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In-feed additives modulate ionotropic receptor genes from the sea louse *Caligus rogercresseyi*: A comparative analysis in two host salmonid species

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ABSTRACT

Recently, a group of chemosensory receptors from the ionotropic receptor family was molecularly characterized in the sea louse *Caligus rogercresseyi*. Nonetheless, understanding the physiological functions of these genes during the sea lice infestation process remains pending. Herein, the aim of this study was to determine the transcriptional modulation of ionotropic receptor genes from *C. rogercresseyi* while infesting *Salmo salar* and *Oncorhynchus kisutch*, as well as to evaluate the effects in-feed additives on sea lice transcriptome. The results revealed significant differences in parasitic load between control diet and the anti-attachment or immunostimulant diet groups. Moreover, there were notable differences in the gene transcription profiles of ionotropic receptors in each group. Under a normal commercial diet, there was a general trend towards higher transcription levels in sea lice infesting *S. salar*, especially at seven days post-infection. This same tendency occurred in sea lice infesting fish fed an immunostimulant diet, but an opposite trend was found in sea lice infesting fish fed with anti-attachment masking compounds. In this case, sea lice infesting *O. kisutch* expressed higher ionotropic receptor levels at seven days post-infection. This study advances the knowledge related to ionotropic receptors and the involvement of these in host-parasite interactions, especially in relation to semiochemical signaling detection.

Statement of relevance

This is a contribution to sea lice control in salmon farms.

1. Introduction

Caligidosis is one of the most notorious and costly ectoparasitic infections affecting wild and farmed salmon species worldwide. The main concerns raised by this disease, especially in the farming industry, are clinical presentations in fish, economic costs, a global presence, and public opinion about aquaculture management (Guo and Woo, 2009). Caligidosis is caused by infestations of sea lice species, which are marine copepods distributed worldwide, with *Lepeophtheirus salmonis* dominant in the Northern Hemisphere and *Caligus rogercresseyi* dominant in southern marine environments (Pike, 1989). Globally, these two species cause high economic losses, which were estimated around €300 million in 2009 alone (Costello, 2009). Since 1970, the control of these parasitic species has been based on the use of chemical antiparasitic compounds, but there is now a trend of reduced efficacy due to emerging resistance and/or lowered susceptibility to these treatments in sea lice species (Bravo et al., 2010). Furthermore, there is increasing concern over the impact of sea lice on wild fish populations and the environment, suggesting that novel control strategies are needed in the salmon industry for caligidosis (Heuschele and Selander, 2014).

In general treatment for caligidosis consists in application of chemotherapeutics, but due to lost in its effectiveness novel methods including vaccine prototypes (Carpio et al., 2011; Ross et al., 2012) and immunostimulation by in-feed additives (Poley et al., 2013) have been applicated. Another proposed method for sea lice control is based on semiochemical traps, which have molecules that disrupt the host identification process by changing the semiochemical cues that the parasite needs to locate host fish (Mordue Luntz and Birkett, 2009). This proposed method is feasible since it is known that Northern-hemisphere

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salmon louse species *L. salmonis* is able to detect chemical cues produced by host species to locate fish in the marine environments (Bailey et al., 2006; Hull et al., 1998). These findings suggest the presence of chemoreceptors and the development of olfactory organs in sea lice (Genna et al., 2005; Ingvarsdóttir et al., 2002; Mordue Luntz and Birkett, 2009).

Regarding chemoreception in *C. rogercresseyi*, a group of *ionotropic glutamate receptors* was found in this species (Núñez-Acuña et al., 2014). These receptors are related to the ionotropic glutamate receptor (IGluR) family, which are important elements in arthropod chemosensory signaling, including the olfactory reception of semiochemicals (Benton et al., 2009). These *C. rogercresseyi* ionotropic receptors are similar to those described in the lobster olfactory organ, which have also been related to semiochemical detection in the marine environment (Corey et al., 2013; Hollins et al., 2003). Furthermore, previous gene expression analyses suggest a role of IGluRs in semiochemical identification, mainly through olfactory reception. In arthropods, the regulation of IGluRs transcription is related to olfactory sensory neurons, thus providing some clues as to the biological functions of ionotropic receptors (Rytz et al., 2013). Moreover, a diverse group of IGluRs exists in the olfactory organ of crustaceans, which is consistent with that found for other arthropod species (Stepanyan et al., 2006).

