheless, the stroma of this hemopoietic tissue showed evidence of increased melano-macrophage-centres (MMCs). The histological sections for 20 randomly chosen fields presented an estimated 88 (control fish) and 506 (infected fish) MMCs respectively (data not shown). The increase in MMCs is in line with the chronic inflammatory lesions associated with *Caligus* infestation (Agius & Roberts, 2003).

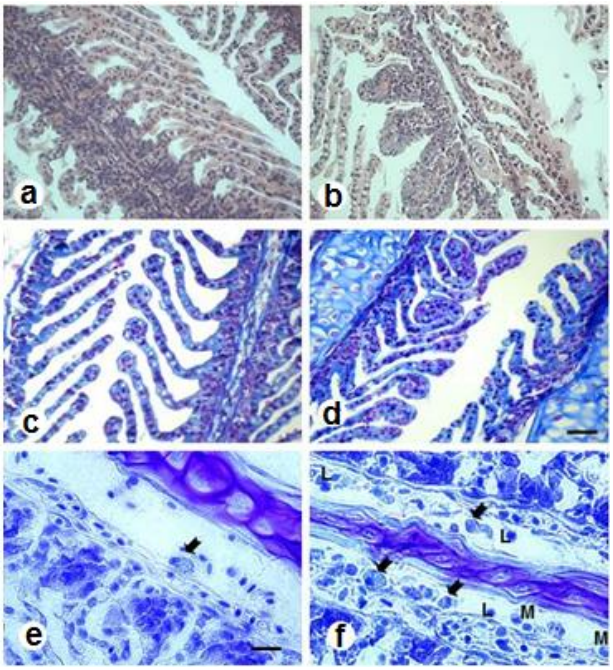

Figure 2. Longitudinal gill cross-sections. Secondary lamellae from infected fish showed hyperplasia, bas, and tip thickening, and/or lamellar fusion (a-b: hematoxylin and eosin; c-d: Masson's trichrome); primary lamellae showed increased MCs/EGCs (arrows), monocytes (M), and lymphocytes (L) ssociated with connective tissue (e-f: Toluidine blue). a,c,e) Control fish, and b,d,f): infected fish. Scale bar: 30 µm (a-d) and 10 µm (e-f).

Both the gills and skin epidermis of the infected fish showed increased goblet cells, which are fundamental for mucus secretion. Positive reactions with PAS revealed the presence of mucin, glycogen, and/or neutral mucopolysaccharides. High mucus production has been described previously for trout and salmon severely infested with *C. rogercresseyi* (González *et al.*, 2016). This production is logical considering the protective function of mucus through the secretion of glycoproteins and glycolipids (Fast *et al.*, 2002). Furthermore, increased mucus coverage of the epidermal surface was correlated with an increase in mucus-producing cells at parasite anchor sites, which may protect fish from osmoregulatory problems (González *et al.*, 2015).

As initiators and effectors of innate immunity, the recruitment of MCs/EGCs to persistent inflammation sites is a general response in teleost's, which is in line with reports for other teleost families, such as cyprinids (Reite & Evensen, 2006). In the gills, MCs/EGCs accumulated in the loose connective tissue of the gill arch, as also reported for mucus-secreting cells in fish infested with the copepod *Ergasillus sieboldi* (Dezfuli & Giari, 2008). The increase in the *lamina propia* of the

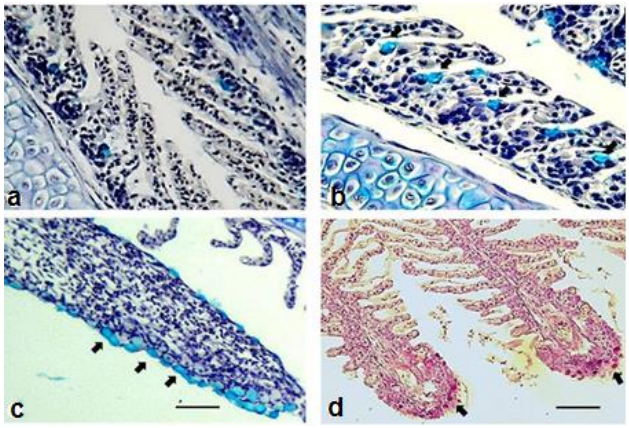

Figure 3. Longitudinal gill cross-sections. Secondary lamellae from infected fish showed a strong increase in secretory cells, which was appreciably higher at the distal end of the primary lamellae (stained blue or purple) containing neutral and acids mucopolysaccharides. a-c) Alcian blue, d) Periodic acid Schiff. a) Control fish, and b,c,d) infected fish. Scale bar: 20 µm (c) and 60 µm (d).