The objective of this study was to evaluate the previously described ionotropic receptors in *C. rogercresseyi* at transcriptional level after infesting *Salmo salar* and *Oncorhynchus kisutch* fed with diets supplemented through anti-attachment compounds or immunostimulant additives. A relationship between the expression levels of IGluRs and parasite load levels was found in both fish species; in addition to differentiated regulation of these in sea lice infesting in-feed salmon species. Overall, the results provide novel knowledge that can contribute towards the successful implementation of integrated pest management practices in the aquaculture industry, specifically for those plans related to the control of caligidosis in salmon farms.

2. Materials and methods

2.1. Experimental design

Specimens of Atlantic salmon (*S. salar*) and Coho salmon (*O. kisutch*) were obtained from salmon farms located in Puerto Montt, Chile (41.4°S; 72.9°W). After rearing in brackish water (15 ppm of salt), salmons were allowed to smolt and then maintained in single-pass flow-through tanks with ultra-violet treated salt-water and a photoperiod system consisting in a 12:12 h light:dark cycle. Fish were fed daily in proportion to 1% of their total biomass. When fish from both species reached an average approximate weight of 280 ± 30 g, specimens were divided between three 500 L salt-water tanks corresponding to each experimental group per species (control diet, anti-attachment diet, and immunostimulant diet). Each group was replicated in triplicate, and each tank contained 60 fish. Fish were acclimated for two weeks with a commercial diet provided by Ewos Company, Chile. Following this, each group was fed with the corresponding experimental diet for three weeks; control group with a normal diet; experimental group with a normal diet plus an anti-attachment additive (3% phytochemical compound); and a second experimental group with a normal diet plus an 1% immunostimulant additive (1% peptidoglycan). The effect of this peptidoglycan-based immunostimulant diet was previously assessed in Atlantic salmon, evidencing immunostimulation in treated fishes (Casadei et al., 2015). On the other hand, the phytochemical compound corresponded to a natural extract for plants, which acts as masking compound to avoid sea lice infestation by disrupting host-recognition process (unpublished data). After three weeks, all groups were determined free of parasites or other pathogens before subsequent infestation by sea lice.

2.2. Sea lice infestation and parasitic load measurements

From the same collection sites previously mentioned, ovigerous adult female *C. rogercresseyi* were collected and transported to the Fundación Chile research center (Chinquihue, Puerto Montt, Chile). Sea lice were transported at 8 °C in sterile collection vessels with aerated seawater. Sea lice without attachment behavior towards the vessels were excluded from the experiment. Sea lice larvae were produced from egg-strings until reaching the copepodid stage. Infestation of the fish species took place after the three-week feeding period with each experimental diet. For this, 60 copepodids per fish were placed in each tank in the dark and without water flow. After infestation, previous experimental conditions, including feed protocols, were continued. Ten fish from each tank were sampled at 1, 3, 7 and 14 days post-infection. Additionally, at all sampling points the parasitic load was measured by counting sea lice abundance in each group. From the ten sampled fish, sea lice specimens were obtained, fixed in the RNAlater® Solution (Ambion, Life Technologies, USA), and stored at −80 °C until subsequent RNA extraction.

2.3. Gene transcription analysis of IGluR

Ionotropic receptor sequences previously reported by Núñez-Acuña et al. (2014) in *C. rogercresseyi* were used to evaluate corresponding gene transcription levels in response to infesting two salmonid species. Herein, contig sequences annotated to ionotropic receptors were identified from the Illumina MiSeq database for *C. rogercresseyi* as described by Gallardo-Escárate et al. (2014), and then used as a template for primer design with the Primer3 Tool (Rozen and Skaletsky, 2000) included in the Geneious Pro software version 8.0 (Drummond, 2009). For gene amplification, total RNA from sea lice (n = 20) were pooled and 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). The qPCR runs were performed with StepOnePlus™ (Applied Biosystems®, Life Technologies, USA) using the comparative ΔCt method. *β-tubulin* was selected as the housekeeping gene (HKG) due to its stable value as inferred through the NormFinder algorithm. The other HKGs assayed were *elongation factor alpha* and *beta actin*. Each reaction was conducted with a volume of

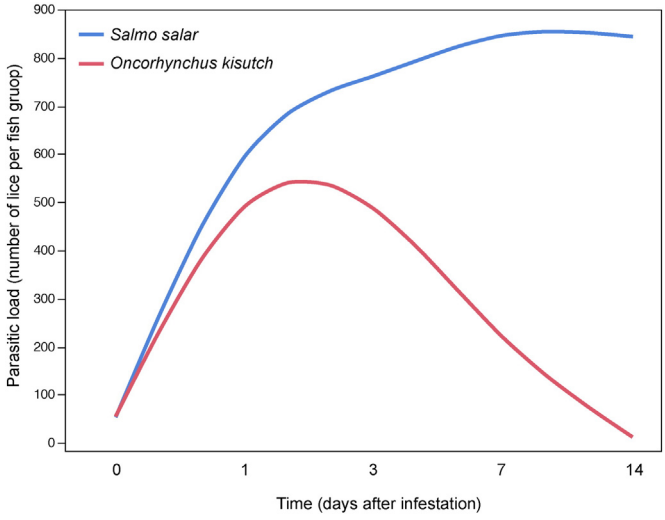


Fig. 1. Parasitic load data. Parasitic load measurements taken after sea lice infestation of Atlantic and Coho salmon. Y-axis corresponds to the total count of sea lice attached to fish for the entire group.