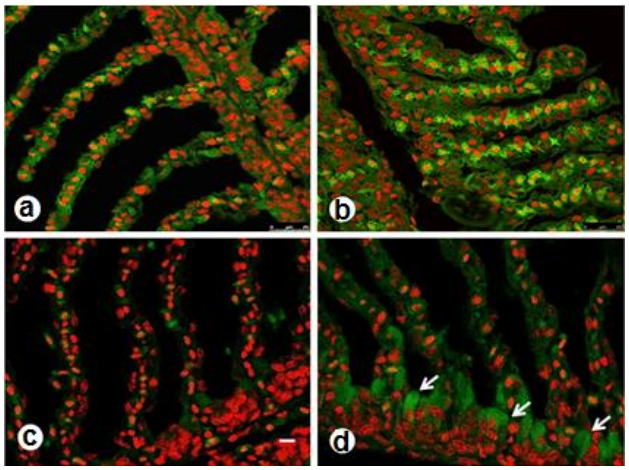

Figure 4. Immunohistochemistry analysis of caspase-1 activation in gill sections. Secondary lamellae from infected fish showed positive reactivity with theanti-caspasee-1 antibody at the filament and base (arrows), indicating an inflammatory reaction. a,c) Control fish, and b,d) infected fish. Scale bar: 10 µm (a-b) and 20 µm (c-d).

intestine has been similarly described for the teleost's intestinal epithelia affected by trematodes or cestodes and in salmonids infested with acanthocephalan parasites (Dezfuli & Giari, 2008). Furthermore, local MC/EGC accumulations in parasitized rainbow trout are associated with persistent inflammatory reactions, and this has also been observed in several salmonids, catostomids, cyprinids, and labroids. While MCs/EGCs were not detected here in the skin of the infected *O. mykiss,* these cells increase in the dermis of rainbow

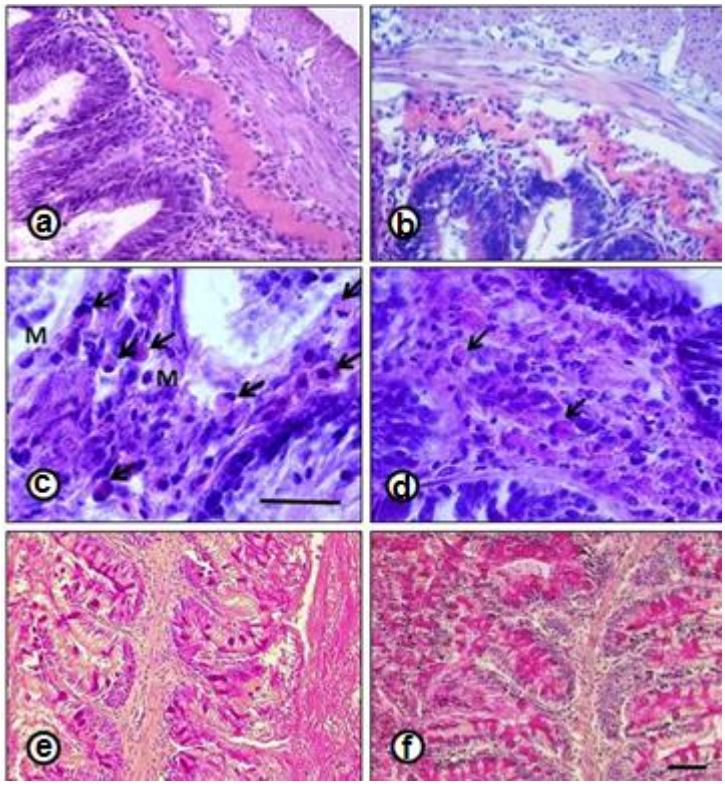

Figure 5. Histological intestine sections. a-d) The intestinall mucosa of infected fish showed a strong increase of MCs/EGCs throughout the *lamina propia* (hematoxylin and eosin), e-f) epithelial goblet cells (stained purple) with mucopolysaccharides (Periodic acid Schiff). a,e) Control fish, and b,c,d,f) infected fish. c-d) Close-ups of infected intestinal tissue with numerous MCs/EGCs (arrows) M: macrophages/monocytes. Scale bar: 25 µm.

trout when exposed to plerocercoid-stage of *Pseudophyllidean cestode*. Various reports postulate that substances released by MCs/EGCs may be involved in the inflammatory reaction, thereby attractting other immune cells (Reite & Evensen, 2006; Lauriano *et al.*, 2012).

In conclusion, this study reports a systemic inflammatory response of *O. mykiss* to *C. rogercresseyi* infestation that was principally characterized by an increased quantity of inflammatory cells in different tissues. The skin and gills were the most affected organs, with both showing evidence of histological changes and gills presenting an activation of pro-inflammatory caspase-1. These data contribute to understanding the response of trout to ectoparasite infestation, a leading issue affecting salmon aquaculture in Chile.

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