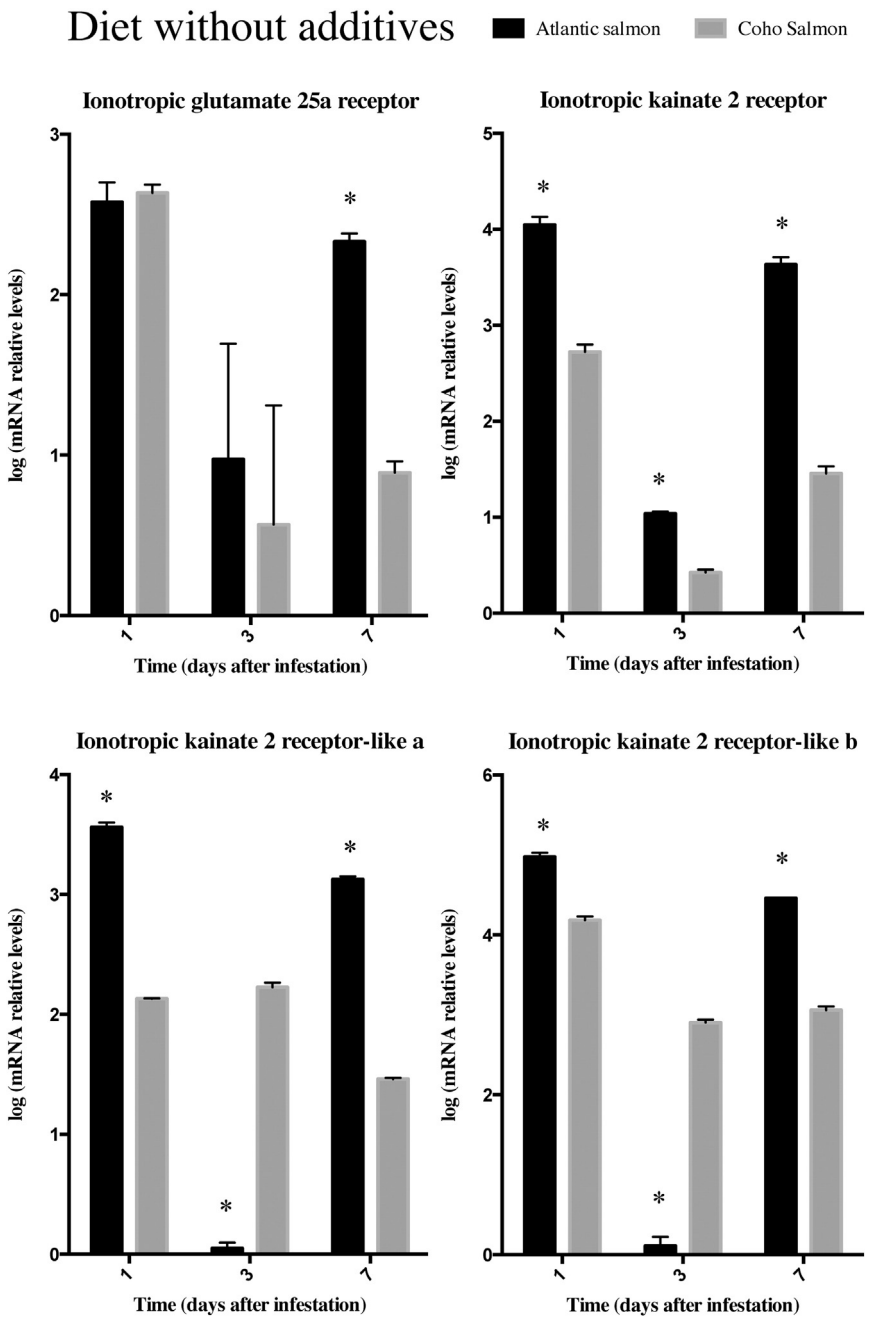


Fig. 2. *C. rogercresseyi* IGluR gene expression following the infestation of two fish species. Relative mRNA levels are presented in a logarithmical scale and correspond to the relative quantification of qPCR data using the *β-tubulin* gene as an endogenous control. Statistically significant differences are shown with a * ($p < 0.05$).

10 μL using the Maxima® SYBR Green/ROX qPCR Master Mix (Thermo Scientific, USA). The amplification cycle was as follows: 95 °C for 10 min, 40 cycles at 95 °C for 15 s, and 60 °C for 1 min, followed by a disassociation curve under these same conditions. Statistical analysis was conducted through one-way ANOVA using the GraphPad Prism software (v6.0, GraphPad Software, Inc. USA). Significant differences were established at $p < 0.05$.

3. Results

3.1. Parasitic load

The amount of total sea lice attached to fish were counted at each sampling point, and the values were averaged to obtain the parasitic load, or number of lice per fish in each group. For all groups, the initial parasitic load was 60 copepodids per fish, corresponding to the moment of infestation (day 0). Parasitic load subsequently increased over time in all groups, but with different trends depending on the species. The parasitic load in control diet Atlantic salmon rapidly increased, reaching ~650 lice per fish group at day 1, ~800 at day 3, and a peak of ~900 sea lice at days 7 and 14. In contrast, control diet Coho salmon evidenced lower infestation levels that increased from 60 to 400 at day 1 and up to 500 at day 3. This load then decreased to 150 sea lice at day 7 before reaching nearly 0 at day 14 (Fig. 1). Regarding the in-feed additive groups, significant differences in infestation rates were congruent with the previously reported by Núñez-Acuña et al. (2014), where all the tested diets showed a decrease of lice infestation up to 25% (Data not shown).

3.2. IGluR gene transcription analyses in C. rogercresseyi

Gene transcription analyses were performed for four inotropic receptor genes from 1, 3 and 7 days after infestation. In the salmonid groups fed with a control non-additive diet, *IR25a* evidenced higher transcription levels in sea lice infecting Atlantic salmon than in Coho salmon, especially at 7 days post-infestation. A similar trend was observed for *ionotropic kainate 2 receptors* (*KAR-2*), where expression levels were higher in sea lice infecting Atlantic salmon at 1 and 7 days post-infestation than those in Coho salmon. The only noticeable difference between the trends of *IR25a* and *KAR-2* expression occurred at 3 days post-infestation, when *KAR-2-like-a* and *b* genes had a higher expression level in Coho salmon-infecting sea lice (Fig. 2).

Differentiated transcript levels were also observed in the anti-attachment diet groups. While a decreasing trend was observed for all four transcripts in both anti-attachment diet groups between 1 and 3 days post-infection (Fig. 3), at 7 days post-infection, Coho salmon expressed increased transcript levels, which was in contrast to the trend observed in control Coho salmon. In regards to the other evaluated transcription patterns in anti-attachment feed fish, *IR25a* showed higher transcription levels at day 1 in Atlantic as compared to Coho salmon.

Concerning fish fed an immunostimulant diet, the transcriptional response of sea lice contrasted with that observed in sea lice sampled from anti-attachment diet salmonids. Sea lice infecting Atlantic salmon given an in-feed immunostimulant additive presented an overexpression

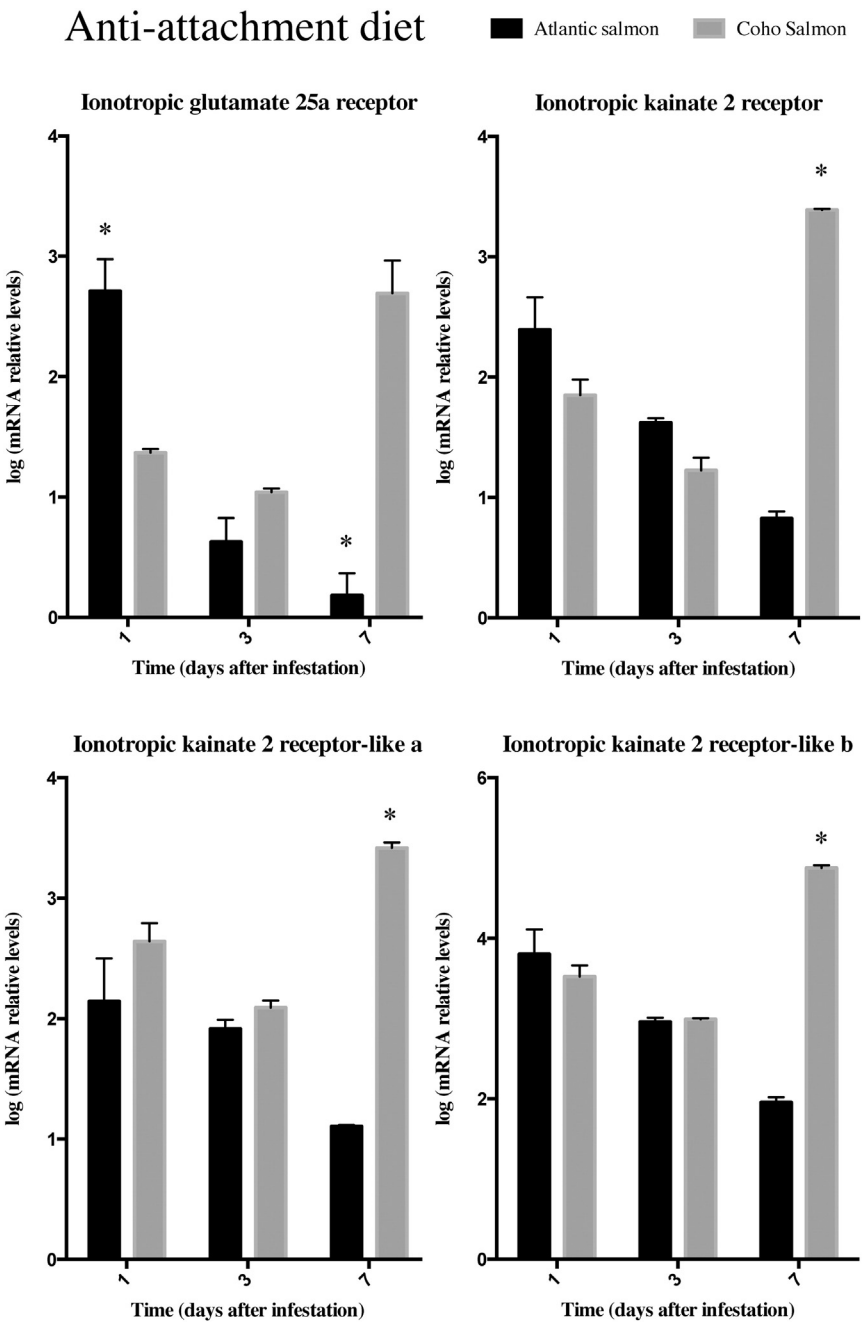


Fig. 3. Sea lice IGluR gene expression after exposure to anti-attachment diet salmon. Results are provided for two host species. The Y-axis corresponds to relative mRNA levels normalized to the *β-tubulin* gene. Statistically significant differences are shown with a * ($p < 0.05$).

of IGluRs over time (Fig. 4). This progressive trend was observed mainly for *IR25a*, *KAR-2*, and *KAR-2 like-a*. Furthermore, *IR25a* and *KAR-2* evidenced higher transcription levels in Atlantic salmon-infecting sea lice than those from Coho salmon at all sampling points. In turn, *KAR-2* like transcripts showed this trend only at 3 and 7 days post-infestation (Fig. 5).

4. Discussion

The present report analyzed the IGluR sequences described for *C. rogercresseyi*, which were the first chemosensory receptors reported in a marine copepod (Núñez-Acuña et al., 2014). While the gene expression of IGluRs from *C. rogercresseyi* was previously evaluated in relation to the incorporation of in-feed additives in the host diet, only the Atlantic salmon as a host species was evaluated. The present results are consistent with this previous study; showing that after the incorporation of immunostimulants in the Atlantic salmon diet, infesting lice overexpressed IGluR transcripts in the days post-infestation. However, the present study also provides two novel contributions regarding IGluR transcriptional information: 1) a description of the early response in sea lice after infestation and 2) a comparative analysis of IGluR genes in response to sea lice infesting different host species.

Regarding the response time of IGluRs, a rapid activation of these genes was expected based on the knowledge that electrophysiological excitation of lice antennule begins just seconds after exposure to semiochemicals (Fields et al., 2007). Nonetheless, it is unpractical to obtain reliable transcriptional data within intervals of seconds; therefore, other aspects must be determined, such as the morphological development

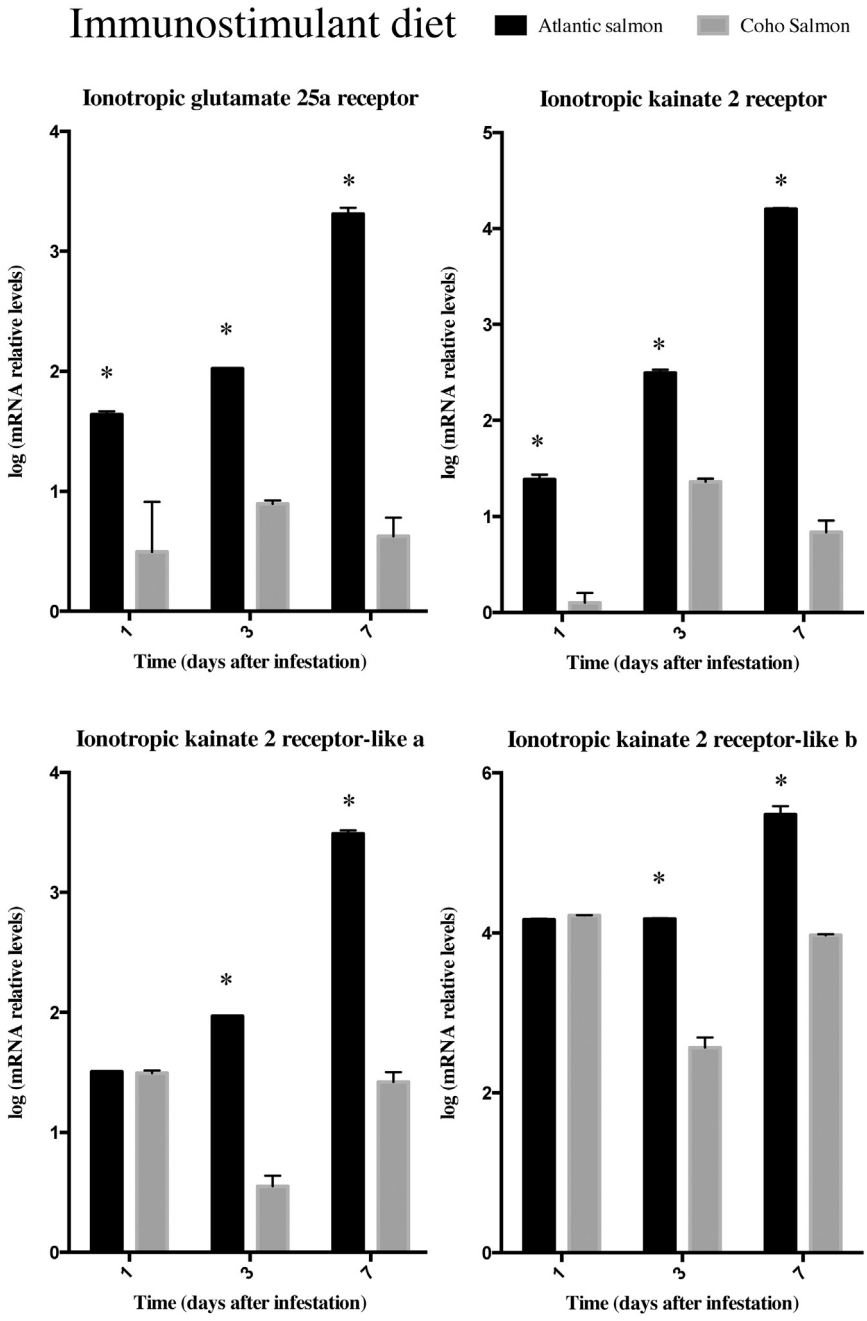
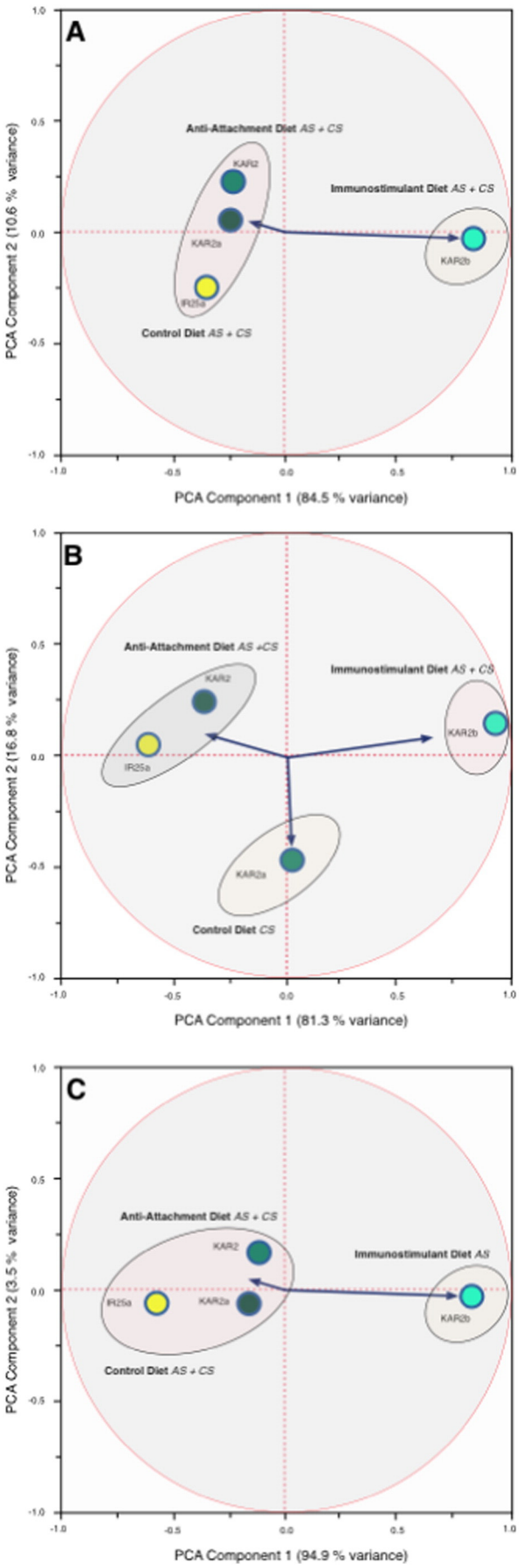


Fig. 4. Sea lice IGluR gene expression after exposure to immunostimulant diet salmon. Results are provided for two host species. Gene transcription levels corresponded to relative mRNA levels normalized to the *β-tubulin* gene in *C. rogercresseyi*. Statistically significant differences are shown with a * ($p < 0.05$).



of sea lice and the physiological response of infested fish. The time frame studied in this work spanned the transition of the infective larval stage (copepodid) to the juvenile stage (chalimus), which is crucial for attachment success in this parasite (González and Carvajal, 2003).

Moreover, this is the first study to provide a comparative analysis of IGluR expression in *C. rogercresseyi* in relation to distinct host salmonids. Different gene transcription patterns were found when comparing Atlantic and Coho salmon in each diet group. According to published literature, Coho salmon are more resistant to sea lice infestation than Atlantic salmon (Fast et al., 2002). The present study supports these prior findings; higher expression levels of the four IGluR were observed in sea lice infesting the more susceptible Atlantic salmon, which is an indicator for the success of the infestation mechanism. This can also be associated with parasitic load data (Fig. 1), in which day 7 marked the trend in infestation levels for both species. Therefore, the observed differences in IGluR gene expression at day 7 could be related to the maintained parasitic load for Atlantic salmon as compared to the decreased parasitic load in Coho salmon.

The results obtained in this work support the hypothesis that IGluR is related to olfactory reception since expression patterns were opposite to those of control diet groups after exposure to anti-attachment compounds. Furthermore, this reverse trend is an important indicator of the effect that masking compounds cause in sea lice infestation. The immunostimulant additive diet caused a similar trend in sea lice IGluR expression as that observed in a prior study, where these genes were overexpressed after exposure to immunostimulant compounds (Núñez-Acuña et al., 2014). However, the reason behind this trend in IGluR remains unclear. One possible explanation is that immunostimulatory molecules could provoke a change in the production of Atlantic salmon semiochemicals. Future studies will aim to resolve this unanswered question.

Given the complexity in controlling caligidosis, and considering the need for multidisciplinary tools to confront this problem, the present study provides novel data that can be applied towards developing additional treatment alternatives to ultimately decrease the detrimental effects of this disease in salmon farming. Currently, caligidosis is controlled mainly through the use of chemotherapeutics, which can unfortunately result in the emergence of pesticide resistance (Bravo et al., 2008; Helgesen et al., 2014). In this context, research groups and managers from the aquaculture industry are discussing novel strategies. While an antiparasitic vaccine exists (Carpio et al., 2011), it has not produced the required effect in salmonids. Other strategies involve changing the environmental conditions, such as the water salinity (Bravo et al., 2015), however these methods have not yet been implemented. Similarly, while semiochemical traps (Mordue Luntz and Birkett, 2009) are a promising alternative oriented towards avoiding sea lice infestations, these traps remain to be applied in the industry.

Despite the existing research, information regarding chemical receptors in sea lice species still remains unclear, and it is difficult to develop new, high efficacy treatment solutions for caligidosis management without a solid basis of data. Therefore, understanding the chemoreception system in sea lice is crucial, and novel studies addressing this topic should be performed. Chemoreception research, and specifically on chemical receptor regulation, such as for ionotropic receptors, contributes towards the development of novel integrated pest management methods in a time when there is an urgent need to control caligidosis in salmon farms worldwide.

Fig. 5. Principal component analyses (PCA) of IGluR transcriptional data. Each PCA corresponds to different infestation times, A) Day 1, B) Day 3 and C) Day 7. Factorial map of the PCA was performed on data from the four IGluR transcripts in each pathway represented as point for each diet: control diet, anti-attachment and immunostimulant diet. The portion of the variance explained by the principal component is indicated in parentheses.

5. Conclusion

This study provides new knowledge on host-parasite interactions and on the modulation of sea lice genes under the effects of a disease-controlling method, the incorporation of in-feed additives in fish diets. The present results provide evidence that IGluR transcription is likely related to sea lice infestation success, which depends on the host species, and also that IGluR appears to have a different transcriptional response in the presence of in-feed additives incorporated into fish diets. This knowledge will be useful towards constructing innovative control methods for caligidosis through the integrated-pest management of *C. rogercresseyi* and similar species.

Acknowledgments

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